UDC 612.748:612.018.2

Yu. DONG[1], H. ZHAO[1], L. YANG[1], Yi. ZHAO[1], Ch. MA[1], and Ch. ZHANG[1]

# EFFECTS OF NEUROTROPHIN-3 PLASMIDS ON MYOCYTE APOPTOSIS AND $CA^{2+}$-ATPase CONTENT IN THE MUSCLE AFTER NERVE INJURY IN RATS

Received April 25, 2014

We estimated the influence of plasmids with DNA carrying the neurotrophin-3 (NT-3) gene on apoptosis in the gastrocnemius muscle and content of $Ca^{2+}$-ATPase in the latter after sciatic nerve injury. Sixty adult Wistar rats were randomly divided into the saline (control) and NT-3 groups. The related indices, such as expression of caspase-3 protein, the rate of apoptosis in the muscle evaluated using a TUNEL technique, and the level of $Ca^{2+}$-ATPase estimated using Western blot, were observed. Expression of caspase-3 protein was elevated at different post-operative times after peripheral nerve injury; NT-3 expression and the rate of muscle cell apoptosis decreased, whereas the level of $Ca^{2+}$-ATPase in the sarcoplasmic reticulum increased; significant differences were observed compared with the saline group ($P < 0.05$). The mitigation mechanism of NT-3 on muscle atrophy after peripheral nerve injury is expressed as inhibition of caspase-3 gene expression, increase in the $Ca^{2+}$-ATPase level, and reduction in the rate of muscle apoptosis.

**Keywords: neurotrophin-3, peripheral nerve injury, muscle cell apoptosis, caspase-3, $Ca^{2+}$-ATPase.**

## INTRODUCTION

Skeletal muscles suffer from muscle atrophy after peripheral nerve injury because loss of the nutritional support and wasting of the nerves, which severely affect the recovery of the limb function [1]. Previous experimental studies showed that passive exercise, medication, and implantation of sensory neurons, as well as some other measures, can prevent skeletal muscle denervation-related atrophy to a considerable extent; however, the experience of the respective clinical applications is limited [2-4]. Muscle apoptosis is a major cause of irreversible denervation-related atrophy. Apoptosis can be inhibited and muscle atrophy can be mitigated by early intervention or effective treatments before neurological recovery; this is a central approach in treating the results of peripheral nerve injury [5, 6].

Muscle apoptosis is irreversible programmed cell death induced by intracellular calcium overloading, excess of free radicals, microcirculation obstruction, and some other reasons. Moreover, apoptosis-related expression of protein products of a few genes, such as caspase, Bcl-2, and p53, also intensifies muscle apoptosis [7, 8].

[1] First Affiliated Hospital of the Xinxiang Medical University, Weihui, China. Correspondence should be addressed to Yu. Dong (e-mail: yuzhendongcnx@126.com).

Many experimental results showed that the secretion of neurotrophic factors is reduced after peripheral nerve injury, and an exogenous neurotrophic factor 3 (neurotrophin 3, NT-3) promotes nerve regeneration and protects against muscle atrophy [9, 10]. However, the mechanism of such effects remains unclear. Therefore, we used NT-3 in the experiment to treat rats in a sciatic nerve injury model. The respective indicators, such as caspase-3 expression, rate of muscle cell apoptosis, and $Ca^{2+}$-ATPase level acting on excitation-contraction coupling, were observed. The protective mechanism of the effects of NT-3 on the nerve post injury repairment and on the prevention of muscle atrophy has been discussed.

## METHODS

**Animal Model and Grouping.** Sixty healthy Wistar rats provided by the Experimental Animal Center of the Xinxiang Medical University were used. These were males and females, with the body mass of 200 to 250 g (animal license number 2008-0001 of SCXK, Henan). The animals were anaesthetized by i.p. injection of 2% sodium pentobarbital (30 mg/kg). The sciatic nerves were exposed under an operation microscope, cut off at 2 cm from the sciatic nerve exit, and sutured

into the surrounding muscles around the proximal back. Distal ends of the nerves were ligated with a 10-0 microscopic suture. After random grouping, saline or a solution containing 2 μl of plasmid DNA carrying gene *NT-3* (concentration 1 μg/μl) were injected into the *m. gastrocnemius* using a microsyringe (plasmid DNA was synthesized and purified by the Beijing Parkson Biological Engineering, China). The injection needles were held for 2 min. All animals were routinely reared separately after the operation.

**Expression of Caspase-3.** Gastrocnemius total RNA was extracted under anaesthesia at post-operative times of 2, 4, or 8 h and 1, 3, 7, 14, or 21 days (d), using the cell protein extraction kit (Sigma, USA), and then amplified by a PCR reaction system for gel electrophoresis. Caspase-3 primer sequences (a caspase-3 upstream primer, 5'-AA G AA G ACC A TA GCA AAA GGA, and a downstream G-3' primer, 5'-CAC AAA GTG ACT GGA TGA ACC-3', an internal reference β-actin upstream primer, 5'-CCA A GG CCA ACCGCG AG-AA GA TG AC-3', and a downstream primer, 5'-A GGGTA CA T GGT GGT GCC GCC A GA C-3'), were used. The *gastrocnemius* muscle was paraffin-embedded and sectioned. Caspase-3 antibody was used for staining; slices were mounted and observed under a light microscope. Then, the slices were treated with 50 μl of 10% goat serum (25°C) for 30 min, with 50 μl of caspase-3 rabbit polyclonal antibody (concentration 1:200) overnight at 4°C, and with 50 μl of secondary antibody (concentration of 1:150) for 20 min at 25°C. This was followed by incubation with 50 μl of HRP-labelled streptavidin for 10 min (25°C). The slices were diaminobenzidine-coloured and hematoxylin-restained; the cyto-immunoplasm of positive units appeared brown-coloured. The caspase-3 protein expression was determined by TJTY-300 Image Analysis software.

**Detection of Apoptosis in the *M. Gastrocnemius*.** Denervated *gastrocnemius* specimens were collected from each group, and paraffin 5-μm-thick sections were prepared at post-operative 1, 4, and 8 weeks. Fluorescent TUNEL assay was used to detect cell apoptosis, in strict accordance with the instructions of the TUNEL *in situ* apoptosis detection kit (Boster Biological Engineering, China). The labelled positive nuclei were brownish-yellow. The 10 selected non-repeated fields of vision were obtained from each slice to count positive cells, and the rate of apoptosis (positive nuclei/total nuclei number · 100%) was calculated. Average values were obtained for each calculation.

**Western Blot.** Similar amounts of protein in the *gastrocnemius* muscle samples were obtained 1, 2, 4, 6, and 8 weeks after operation using gel electrophoresis (Sigma, USA). The proteins were closed using 25 ml of blocking solution for 1 h at room temperature. After the film was transferred, the first antibody was added (incubation for 3 h). Subsequently, horseradish peroxidase-labelled secondary antibody was added, tableted, and punched. The expression of $Ca^{2+}$-ATPase was detected using the corresponding chemical system.

**Statistical Analysis.** SPSS 13.0 software was used for statistical analysis. Results are expressed below as means ± s.d. Mean values were compared between the two experimental groups using the two-sample *t*-test. Differences with $P < 0.05$ were considered statistically significant.

## RESULTS

**Caspase-3 Gene Expression.** The expression changes after amplification of the products of caspase-3 gene were subjected to agarose gel electrophoresis. The products inserted by *NT-3* gene fragments were recycled from hard gel, and the recovery product sequence was verified by restriction of enzyme digestion, showing clear bands of each target gene and internal reference standard (Fig. 1).

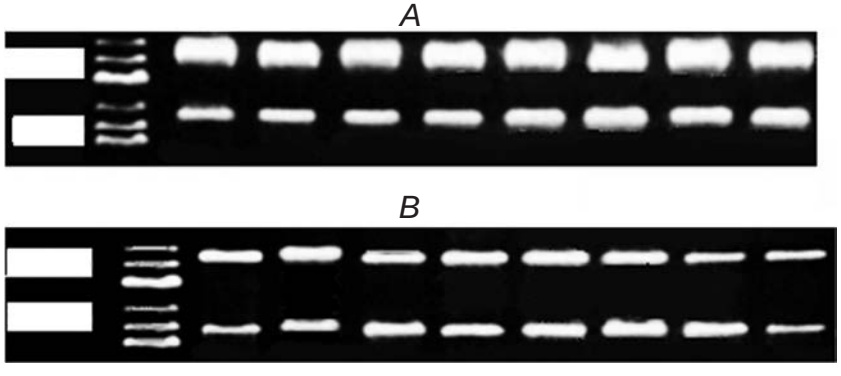


**Fig. 1.** Caspase-3 expression in the saline control group (A) and NT-3 group (B).

**Рис. 1.** Експресія каспази-3 у контрольній групі (*A*) та групі NT-3 (*B*).

Caspase-3 protein expression somewhat increased 2 h after injury, but with no significant difference between the control and experimental groups ($P > 0.05$). The expression continuously increased over time. Meanwhile, the growth rate gradually declined at post-operation d7. From h4, caspase-protein expression in the NT-3 group became significantly lower ($P < 0.05$) than that in the control group (Table 1; Fig. 2).

**Apoptosis Rate.** Muscle cell apoptosis was observed after one week in both groups with no significant intergroup difference ($P > 0.05$). The intensity of apoptosis significantly increased in the control group in 4 and 8 weeks post-operation. The irregular-shape cells appeared shrinked and exhibited a wrinkled nuclear membrane. Brown particles were also observed in the nucleus. Meanwhile, the numbers of positive cells in the NT-3 group were significantly smaller than in the control (Fig. 3). The *gastrocnemius* apoptosis rates in the two groups 4 and 8 weeks post-operation were 32.09 ± 1.02 vs. 19.84 ± 0.63% and 41.67 ± 1.48 vs. 13.83 ± 0.25%, respectively. The means within the groups differed from each other statistically significantly ($P < 0.05$).

**Changes in the $Ca^{2+}$-ATPase Content in the *M. Gastrocnemius*.** $Ca^{2+}$-ATPase expression in the muscle detected in the two groups was measured at postoperative weeks 1, 2, 4, 6, and 8 after peripheral nerve injury. The $Ca^{2+}$-ATPase levels in the control group at weeks 1 and 2 did not significantly differ from those in the NT-3 group. After 4, 6 and 8 weeks, the enzyme expression in the NT-3 group was significantly greater compared with that in the control group ($P < 0.05$).

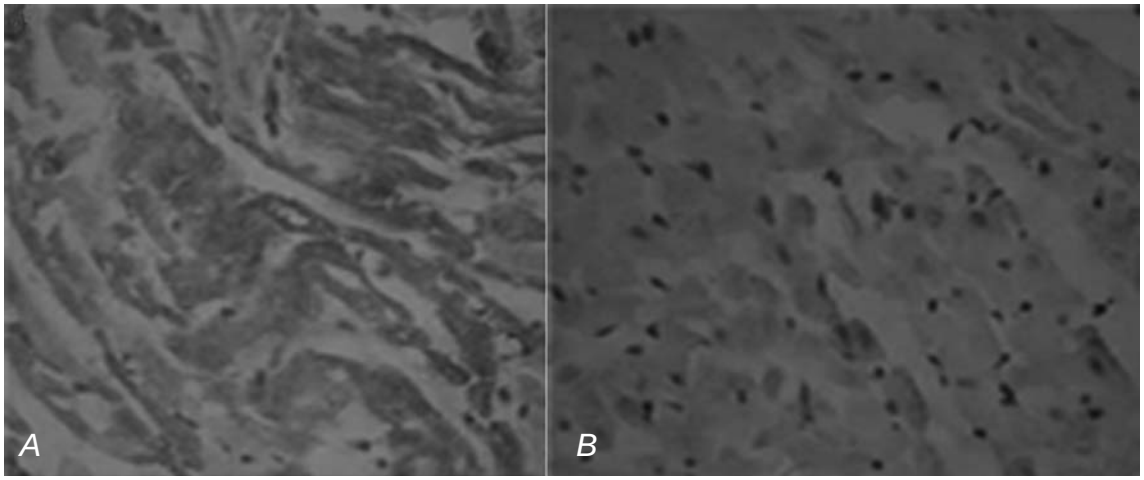


**Fig. 2.** Caspase-3 expression observation on day 21 (×200). A) Control group; B) NT-3 group.

**Рис. 2.** Експресія каспази-3 на 21-й день у контрольній групі (*A*) та групі NT-3 (*B*).

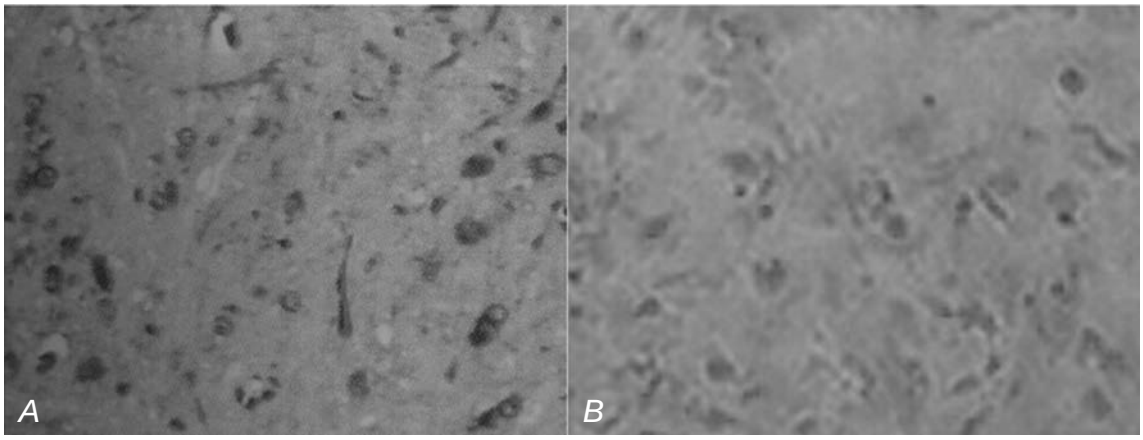


**Fig. 3. O**bservation of TUNEL-visualized muscle cell apoptosis on week 8 (×400). A) Control group; B) NT-3 group.

**Рис. 3**. Візуалізація апоптозу м'язових клітин на восьмий тиждень у контрольній групі (*A*) та групі NT-3 (*B*).

**T a b l e 1.** Expression of caspase-3 protein in two experimental groups (x ± s.e.m.).

**Т а б л и ц я 1.** Експресія протеїну каспази-3 в двох експериментальних групах.

| Group | h2 | h4 | h8 | d1 | d7 | d14 | d21 |
|---|---|---|---|---|---|---|---|
| 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±0.046* | 0.581±0.047* | 0.460±0.037* |

Footnotes: Caspase-3 protein expression in the two groups began to rise 2 h after injury; the intergroup difference at h2 was statistically insignificant ($P > 0.05$). Caspase-3 protein expression of the NT-3 group demonstrated statistically significant differences from h4 after operation compared with that in the control group ($P < 0.05$).

## DISCUSSION

The skeletal muscle loses afferent nerve impulsation and control after peripheral nerve injury, and this results in muscle atrophy because of the absence/decrease of neurotrophic factors in target cells. Mikhail et al. [11, 12] found that NT-3 levels were reduced within a 6 to 12 h interval after peripheral nerve injury. After two weeks, the respective group exhibited NT-3 levels eight times higher than that in the control group. Administration of exogenous NT-3 could promote growth of neuronal axons and myelin, thus maintaining afferent sensory neuronal survival of muscle spindles, tendon receptors, and cutaneous receptors, as well as promoting maturation of nerve-muscle synapses and significantly delaying the effect of skeletal muscle atrophy after nerve injury. However, the exact mechanism of the action of NT-3 has not been examined sufficiently and remains unclear.

Experimental results showed that Trk C is a specific receptor of NT-3, which can achieve the expression of its own signal molecules, such as neuronal $Ca^{2+}$ channels and neurotransmitter receptors, and regulate the functional state through rapid starting of a series of signal transduction pathways. It promotes the function of $Ca^{2+}$-ATPase and prevents atrophy of denervated skeletal muscles [12, 13]. $Ca^{2+}$-ATPase is the major protein involved in the regulation of $Ca^{2+}$ in the sarcoplasmic reticulum and cytoplasm, thus forming the basis for contraction and relaxation of skeletal muscle fibers [14, 15]. Peripheral nerve injury induces an energy deficiency of $Ca^{2+}$-ATPase in the skeletal muscle, in such a way decreasing the absorption capacity for $Ca^{2+}$. Moreover, a large amount of intracellular and mitochondrial $Ca^{2+}$ is accumulated, which results in a decreased contractility of the skeletal muscle; the respective mechanism, however, remains unclear to great extent [16].

Our study showed that the level of *gastrocnemius* $Ca^{2+}$-ATPase decreased significantly one week after denervation, sarcoplasmic $Ca^{2+}$-ATPase increased significantly in the NT-3-treated group (as compared with that in the former group), and the contractile function of the denervated skeletal muscle significantly increased compared with those in the control group. Findlay et al. [17] reported that NT-3 mRNA in a denervated skeletal muscle is downregulated. NT-3 was found to enhance the formation and promote the development of neuromuscular synapses. Regeneration in a mixed culture after NT-3 was locally injected into muscle cells *in vitro* was intensified, thus preventing the effects of muscle atrophy. Sahenk et al. [18] proved that NT-3 is abundant in skeletal muscles. Local endogenous injection can enhance RNA expression in advance, stimulate proliferation of myoblasts, promote the formation of muscle tube fuse into muscle fibers, and accelerate the repair process in the skeletal muscle.

It was found that increased caspase-3 expression is involved in the regulation of apoptosis after spinal cord injury. Caspase-3 was the most important protease involved in the above process. NT-3 may inhibit transcription of the caspase-3 gene, reduce pro-apoptotic factors, and inhibit in such a way neuronal apoptosis [19, 20]. However, many other apoptosis-related proteins, such as the Bcl-2 family and P53 protein, were found [21, 22]. Our experiments showed that caspase-3 gene transcription and the respective protein expression level began to increase 2 h after nerve injury. However, caspase-3 gene transcription in the NT-3 group appeared to be significantly lower at 4 h, whereas the caspase-3 level in the control group remained continuously high (differences between the two groups were statistically significant; $P < 0.05$). After application of exogenous NT-3 for 1 week, no muscle cell apoptosis was observed in both control and NT-3 experimental groups, and differences between the gastrocnemius apoptotic rates in the 4 and 8 week groups were significant ($P < 0.05$).

In our study, caspase-3 gene expression was higher, anti-apoptotic factors were lacking, and the $Ca^{2+}$-ATPase level decreased after nerve injury, which was the major cause of muscle cell apoptosis. Caspase-3 mRNA and protein expression increased the proliferative capacity of skeletal muscle cells by

NT-3 exogenous intervention, Subsequently, the $Ca^{2+}$-ATPase content increased (promoting skeletal muscle contraction and, thereby, reducing the pro-apoptotic factors and inhibiting muscle apoptosis). As a result, post-denervation muscle atrophy was inhibited, and nerve regeneration was promoted. However, muscle atrophy still remained significant despite a sufficient amount of exogenous NT-3. Thus, the lack of neurotrophic factors was not the only cause of muscle atrophy. A search for other reasons and mechanisms of muscle atrophy will be the aim of our future studies.

**Acknowledgment.** This study was supported by the Science and Technology Innovation Grant (2010) from the Department of Education of the Henan Province (2010HASTIT036), China.

The study was carried out in strict accordanse with the existing international ethical standards for the experimental work on animals and instructions of the local Ethical Committee.

The authors, Yu. Dong, H. Zhao, L. Yang, Yi. Zhao, Ch. Ma, and Ch. Zhang, confirm that they have no conflict of interest with any organization or person that may be related to this study; there were also no conflict of interest in interrelations between the authors.

*Ю. Донг[1], Х. Жао[1], Л. Янг[1], Йї. Жао[1], Ч. Ма[1], Ч. Жанг[1]*

## ВПЛИВИ ПЛАЗМІД ІЗ ГЕНОМ НЕЙРОТРОФІНУ-3 НА АПОПТОЗ МІОЦИТІВ ТА ВМІСТ $CA^{2+}$-АТФази У М'ЯЗІ ПІСЛЯ ПОШКОДЖЕННЯ НЕРВА У ЩУРІВ

[1] Перша лікарня при Медичному університеті Ксінкс'янь, Вейху (Китай).

Р е з ю м е

Ми вивчали вплив плазмід із ДНК, що несе ген нейротрофіну-3 (NT-3), на апоптоз міоцитів у *m. gastrocnemius* та вміст $Ca^{2+}$-АТФази в цьому м'язі після перерізання сідничного нерва. 60 дорослих щурів лінії Вістар були рандомізовано поділені на контрольну (введення фізіологічного розчину) та NT-3-групи. Оцінювали відповідні показники – експресію протеїну каспази-3, рівень апоптозу в м'язі (з використанням TUNEL-методики) та рівень $Ca^{2+}$-АТФази (з використанням Вестерн-блотингу). Експресія білка каспази-3 в різні часові інтервали після пошкодження периферичного нерва була підвищеною; експресія NT-3 та рівень апоптозу м'язових клітин були знижені, а вміст $Ca^{2+}$-АТФази в саркоплазматичному ретикулумі – підвищеним. Відмінності цих показників щодо таких у контрольній групі були вірогідними ($P < 0.05$). Обмежуючі впливи NT-3 на процес м'язової атрофії після пошкодження периферичного нерва виявлялись як інгібування експресії гена каспази-3, підвищення вмісту $Ca^{2+}$-АТФази та зниження інтенсивності апоптозу в м'язі.

## REFERENCES

1 A. S. Brack, M. J. Conboy, S. Roy, et al., "Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis," *Science*, **317**, 807-810 (2007).
2 W. Song, H. B. Kwak, and J. M. Lawler, "Exercise training attenuates age-induced changes in apoptotic signaling in rat skeletal muscle," *Antioxid. Redox Signal.*, **8**, 517-528 (2006).
3 L. S. Nierobisz, B. Cheatham, B. M. Buehrer, and J. Z. Sexton, "High-content screening of human primary muscle satellite cells for new therapies for muscular atrophy/dystrophy," *Curr. Chem. Genomics Transl. Med.*, **7**, 21-29 (2013).
4 B. T. Bennett, J. S. Mohamed, and S. E. Always, "Effects of resveratrol on the recovery of muscle mass following disuse in the plantaris muscle of aged rats," *PLoS One*, **8**, e83518 (2013).
5 K. Kaori, Y. Dai, E. Karla, et al., "Caspase-mediated protein kinase C cleavage is necessary for apoptosis of vascular smooth muscle cells," *Am. J. Physiol. Heart Circ. Physiol.*, **297**, 2253-2261 (2009).
6 B. Sishi, B. Loos, B. Ellis, et al., "Diet-induced obesity alters signalling pathways and induces atrophy and apoptosis in skeletal muscle in a prediabetic rat model," *Exp. Physiol.*, **96**, 179-193 (2011).
7 Q. Chen, L. Zhou, and H. D. Shine, "Expression of neurotrophin-3 promotes axonal plasticity in the acute but not chronic injured spinal cord," *J. Neurotrauma*, **23**, 1254-1260 (2006).
8 J. R. Mendell, L. Rodino-Klapac, Z. Sahenk, et al., "Gene therapy for muscular dystrophy: lessons learned and path forward," *Neurosci. Lett.*, **527**, 90-99 (2012).
9 T. Hag, K. A. Baker, J. G. Emsley, et al., "Prolonged local neurotrophin-3 infusion reducs ipsilateral collateral sprouting of spared corticospinal axons in adult rats," *Neuroscience*, **130**, 875-887 (2005).
10 W. Zhang, Y. Miao, Z. Xing, et al., "Growth-associated protein-43 expression in cocultures of dorsal root ganglion neurons and skeletal muscle cells with different neurotrophins," *Muscle Nerve*, **47**, 909-915 (2013).
11 A. K. Mikhail, S. Rachid, R. Khadija, et al., "Pleiotropic effects of neutrophils on myocyte apoptosis and left ventricular remodeling during early volume overload," *J. Mol. Cell. Cardiol.*, **47**, 634-645 (2009).
12 S. A. Kulakowski, S. D. Parker, and K. E. Personius, "Reduced TrkB expression results in precocious age-like changes in neuromuscular structure, neurotransmission, and muscle function," *J. Appl. Physiol.*, **111**, 844-852 (2011).
13 S. Lin, J. Xu, S. Hu, et al., "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.*, **20**, 381-390 (2011).
14 J. H. Hsieh, W. M. Lin, H. Chiang, et al., "Patterns of target tissue reinnervation and trophic factor expression after nerve grafting," *Plast. Reconstr. Surg.*, **131**, 989-1000 (2013).
15 W. Wang, H. Zhang, H. Gao, et al., "$\beta_1$-Adrenergic receptor

activation induces mouse cardiac myocyte death through both L-type calcium channel-dependent and independent pathways," *Am. J. Physiol. Heart Circ. Physiol*., **299**, 322-331 (2010).
16 S. Suzuki, U. Yokoyama, T. Abe, et al., "Differential roles of epac in regulating cell death in neuronal and myocardial cells," *J. Biol. Chem*., **285**, 24248-24259 (2010).
17 A. R. Findlay, S. Lewis, Z. Sahenk, and K. M. Flanigan, "Camptocormia as a late presentation in a manifesting carrier of duchenne muscular dystrophy," *Muscle Nerve*, **47**, 124-127 (2013).
18 Z. Sahenk, G. Galloway, K. R. Clark, et al., "aav1.nt-3 gene therapy for Charcot-Marie tooth neuropathy," *Mol. Ther*., 22, 511-521 (2014).
19 T. T. Tzeng, H. J. Tsay, L. Chang, et al., "Caspase 3 involves in neuroplasticity, microglial activation and neurogenesis in the mice hippocampus after intracerebral injection of kainic acid," *J. Biomed. Sci*., **20**, 90 (2013).
20 S. Zhu, M. Nagashima, M. A. Khan, et al., "Lack of caspase-3 attenuates immobilization-induced muscle atrophy and loss of tension generation along with mitigation of apoptosis and inflammation," *Muscle Nerve*, **47**, 711-721 (2013).
21 H. R. Momeni, M. S. Mehranjani, M. A. Shariatzadeh, and M. Haddadi, "Caspase-mediated apoptosis in sensory neurons of cultured dorsal root ganglia in adult mouse," *Cell J*., **15**, 212-217 (201).
22 Y. Cheng, N. X. Cawley, and Y. P. Loh, "Carboxypeptidase E/NFα1: a new neurotrophic factor against oxidative stress-induced apoptotic cell death mediated by ERK and PI3-K/AKT pathways," *PLoS One*, **8**, e71578 (2013).