Int J Clin Exp Med 2015;8(10):19635-19641
www.ijcem.com /ISSN:1940-5901/IJCEM0012175

Original Article

Effects of exogenous neurotrophin-3 on myocyte apoptosis and Ca^{2+}-ATP enzyme levels following nerve injury in rats

Yu-Zhen Dong, Lin Yang, Tan Lu, Hong-Xing Zhao, Chao Ma, Yi-Lei Zhao

Department of Orthopedics, The First Affiliated Hospital of Xinxiang Medical University, Weihui 453100, China

Received June 30, 2015; Accepted September 30, 2015; Epub October 15, 2015; Published October 30, 2015

Abstract: This study aims to determine the influence of neurotrophin-3 (NT-3) plasmids on neuronal apoptosis and Ca^{2+}-ATP enzyme levels in injured muscles. We also investigated the mechanism underlying the role of NT-3 in delaying muscle atrophy following a peripheral nerve injury. Sixty adult Wistar rats were used to generate the peripheral nerve injury models. The rats were randomly assigned to the saline and NT-3 groups. Related indicators, such as caspase-3 protein expression, skeletal muscle cell apoptosis, and Ca^{2+}-ATP enzyme expression were quantified. The expression levels of caspase-3 and the histone-muscle cell apoptosis rate in the NT-3 group decreased at different post-operative times following peripheral nerve injury, whereas NT-3 expression and the sarcoplasmic reticulum Ca^{2+}-ATP enzyme levels increased. Statistically significant differences were observed in the NT-3 group as compared to the saline group ($P < 0.05$). NT-3 mitigated muscle atrophy following peripheral nerve damage by inhibiting caspase-3 gene expression and increasing Ca^{2+}-ATP enzymatic activity, ultimately reducing muscle apoptosis.

Keywords: Neurotrophin 3, mechanism, muscle cell apoptosis, Caspase-3, peripheral nerve injury

Introduction

Skeletal muscle suffers from muscle atrophy following peripheral nerve injury due to a loss of nutritional support and the wasting of nerves, which severely affects the recovery of limb function [1]. Previous experimental studies have demonstrated that passive exercise, medication, and sensory neuron implantation, amongst other methods, can prevent skeletal muscle denervation atrophy to varying degrees [2-4]. However, the clinical application of these techniques remains limited. Muscle apoptosis is a major cause of irreversible muscle denervation atrophy. Apoptosis can be inhibited and muscle atrophy can be mitigated by early intervention or effective treatment before neurons begin to recover, which is essential in treating peripheral nerve injuries [5, 6].

Muscle apoptosis is an irreversible programmed cell death regulated by intracellular calcium overload, free radicals, microcirculation obstruction, as well as other factors. The expression of apoptosis-related genes such as caspase, Bcl-2, and p53 can also induce muscle apoptosis [7, 8]. Many studies have demonstrated that the secretion of neurotrophic factors is reduced following peripheral nerve damage, and that exogenous neurotrophic factor 3 (neurotrophin 3, NT-3) promotes nerve regeneration and protects against further muscle atrophy [9, 10]. However, the mechanism underlying these actions of NT-3 remains unclear. Therefore, we analyzed the ability of NT-3 to treat rats given sciatic nerve injuries as a model of peripheral nerve damage. We then analyzed the protective mechanisms of NT-3 on nerve injury repair and on the prevention of muscle atrophy. We evaluated the levels of factors associated with atrophy, such as caspase-3 protein expression, muscle cell apoptosis, and Ca^{2+}-ATP enzyme activity and analyzed the overall effects of NT-3 on muscle recovery.

Materials and methods

Animal model preparation and grouping

Sixty healthy Wistar-Kyoto rats of both sexes were obtained from the Experimental Animal Centre of Xinxiang Medical University, with ap-

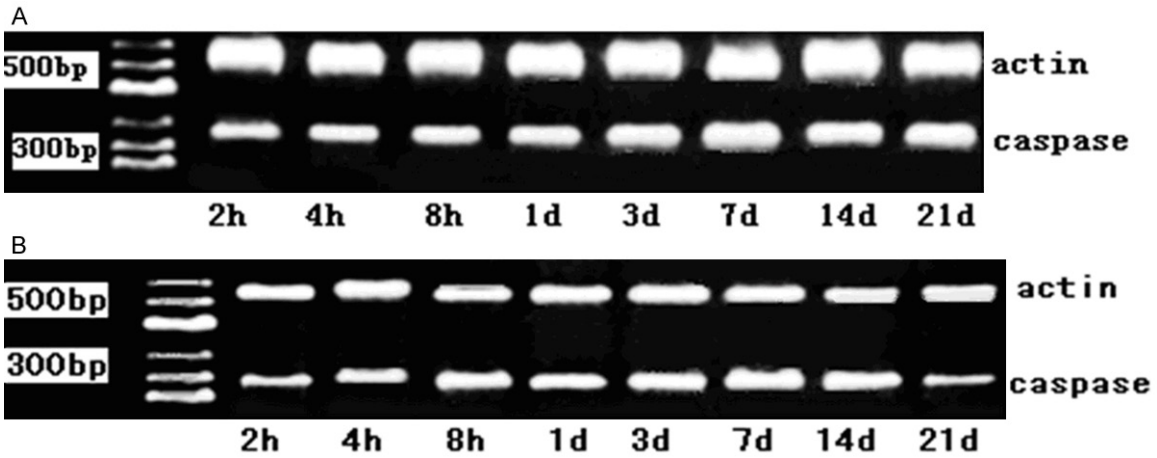

Figure 1. Caspase-3 expression. A. The normal saline control group. B. The NT-3 group.

proximate weights of 200 g to 250 g, using the animal license number of SCXK (Henan) 2008-0001. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Xinxiang Medical University. The rats were deeply anaesthetized with an intra-peritoneal injection of 2% sodium pentobarbital (30 mg/kg). Sciatic nerves were exposed under a dissecting microscope at 16 × magnification, and a 2-cm region of the sciatic nerve was detached and then sutured to the proximal back muscles. The distal ends of the sciatic nerve were ligated with "10-0" microscopic sutures. These rats were used as experimental models of sciatic nerve injury. After randomizing the groups, saline and 2 μL of the NT-3 plasmid (1 μg/μL) solutions were injected into the gastrocnemius using a micro-syringe. NT-3 plasmid DNA was synthesized and purified by the Beijing Parkson Biological Engineering Co., Ltd. The injection needles were held in place for 2 min following the injection. All animals were singly housed separately following the operation.

Expression of caspase-3

Total RNA from the gastrocnemius was extracted under anesthesia at 8 distinct post-operative time points (2, 4, 8 h and 1, 3, 7, 14 and 21 d) using a cell protein extraction kit (Sigma, USA). The target sequences were amplified using a polymerase chain reaction (PCR) system and the resulting amplicons were separated using gel electrophoresis. Caspase-3 was cloned using the following primers: upstream 5'-AAGAAGACCATAGCAAAAGGA-3' and downstream 5'-CACAAAGTGACTGGATGAACC-3'. β-actin was cloned using the following primers: upstream 5'-CCAAGGCCAACCGCGAGAAGATGAC and downstream: 5'-AGGGTACATGGTGGTGCCGCCAGAC-3'.

The gastrocnemius muscle was embedded in paraffin and sectioned. A caspase-3 antibody was used for immunostaining, and the tissue was mounted and visualized with a light microscope. First, 50 μL of 10% goat serum (25°C) was applied to each slide for 30 min. Next, 50 μL of a solution containing a rabbit caspase-3 polyclonal antibody (concentration, 1:200) was treated overnight at 4°C and 50 μL of a biotinylated secondary antibody (concentration, 1:150) was applied to the slides for 20 min at 25°C. Next, 50 μL of a solution containing horseradish peroxidase (HRP) conjugated to streptavidin was applied to the slides for 10 min at 25°C. A colorimetric reaction using diaminobenzidine as the HRP substrate identified caspase-3. A hematoxylin solution was applied to the tissue to identify the nuclei and caspase-3 staining appeared brown in color. Caspase-3 protein expression in the negative control was determined with TJTY-300 image analysis software.

Detection of gastrocnemius apoptosis

Denervated gastrocnemius specimens were collected from each group at post-operative weeks 1, 4, and 8. The tissue was embedded in paraffin and sectioned at a thickness of 5 μm. A fluorescent TUNEL assay was used to detect

Table 1. Comparison of caspase-3 protein expression content in the two groups after transfection ($\bar{x}$ ± s)

Group	2 h	4 h	8 h	1 d	7 d	14 d	21 d
Control	0.427 ± 0.031	0.514 ± 0.036	0.574 ± 0.039	0.636 ± 0.045	0.712 ± 0.051	0.830 ± 0.069	0.941 ± 0.0075
NT-3	0.438 ± 0.030	0.473 ± 0.034*	0.506 ± 0.037*	0.540 ± 0.041*	0.608 ± 0.0046*	0.581 ± 0.047**	0.460 ± 0.037**

*Compared with control group, $P < 0.05$, **$P < 0.01$; Caspase-3 protein expression in the two groups began to rise 2 h after injury, the difference was not statistically significant ($P > 0.05$). The growth rose with time and gradually decreased 7 d after operation. Caspase-3 protein expression of the NT-3 group had statistically significant difference in 4th day after operation compared with that of the control group ($P < 0.05$).

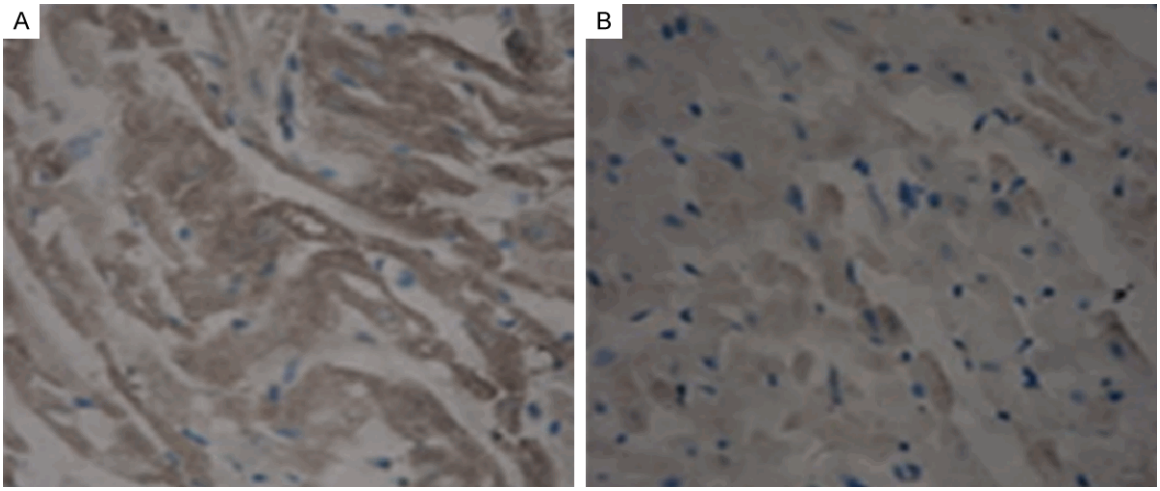

Figure 2. Caspase-3 expression observation in day 21 (× 200). A. The control group; B. The NT-3 group.

Table 2. Comparison of Ca^{2+}-ATP enzyme test in muscle cells of the two groups after denervation ($\bar{x}$ ± s)

Group	1 w	2 w	4 w	6 w	8 w
Control	36.26 ± 0.35	37.54 ± 0.30	22.01 ± 0.24	19.69 ± 0.20	13.85 ± 0.14
NT-3	35.72 ± 0.31	38.39 ± 0.32	40.16 ± 0.43*	43.69 ±0.45*	41.54 ± 0.40**

*Compared with control group, $P < 0.05$, **$P < 0.01$; Ca^{2+}-ATP enzyme levels of the 1 and 2 week control group had no significant difference compared with that of the NT-3 group, while that of the 4, 6 and 8 week NT-3 group increased significantly compared with that of the NS control group, the results of the two groups had statistically significant difference ($P < 0.05$).

cell apoptosis in accordance with the instructions supplied with the TUNEL in situ apoptosis detection kit (Boster Biological Engineering Co., Ltd., Wuhan). Positively labeled nuclei appeared brownish yellow. Ten non-repeating field-of-view images were selected from each slice and the number of positive cells was counted in each image, and the rate of apoptosis was then calculated with the following formula: Apoptosis rate = positive nuclei/total number of nuclei × 100%. The average was obtained for each condition.

Western blot

Similar amounts of protein from the gastrocnemius muscle samples were harvested 1, 2, 4, 6, and 8 weeks after operation and separated with gel electrophoresis (Sigma, USA). After running the proteins on a polyacrylamide gel, the proteins were transferred to a membrane. The blot was blocked with 25 mL of blocking solution for 1 h at room temperature. After blocking, the primary antibody was added and incubated with the blotfor 3 h. Subsequently, horseradish peroxidase-conjugated secondary antibody was added, tableted, and punched. The expression of Ca^{2+}-ATP enzyme was detected using the chemical system.

Statistical analysis

SPSS 13.0 software was used for statistical analysis. Results are expressed as the mean ± standard deviation ($\bar{x}$ ± s). Mean values between the two experimental groups were compared using a two-sample t-test. $P < 0.05$ was considered a statistically significant difference.

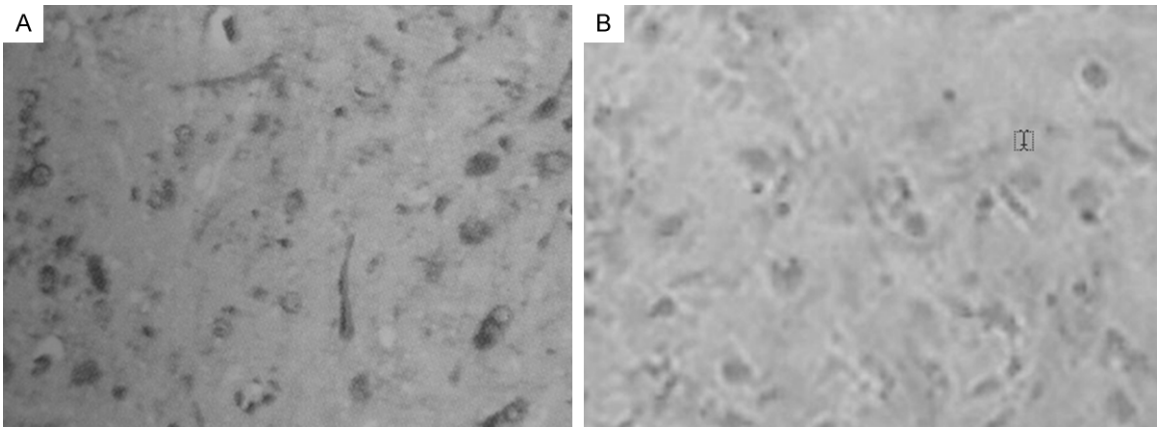

Figure 3. TUNEL muscle cell apoptosis observation in the 8 week (× 400). A. The control group; B. The NT-3 group.

Results

Caspase-3 gene expression

The expression changes in the products through caspase-3 gene amplification were subjected to agarose gel electrophoresis. The products inserted by the NT-3 gene fragments were obtained from the hardened gel and the recovery product sequences were verified by restriction enzyme digestion, showing clear bands from each target gene and an internal reference standard (**Figure 1**).

Caspase-3 protein expression

Caspase-3 protein expression increased 2 h following the injury with no significant difference observed between the control and experimental groups ($P > 0.05$). Expression of caspase-3 gradually increased over time. However, the rate of increase of caspase-3 expression gradually began to decline 7 d post-operation. Caspase-3 protein expression in the NT-3 group was significantly lower than that in the control group 4 h after the operation ($P < 0.05$) (**Table 1**; **Figure 2**).

Apoptosis rate

Muscle cell apoptosis was observed one week following treatment in both groups with no significant differences between the groups ($P > 0.05$). Apoptosis significantly increased in the control group four and eight weeks post-operation as compared to the experimental group. The cells appeared to shrink and had an irregular shape, and the nuclear membrane appeared wrinkled when viewed under high magnification. Brown particles were also observed in the nucleus. Meanwhile, the positive cells from the NT-3 group were significantly reduced (**Table 2**; **Figure 3**). Gastrocnemius apoptosis rates in the two groups at 4 and 8 weeks post-operation were 32.09 ± 1.02% and 19.84 ± 0.63%, and 41.67 ± 1.48% and 13.83 ± 0.25%, respectively. The means between groups were statistically different ($P < 0.05$).

Gastrocnemius Ca^{2+}-ATP enzyme changes

Gastrocnemius Ca^{2+}-ATP enzyme expression was analyzed in the two groups at postoperative weeks 1, 2, 4, 6 and 8 following peripheral nerve injury, and the findings are shown in **Table 2**. Ca^{2+}-ATP enzyme levels in the 1- and 2-week control groups were not entirely different from those in the NT-3 group. After 4, 6, and 8 weeks, enzyme expression in the NT-3 group increased significantly as compared to that in the NS group, and the differences between these two groups was statistically significant ($P < 0.05$).

Discussion

Skeletal muscle loss affects nerve physiology and control following a peripheral nerve injury, resulting in muscle atrophy due to the absence of necessary neurotrophic factors in the target cells. Many experts found that NT-3 levels were reduced within 6 to 12 h of peripheral nerve damage. After two weeks, the NT-3 group exhibited NT-3 levels eightfold higher than those observed in the control group. The administration of exogenous NT-3 treatment was found to promote regrowth of axons and myelin, maintaining neuron populations in the muscle spin-

dles, tendon and skin, as well as promoting nerve-muscle synapse maturation, and significantly delaying the effects of skeletal muscle atrophy following nerve injury [11, 12]. However, the exact mechanism of action remains unclear because of the relative lack of research on NT-3 treatments.

Experimental results indicated that Trk C is an NT-3-specific receptor that can express its own signaling molecules such as neuronal calcium channel and neurotransmitter receptor and regulate its functional state by activating a series of signal transduction pathways, promoting the function of Ca^{2+}-ATP enzymes and preventing denervated skeletal muscle atrophy [13, 14]. The Ca^{2+}-ATP enzyme is the major protein involved in the regulation of Ca^{2+}, which causes the sarcoplasmic reticulum to sequester Ca^{2+} away from the cytoplasm, thus forming the basis of skeletal muscle contraction and relaxation [15]. Peripheral nerve damage induces energy deficiencies due to reductions in the Ca^{2+}-ATP enzyme levels in skeletal muscle, decreasing the absorption capacity of Ca^{2+}. Moreover, this causes intracellular and mitochondrial Ca^{2+} to accumulate, resulting in decreased contractility of the skeletal muscle, but the exact mechanism remains unclear [16].

This study showed that gastrocnemius Ca^{2+}-ATP enzyme levels decreased significantly within one week following denervation. In addition, we found that the sarcoplasmic Ca^{2+}-ATP enzyme level increased significantly in the NT-3 treatment groups, resulting in a significant increase in muscle contractile function following denervation as compared to that in the control group. NT-3 mRNA was downregulated in denervated skeletal muscle. NT-3 was found to enhance the neuromuscular synapse formation, promoting neuromuscular synapse development and regeneration through a mixed culture after NT-3 was locally injected into muscle cells *in vitro*, thus preventing the effects of muscle atrophy [17]. NT-3 is abundant in skeletal muscles. Local injections of NT-3 constructs can enhance RNA expression in advance of denervation, stimulate myoblast proliferation, promote the fusing of muscle tubes to into muscle fibers, and accelerate the skeletal muscle repair process [18].

This study showed increases in caspase-3 expression involved in the regulation of apoptosis following spinal cord injury. Caspase-3 is the most abundant protease involved in apoptosis. NT-3 may act by inhibiting the transcription of the caspase-3 gene, reducing pro-apoptotic factors and subsequently inhibiting neuronal apoptosis [19, 20]. However, several studies have found many other apoptosis proteins, such as BCL-2 family and P53 protein [21, 22]. This experiment demonstrated that caspase-3 gene transcription and protein expression levels begin to increase 2 h following nerve injury and peaked at 7 d. However, caspase-3 gene transcription in the NT-3 group was significantly lower at 4 h, whereas caspase-3 levels in the control group were consistently higher. The difference in caspase-3 levels between the two groups was statistically significant ($P < 0.05$). After application of exogenous NT-3 for 1 week, there was no apparent muscle cell apoptosis occurring in either the control group or NT-3 experimental group, whereas the gastrocnemius apoptosis rates at the 4- and 8-week time points were significantly different ($P < 0.05$).

In this study, we found that caspase-3 gene expression was higher, anti-apoptotic factors were reduced, and Ca^{2+}-ATP enzyme activity decreased following nerve injury, which was the major cause of the reduction in muscle cell apoptosis. Caspase-3 mRNA and protein expression was reduced by NT-3 exogenous intervention, which increased the proliferative capacity of skeletal muscle cells. Subsequently, Ca^{2+}-ATP enzyme activity increased, promoting skeletal muscle contractions, reducing pro-apoptotic factors, and inhibiting muscle apoptosis. As a result, muscle atrophy was inhibited following denervation, which promoted nerve regeneration, providing a theoretical basis for repair in the brain, spinal cord, and peripheral nerves following injury. However, despite sufficient exogenous NT-3 treatment, muscle atrophy still occurred. Thus, a lack of neurotrophic factors was not the only cause of muscle atrophy. Future studies will look for alternative causes and mechanisms underlying the observed muscle atrophy.

Acknowledgements

This study was supported by Science and Technology Innovation Grant in 2010 from Department of Education of Henan Province (2010HASTIT036), Scientific Research Fund of Xinxiang Medical University, No. 2013ZD120, China.

Disclosure of conflict of interest

None.

Address correspondence to: Yu-Zhen Dong, Department of Orthopedics, The First Affiliated Hospital of Xinxiang Medical University, No. 88 Jiankang Road, Weihui 453100, China. Tel: +86 373 3831349; E-mail: yuzhendongcnx@126.com

References

[1] Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C and Rando TA. Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. Science 2007; 317: 807-810.

[2] Song W, Kwak HB and Lawler JM. Exercise training attenuates age-induced changes in apoptotic signaling in rat skeletal muscle. Antioxid Redox Signal 2006; 8: 517-528.

[3] Nierobisz LS, Cheatham B, Buehrer BM and Sexton JZ. High-content screening of human primary muscle satellite cells for new therapies for muscular atrophy/dystrophy. Curr Chem Genomics Transl Med 2013; 7: 21-29.

[4] Bennett BT, Mohamed JS and Alway SE. Effects of resveratrol on the recovery of muscle mass following disuse in the plantaris muscle of aged rats. PLoS One 2013; 8: e83518.

[5] Kato K, Yamanouchi D, Esbona K, Kamiya K, Zhang F, Kent KC and Liu B. Caspase-mediated protein kinase C cleavage is necessary for apoptosis of vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 2009; 297: 2253-2261.

[6] Sishi B, Loos B, Ellis B, Smith W, du Toit EF and Engelbrecht AM. Diet-induced obesity alters signalling pathways and induces atrophy and apoptosis in skeletal muscle in a prediabetic rat model. Exp Physiol 2011; 96: 179-193.

[7] Chen Q, Zhou L and Shine HD. Expression of neurotrophin-3 promotes axonal plasticity in the acute but not chronic injured spinal cord. J Neurotrauma 2006; 23: 1254-1260.

[8] Mendell JR, Rodino-Klapac L, Sahenk Z, Malik V, Kaspar BK, Walker CM and Clark KR. Gene therapy for muscular dystrophy: lessons learned and path forward. Neurosci Lett 2012; 527: 90-99.

[9] Hagg T, Baker KA, Emsley JG and Tetzlaff W. Prolonged local neurotrophin-3 infusion reducs ipsilateral collateral sprouting of spared corticospinal axons in adult rats. Neuroscience 2005; 130: 875-887.

[10] Zhang W, Miao Y, Xing Z, Li H, Liu H and Li Z. Growth-associated protein-43 expression in cocultures of dorsal root ganglion neurons and skeletal muscle cells with different neurotrophins. Muscle Nerve 2013; 47: 909-915.

[11] Kolpakov MA, Seqqat R, Rafiq K, Xi H, Margulies KB, Libonati JR, Powel P, Houser SR, Dell'italia LJ and Sabri A. Pleiotropic effects of neutrophils on myocyte apoptosis and left ventricular remodeling during early volume overload. J Mol Cell Cardiol 2009; 47: 634-645.

[12] Kulakowski SA, Parker SD and Personius KE. Reduced TrkB expression results in precocious age-like changes in neuromuscular structure, neurotransmission, and muscle function. J Appl Physiol 2011; 111: 844-852.

[13] Lin S, Xu J, Hu S, Xu L, Zhang C, Wang Y and Gu Y. Combined application of neutrophin-3 gene and neural stem cells is ameliorative to delay of denervated skeletal muscular atrophy after tibial nerve transection in rats. Cell Transplant 2011; 20: 381-390.

[14] Hsieh JH, Lin WM, Chiang H, Chang LY, Wu CT, Pu CM, Wu JT and Hsieh ST. Patterns of target tissue reinnervation and trophic factor expression after nerve grafting. Plast Reconstr Surg 2013; 131: 989-1000.

[15] Wang W, Zhang H, Gao H, Kubo H, Berretta RM, Chen X and Houser SR. β1-Adrenergic receptor activation induces mouse cardiac myocyte death through both L-type calcium channel-dependent and -independent pathways. Am J Physiol Heart Circ Physiol 2010; 299: 322-331.

[16] Suzuki S, Yokoyama U, Abe T, Kiyonari H, Yamashita N, Kato Y, Kurotani R, Sato M, Okumura S and Ishikawa Y. Differential roles of epac in regulating cell death in neuronal and myocardial. Cells J Biol Chem 2010; 285: 24248-24259.

[17] Findlay AR, Lewis S, Sahenk Z and Flanigan KM. Camptocormia as a late presentation in a manifesting carrier of duchenne muscular dystrophy. Muscle Nerve 2013; 47: 124-127.

[18] Sahenk Z, Galloway G, Clark KR, Malik V, Rodino-Klapac LR, Kaspar BK, Chen L, Braganza C, Montgomery C and Mendell JR. AAV1.NT-3 Gene Therapy for Charcot-Marie-Tooth Neuropathy. Mol Ther 2014; 22: 511-521.

[19] Tzeng TT, Tsay HJ, Chang L, Hsu CL, Lai TH, Huang FL and Shiao YJ. Caspase 3 involves in neuroplasticity, microglial activation and neurogenesis in the mice hippocampus after intracerebral injection of kainic acid. J Biomed Sci 2013; 20: 90.

[20] Zhu S, Nagashima M, Khan MA, Yasuhara S, Kaneki M and Martyn JA. Lack of caspase-3 attenuates immobilization-induced muscle atrophy and loss of tension generation along with mitigation of apoptosis and inflammation. Muscle Nerve 2013; 47: 711-721.

[21] Momeni HR, Soleimani Mehranjani M, Shariatzadeh MA and Haddadi M. Caspase-mediated apoptosis in sensory neurons of cultured dor-

sal root Ganglia in adult mouse. Cell J 2013; 15: 212-217.
[22] Cheng Y, Cawley NX and Loh YP. Carboxypeptidase E/NFα1: a new neurotrophic factor against oxidative stress-induced apoptotic cell death mediated by ERK and PI3-K/AKT pathways. PLoS One 2013; 8: e71578.