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Investigation of decreased activity on NT3 gene expression of male Wistar rat's sciatic nerve fiber

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ABSTRACT: Painful neuropathy is a state that resulted from somatosensory disease or injury and expose affected patients to the various functional complications such as decreased physical activity and its complication like muscular and cardiovascular diseases. Recently, it has been demonstrated that neurotrophin such as NT-3 are vitally for neural growth and development and protect integrity of function and structure of nervous system. In respect to the importance of physical activity in neural plasticity, the object of present study is investigation of chronic effect of decreased activity in the form of spinal nerve ligation on NT-3 gene expression of male Wistar rat's sciatic nerve fiber. Ten adult male Wistar rats in the weight range of 250±30 gr randomly divided into two groups including healthy control (C), Decreased physical activity (SNL). Over the six weeks, neuropathic pain behavior tests conducted continually in groups. In the end of Sixth weeks, change of NT-3 gene expression in sciatic nerve measured with Real time technique. Behavioral tests demonstrated that spinal nerve ligation induce thermal hyperalgesia and mechanical allodynia in the SNL group which this decreased pain threshold seen in allover period of study(P≥o.o5). Also in compare to the C group, NT-3 gene expression in sciatic nerve fiber was higher in SNL group significantly (P≥o.o5).In the present study, it was detected that decreased activity in the form of SNL is associated with increased NT-3 gene expression in experimental group. Although it has been demonstrated that increased physical activity induce overexpression this protein in nerve injury models but it isn't clear what is the difference between increased NT-3 expression in nerve injuries and neuropathic pain with the elevated expression of this protein in effect of exercise training.

***Keywords*:** Neuropathic pain, Decreased physical activity, Neurotrophin, NT3.

INTRODUCTION

Neuropathic pain is a chronic pain that is defined as a pain caused by damages to or dysfunction of somatosensory system and can expresses itself in following forms: allodynia, hyperalgesia and spontaneous pains [1]. Along with causing changes to nervous system, neuropathic pain can decrease physical activity levels [2, 3]. Furthermore neuropathic pain can effect on structure and function of muscles through muscular atrophy [4, 5]. Many studies have proven that neuropathic pains' models will be followed with muscle atrophy [5-8]. However, exact cellular mechanisms which cause changes after nervous damages are still unknown [8].

Among the most prevalent complication of diabetes, neuropathy develops as an irreversible complication in more than half of with diabetes type 1 or 2 [9]. Diabetes is associated with slowing of motor nerve conduction velocity and reduced muscle contractile properties [10-11]. In contrast, on the sensory side, there is not only slowing of sensory nerve conduction velocity but also atrophy of primary and axons associated with down-regulation of structural protein synthesis and loss of terminal epidermal axons [12-13].

NTs regulate the growth, maintenance and apoptosis of neurons in the neurogenesis process as well as injured neurons (14–16). The neurotrophins (NTs) particularly NGF, BDNF and NT-3 are synthesized mainly in the dorsal root ganglia (DRG) with anterograde transported into the dorsal horn of the spinal cord (17). Recently, NTs have

been shown to be involved in the neuronal mechanism underlying neuropathic pain development and transmission (18). Apart from their roles in various physiological functions, they modulate central sensitization in the spinal cord that underpins maintenance of neuropathic pain (17). Since NTs have key roles in the complex mechanisms of peripheral and central sensitization, it seems that they contribute to the pathogenesis of neuropathic pain (19–21). However, the specific contribution of individual neurotrophins signaling through a particular Trk receptor and/or the p75NTR in the pathobiology of neuropathic pain is remaining elusive (17).

The present study utilized spinal nerve ligation (SNL) as neuropathic pain and investigated whether neurotrophins and their receptors were activated after SNL and then determined whether the activated neurotrophins played a role in neuropathic pain. These findings raised the possibility of targeting neurotrophins having a specific role in the treatment of neuropathic pain.

MATERIALS AND METHODS

Animals

In present study 10 mature Wistar male rats with 10 weeks age and weight range of 250 g were provided by Animal Maintenance Unit of Razi Research Center (Razi institute Animal Center, Karaj, Iran) and conveyed to Animal Laboratory of Tarbiat Modares University. All rats were kept under a controlled environment condition with mean temperature of 22±3 C degrees, dark-light cycle of 12:12 hours, relative humidity of 40 % and free access to food and water ad libitum. The experimental protocols to perform this study were approved by the Ethics Committee on the use of animals of Tarbiat Modares University, Tehran, Iran. All efforts were made to minimize discomfort of the animals and reduce the number of experimental animals. All procedures conformed to the ethical guidelines for the care and use of laboratory animals, published by the International Association for the Study of Pain and the National Institutes of Health. After two weeks of acclimatization of animal with new environment, experimental protocols were initiated and the rats were randomly (simple randomization) allocated in three groups (5 rats in each group): (1) detraining (n=5); (2) sham surgery (Sham: n=5).

Induction of neuropathic pain

Animals were anesthetized by pentobarbital sodium (60 mg/Kg, intraperitoneal). Then the L_5 spinal nerve was tightly ligated according to the method of Kim and Chung (1992) [22]. Briefly the left Para spinal muscles were separated at the L_5-S_2 levels and the left transverse process of the L_6 vertebra was removed. The left L_5 spinal nerve was identified and gently separated from adjacent L_4 spinal nerve. The L_5 spinal nerve was tightly ligated using silk threads (6-0) and was transected just distal to ligature to ensure that all fibers were interrupted. Then the wound closed with 3-0 silk threads. Great care was taken to avoid any damages to L_4 nerve. In a control sham group, the surgical procedure was identical to that described above, except for the left L_5 spinal nerve that was not ligated and transected. Only animals showing no signs of motor deficiencies were considered to be used for further experimentations. Only animals were chosen to continue the experiment with that had shown neuropathic pains in their behavioral tests. Then the rats were divided in 2 groups with 5 members in each: sham and detraining groups. After 6 weeks, rats anesthetized to take samples after injecting intraperitoneal ketamine (90 mL in Kg) and xialyzine (10 mL in Kg) and the muscle tissue samples separated from the left soleus muscle and were situated in -80 nitrogen for future analysis.

RNA extraction and cDNA synthesis

RNA extraction was done by QlAzol® Lysis Reagent (Germany, Qiagen) and chloroform (Germany, Qiagen) and in accordance to its manufacturer's instructions. So about 50 Mg of the muscle tissue homogenized separately in 1 to 10 portions in QlAzol® Lysis Reagent for total RNA extraction and for removing protein components. The final product was centrifuged at 12000×g for 10 minutes at 4 degrees C. Then mixed with chloroform in 1 to 5 portions and shaken severely for 15 seconds. Then the supernatant was at 12000×g for 10 minutes at 4 degrees C and its mineral part and its water removed. Finally, its RNA contained portion removed and mixed with isopropanol in 1 to 5 portions. It left for 10 minutes in room temperature and then centrifuged in 4 C degrees for 10 minutes with 12000 g revolution. RNA contained Pellet was washed and resolved in 20 microlitre RNsa-free water. RNA concentration was measured by UV spectrophotometry method (Eppendorff, Germany), and 260 to 280 portions in 1.8 -2 were determined as the desired purification. cDNA synthesis was done by using Quanti Tect Reverse Transcription Kit (Qiagen, Germany) in accordance to manufacturer's manual.

Real-Time PCR

Real-Time PCR quantity method was used by Premix SYBR Green II (Qiagen, Germany) for measuring NT-3 mRNA expression levels (Applied Biosystems Step One, America). Reaction mixture was done in final volume in 20 microliters (includes 1 microliter of cDNA, 1 microliter of forward primer, 1 microliter of reverse primer, 7 microliters of DEPC water and 10 microliters of Syber Green) and each reaction in a duplicate form. Designing primers was done according to NT-3 and GAPDH genes in gene bank of NCBI and by German company, Qiagen. Usable primer sequences have been reported. Thermal program used in Real Time-PCR included: 95 degrees C for 10 minutes, 95 degrees C for 15 seconds, 60 degrees C for 1 minute (40 cycle repetitions). Melt curve and standard curve were drawn and considered for evaluating data authenticity and optimization experiment conditions respectively and NT-3 expression data were normalized using GAPDH (reference gene). Fold change of genes was measured by formula $R=2^{-ct\Delta\Delta}$ [23].

Statistical analysis

All statistical analyses were done by using SPSS software (version 19, SPSS Inc., Chicago, IL, USA). Normal assumption was examined using one-sample Kolmogorov-Smirnov test. T-tests were used to comparing groups in under study variables. Significant level was determined at $\alpha=0.05$.

RESULTS AND DISCUSSION

To elucidate the possible regulation of NT-3 at mRNA level in rat models with neuropathic pain, we examined NT-3 at mRNA level in soleus muscle after 6 weeks of tight ligation of L5 spinal nerves. Indicated in Figure **1** NT-3 levels were upragulated.

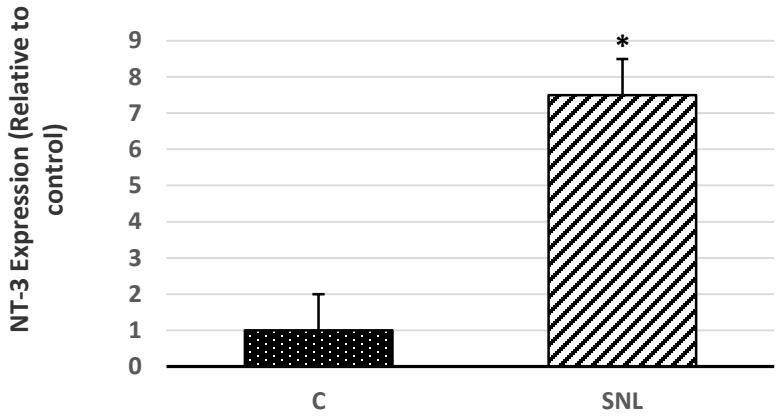

Figure 1. Real-Time amplification of NT-3 mRNA in Sciatic nerve. Data are shown as mean * which indicates significant differences with another groups ($p< 0.05$).

Discussion and conclusion

The results of present study are the first to demonstrate that SNL up-regulates NT-3 mRNA at sciatic nerves of rats. Moreover, physical activity modulated both NT-3 in sciatic nerves of active rats. These results indicated that sciatic nerves' NT-3 levels in SNL state could be changed and probably exercise training can modify it.

In summary, the present study provided new evidence that neuropathic pain induced by spinal nerve ligation (SNL) could activate neurotrophins and Trk receptors expression in DRG. However, this effect may be attributed to neural protection role of neurotrophins. Moreover, the results of this study provide a new interesting finding that the elevated neurotrophins expression following SNL indicates its neuropathic pain mediator role or its neuroprotective role. Thus, further research is needed to better elucidate the role of NTs in neuropathic pain.

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