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Original Article

Brain-derived neurotrophic factor is up-regulated in severe acute cauda equina syndrome dog model

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Abstract: To determine the level of brain-derived neurotrophic factor (BDNF) in experimental dog model of severe acute cauda equina syndrome, which was induced by multiple cauda equina constrictions throughout the entire lumbar (L), sacral (S) and coccygeal (Co) spinal cord and their central processes of the dorsal root ganglia neurons. Adult male mongrel dogs were randomly divided into 2 groups. The experiment group (n=4) was subjected to multiple cauda equina constrictions. The control group (n=4) was subjected to cauda equina exposure without constrictions. Level of BDNF in the spinal cord and the dorsal root ganglion cells (L_7, S_1-S_3) was assessed 48 hours after multiple constrictions by immunohistochemical and histopathological analyses. 48 hours after multiple constrictions of cauda equina, up-regulation of BDNF within lumbosacral (L_7-S_3) spinal cord and dorsal root ganglion was observed in experimental group as compared to control group. Our result suggests that BDNF might play a role in the inflammatory and neuropathic pain as a result of multiple cauda equina constrictions. Regulation of BDNF level could potentially provide a therapy for treating cauda equina syndrome.

Keywords: Cauda equina syndrome (CES), dorsal root ganglion (DRG), brain-derived neurotrophic factor (BDNF), multiple cauda equina constrictions (MCEC), neurotrophic factors (NFs)

Introduction

The cauda equina is often involved in the development and progression of several diseases, such as disc herniation, spinal stenosis, tumor and vertebral fracture (references). Cauda equina syndrome (CES) is a pathological condition caused by mechanical constriction of cauda equina, which results in changes to the intraradicular circulation [1] and nerve fiber dysfunction (reference). The mechanical constriction may also lead to a series of intraneural tissue reactions, including edema formation, demyelination, and fibrosis [2-4]. Fully developed CES is associated with sensory and motor disorders, which results in lower-back pain, saddle anesthesia, and motor weakness of lower extremities leading sometimes to paraplegia or bladder dysfunction [5-7].

Neurotrophic factors (NFs), which are important for the growth and survival of neurons during the development of nervous system, have also been shown to function in the transmission of physiological and pathological pain. BDNF, one of the NFs, is synthesized in the primary sensory neurons and then anterogradely transported to the central terminals of the primary afferents in the spinal dorsal horn, It has been shown to be involved in the modulation of pain stimuli [8]. BDNF is also an important survival factor for spinal motor neurons [9] and one of the most important drivers of neurite outgrowth [10]. BDNF, together with nerve growth factor (NGF), could stimulate the growth of axons and salvage the injured neurons of spinal cord. Recent study by Xu *et al* [11] has also demonstrated the neuroprotective effect of BDNF in the twy

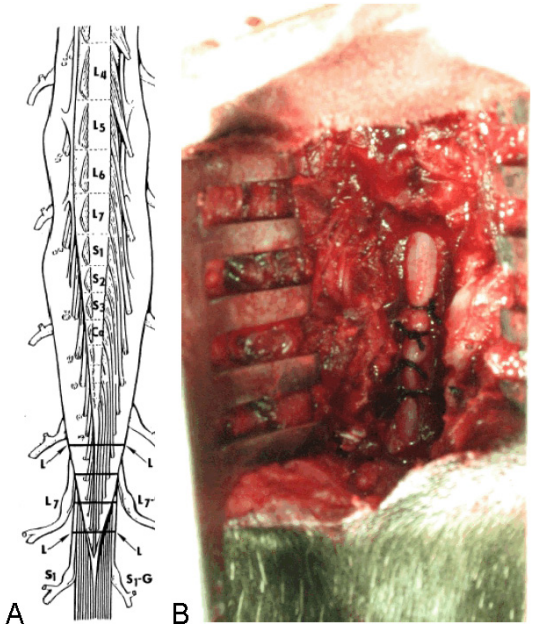

Figure 1. A: Schematic drawing depicting the position of four tightened constrictions (about 2.0 mm wide) around the cauda equina (L, L); L_7-G and S_1-G point to the corresponding dorsal root ganglia to produce acute and severe compression. The entire cauda equina was constricted by 50%-75% by the first tightened constriction (from the top), and the lower cauda equina was constricted by 25%-50% by the other three tightened constrictions. B: Dog model of multiple protracted cauda equina constrictions (MCEC) during surgery.

mouse with spontaneous chronic compression of the spinal cord motoneuron.

Clinical symptoms of CES are related to sustained stimulation of the cutaneous, muscular and visceral nociceptive afferents [5-7]. Thus, BDNF expression in the neurons of corresponding spinal cord segments could be expected as intrinsic spinal cord neurons are involved in the compression-induced CES including anterograde, retrograde and transneuronal degeneration in the lumbosacral segments [6].

Our current study is to investigate the role of BDNF in the development and progression of experimental model of CES by looking at the levels of BDNF after multiple cauda equina constrictions (MCEC) [12]. BDNF expression in the lower lumbosacral spinal cord segments and corresponding DRGs 48 hours following MCEC was upregulated, which might suggest a role in the neuroprotective processes.

Material and methods

Animals

A total of 8 adult mongrel male dogs, aged 18–48 months and weighing 10–15 kg, were purchased from the Institutional Animal Care and Use Committee of the Institute of Navy (license No. SYXK (Hu) 2007-0003). Animals were housed in individual runs, given free access to water and fed a dry certified canine diet. Animal room temperature and light cycle were controlled (targeted conditions: temperature range 18.3–25.5°C, 12-hour light/dark cycle). Humidity was not controlled but recorded regularly. Dogs were allowed to acclimate for a minimum of 7 days after receipt and conditioned to vests for 3 days prior to surgery. All protocols were conducted in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China [13].

Antibodies and reagents

Rabbit anti-BDNF antibody of low-density lipoprotein was from Boster Biotech Corp (Wuhan, Hubei Province, China).

Establishing CES model

Each animal received penicillin G procaine (20000 U/kg, i.m.) and atropine (0.04 mg/kg, i.m.) before operation. Dogs were anesthetized with parenteral solution of the Su-mian-xinII (0.08–0.10 mL/kg, i.m., made by Military Veterinarian Institute of Academy of Military Medical Sciences, Changchun, Jilin Province, China), endotracheally intubated and artificially ventilated on a respirator with halothane (1–2%) in a mixture of oxygen and nitrous oxide (1:1). Lumbar laminectomy was performed and the dural sac of the exposed cauda equina comprising dorsal and ventral roots of L_7, S_1–S_3, Co_1–Co_5 segments was tied by four loose 4-0 ligatures (Shanghai Jade Weaver Co., Ltd., Shanghai, China) with about 2 mm spacing, and the entire cauda equina was constricted by 50–75% by the first tightened constriction, and the lower cauda equina was constricted by 25–50% by the other three tightened constrictions (**Figure 1**). The central processes of the L_7-Co_5 DRG neurons were permanently constricted. Control dogs went through the cauda

equina exposure without ligation. 48 hours after the surgery, both groups of dogs, under anesthetization, were transcardially perfused through the heart with 2000 mL PBS followed by 2000 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The spinal cord and the corresponding DRGs (L_7, S_1-S_3) were removed and sliced into 5-μm transverse paraffin sections throughout the entire lumbar (L), sacral (S) spinal cord segments and DRGs.

Histopathology and morphometric evaluation

Samples taken from the level of spinal cord and the corresponding DRGs (L_7, S_1-S_3) were fixed in formalin an d embedded in paraffin. Transverse sections of paraffin block measuring 5 μm in thickness were stained with hematoxylin and eosin (H&E) and images were taken using light microscope. Morphology and density of neurons within spinal cord and DRG were assessed. To avoid examining the same neurons twice, we left more than 8 μm gap in-between sections.

Immunohistochemical staining

For cryoprotection, samples were immersed in PBS containing 30% sucrose for 24 hours at 4°C. Free floating sections were immediately sliced into 5-μm paraffin sections, deparaffinized and dehydrated, washed in distilled water and then PBS, followed by microwave repairing with citrate buffer solution (pH 6.0) for 3 minutes and 30 seconds, rinsing in the distilled water and PBS again after natural cooling. The specimens were exposed to 0.3% H_2O_2 in PBS for 10 minutes to inactivate endogenous peroxidase. Sections were subsequently incubated with a rabbit monoclonal anti-BDNF antibody of low-density lipoprotein (1:50; Boster Biotech Corp, Wuhan, Hubei Province, China) for 1 hour at 37°C. BDNF was immunostained using the ENVISION system (DAKO, Carpinteria, CA, USA) according to the manufacturer's instructions. Staining therefore continued by rinsing in the PBS, incubation with secondary sheep anti-rabbit IgG and labeled with horseradish peroxidase (1:250; Sigma, St. Louis, MO, USA). After washing in PBS, the sections were developed with 0.05% 3, 3' diaminobenzidine tetrahydrochloride (Sigma) and 0.006% H_2O_2 in PBS for 10 minutes. The slides were counterstained with Meyer's hematoxylin for 10 minutes, differentiated with 75% hydrochloric acid-alcohol for 30 seconds, and then the sections were washed, dried, dehydrated and mounted on neutral resin. Positive signal was located in the cytoplasm of neurons and gliocytes and stained with buffy [14] under light microscopy (Toshiba, Tokyo, Japan). BDNF expression was graded by a blinded observer and a semiquantitative score was used.

Results

MCEC

To establish experimental dog model of CES, we performed MCEC. (**Figure 1A**) shows the location of cauda equina along spinal cord. Four positions of cauda equina were tied with loose ligature and with 2 mm spacing (**Figure 1B**).

MCEC leads to severe acute CES in dog

To assess the effects of MCEC, we performed H&E stainings of the cauda equina from control and experimental dogs. Most of neurons in the spinal cord of the experimental group showed degeneration of nuclei, partial loss of perinuclear Nissl bodies, extensive infiltration of lymphocytes and presence of blood vessel mouthpiece (**Figure 2**). Similar changes were also observed in the DRG neurons (**Figure 3**). On the other hand, neurons in the spinal cord and DRG from control animals showed normal histology with intact nuclei, which were light colored and centrally located with distinct nucleoli (**Figures 2** and **3**).

BDNF expression was up-regulated following MCEC

To determine BDNF expression level in relation to CES, we examined BDNF stainings in the neurons of DRG 48 hours after MCEC, We observed extensive and different degree of BDNF expression in the neurons of the lumbosacral spinal cord and DRGs from experimental animals (**Figures 2** and **3**). BDNF expression was also clearly detectable in the satellite cells surrounding the DRG cells and neurons of spinal cord. However, no specific BDNF-positive neuronal cells could be detected in the DRGs or the spinal cord of control animals, although the

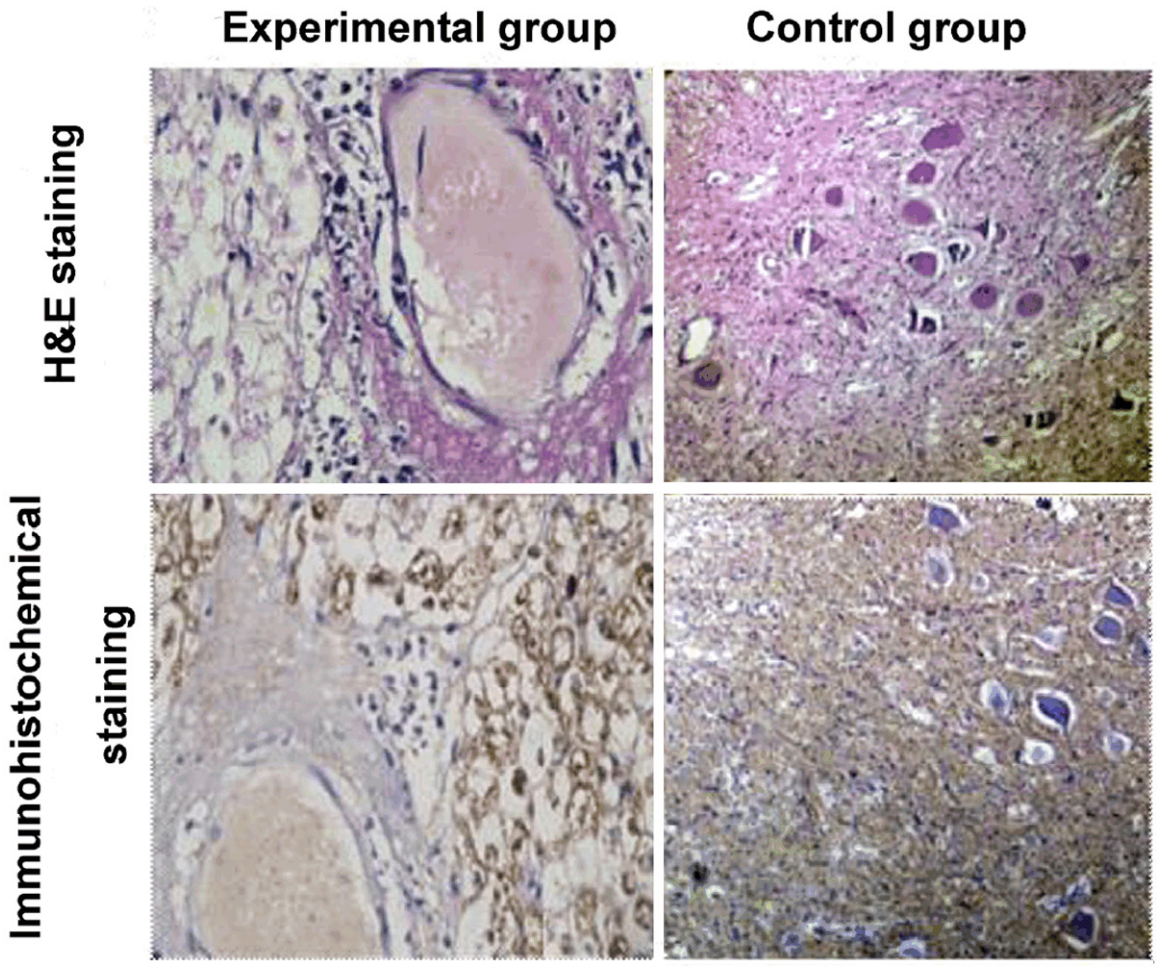

Figure 2. Morphology of the spinal cord sections at L_7 between experimental group and control group (hematoxylin-eosin staining and immunohistochemical staining, light microscope, × 100). Most neurons have degeneration of nucleus, partial loss of perinuclear Nissl bodies, extensive infiltration of generous lymphocytes and present of blood vessel mouthpiece of experimental group comparing with the control group. Note the different degree of BDNF expression in the endochylema of neurons of experimental group, while negative in the control group. There is an obviously increase of BDNF expression in the glial cells compared with the control group.

adjacent glia and nerve fibers of L_7 and sacral spinal cord and DRGs showed different degree of BDNF expression (**Figures 2** and **3**). BDNF expression in the neuronal population throughout the entire corresponding lumbosacral (L_7-S_3) spinal cord and DRG following MCEC was significantly higher than that in control animals (**Table 1**). In addition, we also observed some strong BDNF stainings in the spinal cord, nerve roots and DRG, which may represent activated glial cells.

Discussion

Several animal models mimicking the cauda equina syndrome have been used to study and explain the pathophysiology of the polyradicular symptomatology of the syndrome [6, 15]. A model of lumbar spinal stenosis in dogs [16] was developed consisting of the entire cauda equina constriction at the seventh lumbar level with a nylon electrical-cable tie, 2.8 mm wide, placed circumferentially around the dura and, after a laminectomy of the sixth and seventh lumbar vertebrae, the cauda equina was constricted by 25, 50, or 75% to produce chronic compression. While multiple protracted cauda equina constrictions (MCEC), characterized as a model of somatovisceral pain model in dogs [12, 14], is more comparable with pain models using peripheral nerve ligation. In MCEC, lumbar laminectomy of the sixth and seventh lami-

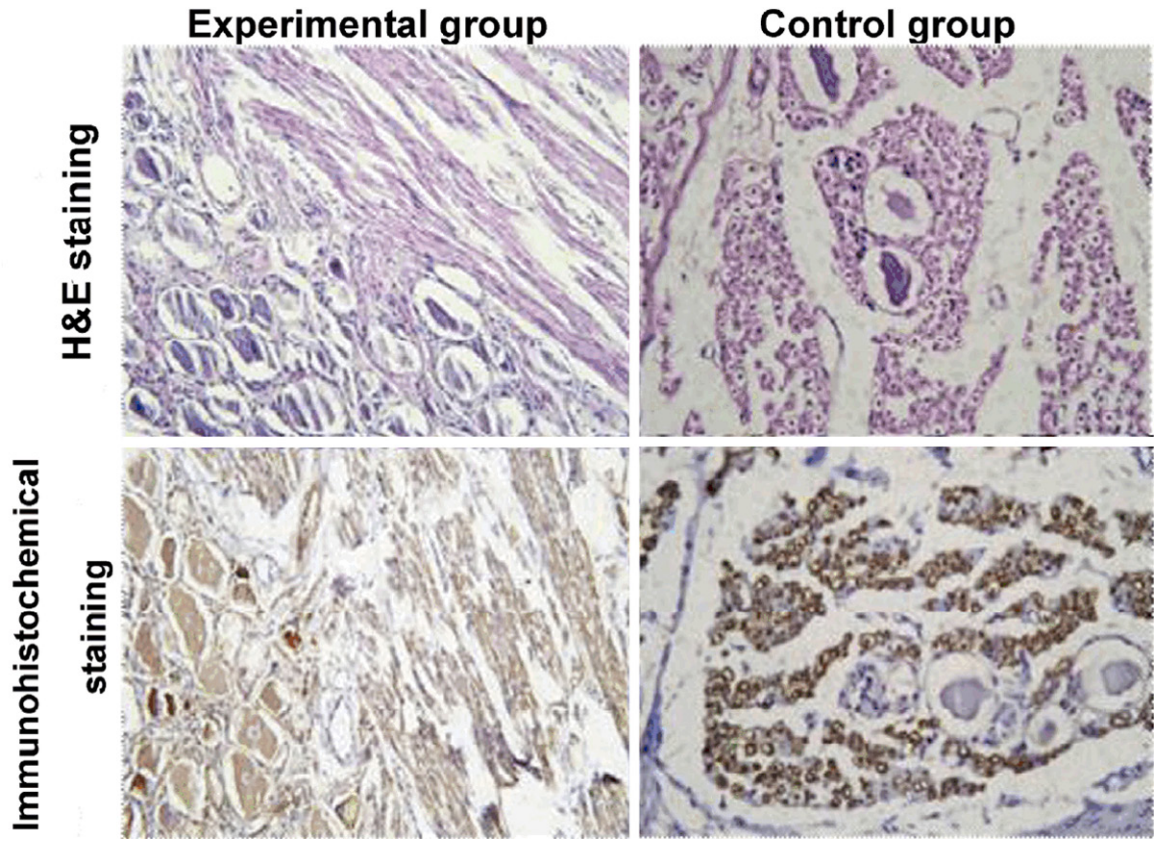

Figure 3. Morphology of dorsal root ganglion of L_7 level between experimental group and cortrol group (hematoxylin-eosin staining and immunohistochemical staining, light microscope, × 100). Most of the nucleus of sensory neurons in the experimental group have deformed and moved to the periphery of the cell, partial loss of perinuclear Nissl bodies and extensive infiltration of generous lymphocytes, and central chromatolysis had started comparing with the control group. There is an enhanced BDNF expression in the endochylema of sensory neurons in the experimental group, while negative in the control group.

Table 1. Semiquantitative BDNF expression in the neurons and glial cells of the spinal cord and dorsal root ganglion at L_7 level of each dog

BDNF expression		Dog 1	dog2	dog3	dog4
		Exp/contr	Exp/contr	Exp/contr	Exp/contr
spinal cord	Neurons	++/-	++/-	++/-	++/-
	glial cells	++/+	++/±	++/+	++/+
DRG	Neurons	++/-	++/-	++/-	++/-
	glial cells	++/++	++/+	++/++	++/++

Note: Exp/contr: dog from experiment group/dog from control group; -: no BDNF expression in the endochylema of neurons of spinal cord and DRG at the L_7 level; +-: low/intermediate BDNF expression in the glial cells of spinal cord at the L_7 level; +/++: high BDNF expression in the endochylema of neurons of spinal cord and DRG at the L_7 level.

nae is carried out thus gaining access to the cauda equina. Constrictions of the dural sac are produced by tying four loosely constrictive ligatures with about 2 mm spacing causing protracted constrictions of the central processes of the DRG cells of L_7, S_1-S_3, and Co_1-Co_5 segments along with the ventral roots of the same segments. In dog models of MCEC-induced CES, constrictions of entire cauda equina with different degrees can cause different neurological deficits, cortical evoked potentials and histological abnormalities [12, 14].

This study has proved that the cauda equina injury will be at a significantly greater ischemic risk from a multi-level compression than from a one-level or two-level lesion, just as this investigation have identified that cauda equina had significant disorders of blood circulation and cerebrospinal fluid circulation, and pathological changes of Wallerian degeneration and demyelination after MCEC in experimental dogs of control and experimental

groups. So we suggested that mechanical and vascular factors may participate in the development of the CES at least. In the present study, we successfully generated an experimental dog model of CES using MCEC. 48 hours after MCEC, dogs displayed typical pathological features of CES. And we observed up-regulation of BDNF expression in the endochylema of the lower lumbosacral spinal cord segments and corresponding DRGs neurons as a result of MCEC. Our studies shed light on the possible roles of BDNF in the neuroprotective processes by preventing the neuronal degeneration and in the development of persistent inflammatory and neuropathic pain of experimental dogs.

BDNF has been known to be one of the powerful survival factors for spinal motoneurons [9]. Also it has been well demonstrated, in rat acute and chronic spinal cord injury (SCI) model, that immediate administration of BDNF into the SCI site promotes significant rubrospinal axonal regeneration and prevents axotomy-induced atrophy and/or death of rubrospinal neurons [17]. Our current study demonstrated that BDNF expression in the spinal cord and DRG was up-regulated significantly 48 hours after MCEC as compared to normal control group. It is the possible that BDNF serves as one of the survival factors for various neurons during the early phase of severe compression. BDNF could potentially be used as therapy to prevent neuron degeneration during the pathophysiologic processes of CES.

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