Expression of vascular endothelial growth factor and neural repair after alprostadil treatment of sciatic nerve crush injury

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Abstract

Background: Vasoactive drug alprostadil improves microcirculation and can be effective in treating disorders of peripheral nerves. Vascular endothelial growth factor (VEGF) has been shown to have protective action in cerebral ischemia, disorders of spinal cord, and also peripheral nerves. However, the mechanism of action of VEGF in peripheral nerve injuries is uncertain. Objectives: To study the effect of application of alprostadil on the pathological and functional repair of crush nerve injuries and also the expression of VEGF.

Materials and Methods: Rat sciatic nerves were crushed by pincers to establish the model of crush injury. All of the 400 Sprague Dawley (SD) rats were randomly divided into: Control; saline; saline + VEGF-antibody; alprostadil; and alprostadil + VEGF antibody groups. The SPSS 11.5 software was used for statistical analysis. The expression of VEGF in dorsal root ganglia (DRGs), following crush injury to sciatic nerves, was studied by reverse transcribed-polymerase chain reaction (RT-PCR), immunohistochemistry, electromicroscope, and electrophysiology. The effects of alprostadil on expression of VEGF, repair of neural pathology, and recovery of neural function were also evaluated.

Results: We found that VEGF messenger ribonucleic acid (mRNA) was significantly increased in alprostadil and alprostadil + VEGF-antibody groups, compared to the saline and saline + VEGF antibody groups. The number of VEGF-positive neurons was significantly increased in the alprostadil group, compared to the saline, saline + VEGF antibody, and alprostadil + VEGF antibody groups. Besides, addition of this drug also caused less pathological changes in DRGs, better improvement of nerve conduction velocities of sciatic nerves, and more increase of toe spaces of right hind limbs of rats.

Conclusions: The vasoactive agent alprostadil may reduce the pathological lesion of peripheral nerves and improve the rehabilitation of the neural function, which may relate to upregulation of the expression of VEGF, following crush injury to the peripheral nerves.

Key words: Crush injury, dorsal root ganglion, sciatic nerve, vasoactive agent, vascular endothelium growth factor

Introduction

Crush injury to peripheral nerves can adversely affect neural microcirculation and capillary occlusion. Consequently, ischemia and oxygen deficiency in nerves, destruction of the blood-nerve barrier, neural edema, disorder of the neural internal environment, and neural dystrophy appear, and can result in dysfunction of neural conduction. It has been reported by clinical and experimental studies that vasoactive treatment can alleviate the effects of lesions in peripheral nerves. Alprostadil is an effective agent for treating disorders of peripheral nerves. It is known that the vascular endothelial growth factor (VEGF) can protect...
against neuronal lesions. However, the changes in VEGF following crush injury to peripheral nerves, and the mechanisms of vasoactive treatment are incompletely understood. In order to explore the protective mechanisms involved in vasoactive treatment on peripheral nerves after injury, the sciatic nerves of rats were crushed, and the effect of the vasoactive agent, alprostadil, on expression of VEGF was assessed.

Materials and Methods

Sciatic nerve injury in rats
After fasting for 12 hours, 400 Sprague Dawley (SD) rats (200 males and 200 females, SPF grade, body weight 180-220g, Shanghai Laboratory Animal Center, Shanghai, China) were randomly divided into: Control; saline; saline + VEGF antibody; alprostadil; and alprostadil + VEGF antibody groups. Each group included 80 rats (40 males and 40 females). All procedures were performed in accordance with the animal care guidelines of the Nanjing Medical University, which conform to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised, 1985). The rats were fixed onto rat plates after they were anesthetized with 10 ml/kg of 3% pentobarbital via intraperitoneal (ip) injection. An incision was made in the right hind limb to expose the sciatic nerve. In the control group, the incision was closed after exposure of the sciatic nerve. The sciatic nerves of the rats in other groups were clipped using pressure equal to half of the body weight for 30s, the pressure was then released and the incisions were closed. Routine antibiotics were given to prevent infections. The rats in the alprostadil (Beijing Taide, batch number 2044T, Beijing, China) and VEGF antibody (Beijing Zhongsan Jingqiao Biotechnology Ltd., Beijing, China) groups were administered drugs (4 μg/kg/d, ip) and rabbit IgG polyclonal VEGF antibody (diluted with saline to 1:100, 2 ml/d, ip), and an equivalent volume of saline (ip) was given to the rats in the saline group.

Separation of dorsal root ganglia and sciatic nerve trunks
The dorsal root ganglia (DRGs) and sciatic nerve trunks were separated immediately after the rats were sacrificed. The ribs were sheared along both sides of the spine, and the spine’s thoracic and lumbar segments were removed. The section was cut along the median line, the spine segments, DRGs, neural roots and the sciatic nerve trunks were placed in culture dishes containing oxygen-enriched, saturated Dulbecco’s Modified Eagle’s Medium (DMEM), pH 7.4, the osmotic pressure was 340 mOsm/L. DRGs, nerve roots (anterior and posterior roots) and the sciatic nerve trunks were removed from the neural canal. The membrane of the peripheral connective tissue, which connects to the nerve trunk, was removed under a stereoscopic microscope using fine corneal scissors, wire forceps and scissors.

Detection of vascular endothelial growth factor mRNA in dorsal root ganglia cells
The DRGs were taken at time points including: 0, 3, 6, 12, 24, 48, 72, and 96 hours, as well as at day seven after crushing the sciatic nerves. Reverse transcribed-polymerase chain reaction (RT-PCR) was performed as follows (reverse transcribed kit from Promega, Madison, WI, TRIzol from Invitrogen, Carlsbad, CA):

1. Total ribonucleic acid (RNA) was extracted from DRGs according to the manufacturer’s instructions. The extracted RNA was incubated at 70°C for 10 min and then placed on ice.
2. The oligonucleotide primers for VEGF were 5’-GAGGTGTTACATGGATGTC-3’ (forward) and 5’-CGATCCTCGATACGTCCCTTT (reverse), and amplified a 541 bp fragment; and for β-actin the primers were 5’-CCGTCCGCTGTCTGAC-3’ (forward) and 5’-GTCACGCAGATTCCCCGT-3’ (reverse), amplified a 619 bp fragment (Shanghai Shenggong Biotechnology limited-liability company, Shanghai, China).
3. Amplification conditions consisted of an initial denaturation at 94°C for 2 mins, followed by, 32 cycles of denaturation (94°C for 0.5 min), annealing (64°C for 1 min), extension (72°C for 1 min), and a final elongation step (72°C for 2 mins).
4. Amplification products were resolved by agarose (2%) gel homeothermic electrophoresis at 80 V for 30 min. The predicted sizes of the amplification products were observed after electrophoresis, under an ultraviolet lamp, and densitometry was done. Then, the index of mRNA (RI) was calculated (RI = VEGF mRNA density/β-actin density × 100%).

Detection of vascular endothelial growth factor positive neurons in dorsal root ganglia
The DRGs of each group were taken at 0, 1, 3, 6, 12, 24, 48, 72, 96 hours and day seven after crushing the sciatic nerves. They were treated as follows (pv-6001/6002 immunohistochemistry kit from Beijing Zhongsan Jingqiao Biotechnology Ltd. China):

Immunohistochemistry
(1) APES (3-aminopropyl triethoxysilane) smear was conducted on glass slides; (2) Routine deparaffinizing was performed on the sections; (3) The sections were incubated in 3% H2O2 in deionized water for 5-10 mins to block endogenous peroxidase; (4) Hot-fix of the antigens was performed; (5) The primary antibody (Immediate-use VEGF rat anti-human monoclonal antibody from the immediate-use non-biotin pv-6001/6002 reagent kit, Beijing Zhongsan Golden Bridge Biotechnology
Co. Ltd. Beijing, China) was added. The sections were placed in 4°C refrigerator overnight and then rinsed with phosphate buffered saline (PBS) three times for 2 mins each; (6) The secondary antibody (Immediate-use goat antimouse IgG-horse radish peroxidase (HRP) polymers from the immediate-use nonbiotin pv-6001/6002 reagent kit, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) was added. The sections were incubated at room temperature for 20-30 mins, and then rinsed with PBS three times for 2 mins each; (7) Diaminobenzidine (DAB) color development was conducted; (8) Washing with tap water was performed; (9) The samples were stained with hematoxylin, dehydrated, cleared and mounted. The sections were then observed under a microscope (50i, Nikon, Shanghai China) and the cells containing brown-yellow granules were considered to be positive.

**Image analysis**

Two specimens were randomly selected from each group at each time point and VEGF immunohistochemistry was performed. A light microscope (×400) was used to observe the number of cells with positive expression in each sample (five fields were randomly taken from each section to count the positive cells and the average value was calculated), and a comparison was conducted.

**Pathological examination**

The rats in each group were sacrificed at week four after crush. The DRGs and sciatic nerves were separated according to the method described. Examination of the DRGs and sciatic nerves was performed by electromicroscope.

**Nerve conduction velocities of sciatic nerves**

Eight rats from each group were killed at week four after crush. Then a 6-cm length of sciatic nerve trunk was separated. The Nerve conduction velocities (NCVs) of sciatic nerves in the NCV determination box (constant temperature 35ºC) were detected at 2 mins, 3 mins, and 4 mins, respectively (D95Super Lab determinator of NCV from Medical Electrons Institute of Academe of Jiangsu Biomedical Engineering). Different parameters of a stimulus impulse were: 0.04 ms, 2.7 voltage, and scanner speed 50,000 mm/s. The distance between the two recording electrodes on every sciatic nerve trunk was 3.5 cm. The time of action potential peak of two recording electrodes was detected.

**Toe spaces**

The toe spaces of rats in each group were determined at week four after crush. We stained ink onto the rats' toes and made them walk on papers of 60 cm length and 10 cm width. The space of three to four toes of the right hind limbs was determined with vernier calipers.

**Statistics**

Statistical Package for the Social Sciences (SPSS, Bizinsight, Beijing China) 11.5 was used, and P values < 0.05 were considered to be significant. Analysis of variance (ANOVA) was used for comparison. The number of spinal cord neurons and the number of cells expressing VEGF were consistent with normal distributions. The Student-Newman-Keuls analysis (SNK) method was used.

**Results**

**Vascular endothelial growth factor mRNA by RT-PCR**

There was little expression of VEGF in all groups, 0 h after crushing, and no significant difference among them (P > 0.05). Compared with 0 h: (1) the VEGF mRNA levels were not different from the control group (P > 0.05); (2) VEGF mRNA from other groups increased at 3 h and 6 h (P < 0.01 at 6 h) after crushing, and the VEGF mRNA from the saline, saline + VEGF antibody, alprostadil and alprostadil + VEGF antibody groups at 6, 12, 24, 48, 72, and 96 hours were more than that at 0 h (P < 0.01); (3) VEGF mRNA peaked at 72 h and then decreased to baseline, such that there was no difference between 0 h and day seven in each group (P > 0.05); (4) VEGF mRNA from the saline, saline + VEGF antibody, alprostadil and alprostadil + VEGF antibody groups were significantly more than that of the control group at 6, 12, 24, 48, 72, and 96 hours (P < 0.01); (5) VEGF mRNA from the alprostadil and alprostadil + VEGF antibody groups were significantly more than that of the saline group at 6, 12, 24, 48, 72, and 96 hours (P < 0.05), but there was no intragroup difference (P > 0.05). There was no difference between the saline + VEGF antibody group and the saline group (P > 0.05) [Figure 1, Table 1].

**VEGF-positive neurons in DRGs**

There were only a few VEGF-positive neurons in the DRGs in the control group. In the alprostadil group, the

![Figure 1a: VEGF mRNA in control group (M marker, N negative)](image1)

![Figure 1b: VEGF mRNA in saline group (M marker, N negative)](image2)
The number of VEGF-positive neurons began to increase at 6 h ($P < 0.05$), and at 12 h in the other groups ($P < 0.01$); the numbers peaked at 72 h ($P < 0.01$) and then started to decrease, returning to normal on day seven. The VEGF-positive neurons in the alprostadil group were more than that of the saline group at the same time ($P < 0.05$). The VEGF-positive neurons in saline + VEGF antibody group were less than that of the saline group ($P < 0.05$).

Pathological changes of DRGs electromicroscopically

In the saline and saline + VEGF-antibody groups, the density of electrons of numerous nuclei decreased,
Vacuolar degeneration and pyknosis appeared, the nucleoli or even nuclear structure disappeared, and the nuclear membrane was incomplete. The density of cytoplasm increased. The change of patching homogeneity, vacuolar degeneration, and many apoptosis body appeared in the cytoplasm. The pathological changes of the saline + VEGF-antibody group were more severe than those of saline group. In the alprostadil group, the density of electrons of some nuclei decreased and few apoptosis bodies appeared in the cytoplasm, and the other pathological changes were also less than those in the saline group. The pathological changes in alprostadil + VEGF-antibody group were severe than those in the alprostadil group. There was no pathological change of DRGs in the control group [Figure 3].

**NCVs of sciatic nerves**

The NCVs of sciatic nerves in the alprostadil group were faster than that in the saline group ($P < 0.05$) and slower than that in the control group ($P < 0.01$) at week four after crush injury. The NCVs of sciatic nerves in the saline + VEGF-antibody group were slower than that in the saline group ($P < 0.05$) and the NCVs of sciatic nerves in the alprostadil + VEGF-antibody group was slower than that in the alprostadil group ($P < 0.05$) at week four after crush injuries [Table 3].
At week four after crush, the toe space in the saline group was significantly shorter than those in the control and alprostadil groups ($P < 0.01$) and the toe space in the alprostadil group was not different to that in the control group ($P > 0.05$). The toe space of the saline + VEGF-antibody group was shorter than that of the saline group, but there was no statistical value ($P > 0.05$). The toe space of the alprostadil + VEGF-antibody group was shorter than that of the alprostadil group ($P < 0.01$) [Table 4].

**Discussion**

Relevant research indicated that the expression of VEGF could be upregulated by anoxia. Following traumatic brain injury, there was an early (within 4 h postinjury) increase in the expression of VEGF that was associated with neutrophilic invasion in the traumatized parenchyma. The maximum number of astrocytes expressing VEGF was observed on the fourth day after traumatic brain injury. Macrophages in the periphery and the core of infarct represented the major source of

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VEGF during the early stages, 18 h to day 2 postinjury. The number of immunoreactive macrophages decreased gradually from day 2, in both the periphery and core. VEGF was detected in neurons from 18 h after the lesion to day five in the core, and up to day ten in the periphery. In the early stages, intracytoplasmic granular immunolabeling in the neurons and dendrites was observed. Immunostaining in glial cells was observed during the entire active period, from 18 h after the lesion to day 14. However, their peak activities were occurred from day five to seven in the periphery.[10] Minamino reported that the expression of VEGF and other growth factors could be induced by hypoxia.[11] Gaumann found that the presence of VEGF was associated with tumor necrosis. Local hypoxia of tumors could induce VEGF and result in higher VEGFR-1 and VEGFR-2 levels in vascular endothelial cells.[12] Hypoxia increased the expression of VEGF mRNA and protein via the following mechanisms: (1) the regional promoter of VEGF included a response element of the hypoxia-inducible factor-1 (HIF-1). Hypoxia directly induced the expression of VEGF; (2) Stimulation of hypoxia activated c-Src, which increased the expression of VEGF; (3) During hypoxic induction, stability of VEGF was enhanced, and its half-life increased from 30-40 min to 6-8 h. This indicated that the increase in hypoxic-induced VEGF occurred on the transcriptional and post-transcriptional levels; (4) Mutations in the p53 gene cause the survival of hypoxic tumors; (5) Cui et al. reported that the expression of VEGF was increased, the microcirculatory disturbance was further aggravated, the crush of nerves was aggravated, and the dysfunction of neural metabolism and conduction occurred.[12] Li[27] found that when neurolemma was dissected during facial nerve decompression, the

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>NCV</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>45.9578 ± 1.9390</td>
</tr>
<tr>
<td>Saline</td>
<td>8</td>
<td>25.1211 ± 5.0592**</td>
</tr>
<tr>
<td>Alprostadil</td>
<td>8</td>
<td>38.08 ± 4.7166**±±</td>
</tr>
<tr>
<td>Saline + Ab</td>
<td>8</td>
<td>22.0938 ± 4.2217**±±</td>
</tr>
<tr>
<td>Alprostadil + Ab</td>
<td>8</td>
<td>22.9313 ± 4.7315**±±</td>
</tr>
</tbody>
</table>

Compared with the control group: * < 0.05**, ** 0.01; compared with the saline group: * < 0.05**, ** 0.01; compared with the Alprostadil group: * < 0.05**, ** 0.01, (Ab = VEGF-antibody)
oedematous nerves would immediately bulge, the blood vessels of the epineurium became dilate, the color of the nerves turned from pale to red, and facial movement would recover dramatically. The results of pathology, NCVs and toe spaces in this experiment indicated that alprostadil could reduce the pathological injury and improve the functional recovery after crushing injury to sciatic nerves.

This study indicated that when the expressions of VEGF and the number of the VEGF- plus VEGF receptor-positive DRG neurons increased with alprostadil treatment, the pathological lesion of DRGs was alleviated and the functional rehabilitation of sciatic nerves was accelerated. In addition, the benefit of alprostadil was decreased by application of VEGF-antibody. It was suggested that the overexpressations of VEGF was important in pathological and functional repair of crush injury to sciatic nerves by alprostadil treatment.

Conclusions

The results of this experiment indicate that the vasooactive agent alprostadil may reduce the pathological lesion of peripheral nerves and improve the rehabilitation of the neural function, which may relate to upregulation of the expression of VEGF, following crush injury to peripheral nerves.

Acknowledgment

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