

NGF and CNTF Expression and Regulation Mechanism by miRNA in Acute Paralytic Strabismus

Hua Liu, Nian Tan, Duo Xu*, Chong-Yi Li and Guang-Jun Xian

Department of Ophthalmology, Daping Hospital, Army Medical Center of PLA, Chongqing, P.R. China

Abstract

Nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF) are well known neurotrophic factors and widely used in the clinical treatment for its promotion effect on peripheral nerve regeneration. And they were also recommended for the acute paralytic strabismus treatment. However, whether the NGF and CNTF have protective effect for the extraocular muscles of acute paralytic strabismus patients is still poorly understood. Thus, in this study, we want to evaluate the biological function of NGF and CNTF on the extraocular muscle cells and revealed the regulation mechanism behind it. Firstly, the relative expression of *ngf* and *cntf* was assessed by Quantitative Real-time Polymerase Chain Reaction (RT-PCR). Then, the influence of NGF and CNTF on the extraocular muscle cell proliferation was determined by Cell Counting Kit-8 (CCK-8). The inflammatory response in muscle cells after NGF and CNTF treatment was evaluated by ELISA and Reactive Oxygen Species (ROS) detection. In addition to this, the up-stream regulation of the *ngf* and *cntf* expression was also studied. The TargetScan was used for the predication of potential miRNAs targeting with *ngf* and *cntf* 3'-UTR, which is soon confirmed by luciferase activity assay. Taken together, all the results above indicated that NGF and CNTF could promote the muscle cells proliferation and inhibit the inflammatory levels, then exert protective effect on the muscle cells function. It was conceivable that let 7-5p was the up-stream regulator of *ngf* and *cntf*, and let 7-5p might serve as a potential molecular target for acute paralytic strabismus treatment.

Keywords: Acute paralytic strabismus; miRNAs; *let 7-5p*; NGF; CNTF

Introduction

Paralytic strabismus is a complete or partial paralysis of the single or multiple extraocular muscles caused by the nucleus that controls the movement of the eye muscles or the external of the extraocular muscles (extraocular muscle in dysfunctional condition but not complete paralysis) [1-3]. Paralytic strabismus is divided into congenital and acquired paralytic strabismus. Acquired paralytic strabismus is mostly acute paralytic strabismus, and its clinical causes can be divided into neurogenic, myogenic and mechanical. At present, the commonly drugs used for acute paralytic strabismus in the clinic include vitamin B3, vitamin C, creatinine, adenosine triphosphate, and coenzyme A, and so on [4]. These drugs can improve neuromuscular function. However, the current clinical data show that the cure rate of acute paralytic strabismus is not very ideal, so we need to further reveal the pathogenesis of acute paralytic strabismus, and provide new targets for drug development in the future.

Nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF) are two kinds of neurotrophic factors which are deeply and widely reported, and its promotion effect on peripheral nerve regeneration has been widely confirmed [5,6]. In the previous study, we can see the NGF has a relatively strong promoting effect on the fiber regeneration, while the CNTF shows a relative stronger promotion on the motor fiber regeneration. A large number of studies have shown that NGF and CNTF have protective effects on sympathetic, sensory and motor nerves. However, the current research on NGF and CNTF pays more attention to its nutritional effects on neurons. For example, the NGF and CNTF are given after the peripheral nerve injury treatment. Generally, only the protective effect of NGF and CNTF on neurons is noticed, its Non-neuronal protection was needed to be explored, especially the protection on skeletal muscle.

In recent years, more and more researches focused on the study of

microRNAs (miRNAs) regulation on target genes expression. miRNAs are endogenous RNAs with 20-24 nucleotides [7], which could directly bind to the 3'-untranslated region (UTR) of its target gene at the posttranscriptional level and involved in post-transcriptional regulation, affecting both the stability and translation of mRNAs, then regulating various cellular functions [8]. A growing number of studies have demonstrated that miRNAs participate in the progression processes of multiple diseases, and have the potential to be a promising target for the target for disease treatment.

In this present research, we aimed to explore the role of NGF and CNTF on the acute paralytic strabismus treatment [9]. Firstly, the relative expression of NGF and CNTF in the extraocular muscle samples was measured in both the mRNA and protein levels. The influence of NGF and CNTF on extraocular muscle cell proliferation and inflammatory level was measured to prove the protective effect on extraocular muscle. Subsequently, the bioinformatics prediction was performed for the searching of miRNA which could regulate the expression NGF and CNTF, and its binding with target genes was confirmed by luciferase activity assay. Finally, we want to clarify the potential mechanisms of

***Corresponding author:** Dr. Duo Xu, Department of Ophthalmology, Daping Hospital, Army Medical Center of PLA, Chongqing, P.R. China, Tel: +1832322321; E-mail: 541460765@qq.com

Received September 15, 2019; **Accepted** September 24, 2019; **Published** September 30, 2019

Citation: Liu H, Tan N, Xu D, Li C, Xian G (2019) NGF and CNTF Expression and Regulation Mechanism by miRNA in Acute Paralytic Strabismus. Cell Mol Biol 65: 156.

Copyright: © 2019 Liu H, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

NGF and CNTF protective effect on extraocular muscle. In conclusion, we convinced the important role of the *let 7-5p* in the acute paralytic strabismus by regulating NGF and CNTF expression.

Materials and Methods

Patients and tissue specimens collection

In this present research, the extraocular muscle samples were collected from 75 patients who were diagnosed as acute paralytic strabismus and underwent surgical resection treatment in our hospital from March 2018 to May 2019. All patients were informed about the detail perforation of this research and signed the patient consents before the collection of extraocular muscle samples. This experiment was approved by the Ethics Committee of Daping Hospital. All tissue specimens harvested were stored in liquid nitrogen at -80°C immediately after surgical resection.

Cell cultures

Human 293-T cell line was obtained from American Type Culture Collection. The HeLa cells were maintained in Dulbecco's Modified Eagle's Medium medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1% Penicillin and Streptomycin sulfate solution, 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco, Life Technologies), as well as 2% L-glutamine. The 293-T cells were incubated in an incubator at 37°C , 5% CO_2 , the culture medium was changed according to the cell state.

Cell transfection

The *let 7-5p* mimic, *let 7-5p* inhibitor, as well as the mimic-control and inhibitor-control were designed according to the sequence of NGF and CNTF mRNA, and then the *let 7-5p* mimics and inhibitors were synthesized by ShengGongPharma, Shanghai, P.R. China.

The PCR was performed to get the cDNA fragment sequence of the 293-T after amplification the *ngf* and *cntf* sequence was cloned into pcDNA3.1 vector (Ruibobio, Guangzhou, China). In the parallel test group, the empty pcDNA-3.1 plasmid was used as negative control [10]. For miRNAs or pcDNA-3.1 plasmids transfection, the Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used following the manufacturer's instruction with little modification [11].

RNA isolation and quantitative RT-PCR

The transfection efficiency of *let 7-5p* and plasmid, as well as the relative expression of *ngf* and *cntf* was assessed by quantitative real-time RT-PCR according to the manufacture's protocol with some modification [12]. Generally, the TRIzol Reagent (Sigma, St. Louis, MO, USA) was used to extract the total RNA sample in the cells after transfection or treatment under the instructions. Then, the total RNA sample was qualified and quantified with spectrophotometer, followed by reverse transcribed into cDNA following the manufacturer's proposal. Finally, to measure the transfection efficiency and genes relative expression, the RT-PCR was performed using SYBR Green Master Mix (Taraka), using the *gapdh* as the internal reference. The

Genes	Sequences
<i>let 7-5p</i>	TCTGGCAAATTGAGGTAGTAGGT
	TCAAGCAAAGAAAGCTAGCACAA
<i>ngf</i>	GGGAGCGCAGCGAGTTTGG
	GAGTGTGGTTCCGCCTGTAT
<i>cntf</i>	TGATTAGGCCCGCCAACTT
	AACCTGGTATAAGCCGTGCC
<i>gapdh</i>	ATGTTGCAACCGGGAAGGAA
	AGGAAAAGCATCACCCGGAG

Table 1: The primers used in this experiment.

sequence of *ngf* and *cntf* were obtained from PUBMED/Nucleotide GeneBank, and the primer sequence was designed by Primer Premier 5.0 and synthesized by ShengGong (ShangHai, China). The primers used in this experiment are listed in Table 1. $2^{-\Delta\Delta\text{Ct}}$ method was recommended for the calculation of genes relative expression.

ELISA assay

The release of IL-1 β and TNF- α in extraocular muscle tissues of acute paralytic strabismus patients was detected in this experiment performed according to the protocols with some modification [13]. In brief, the extraocular muscle was isolated and cultured at 37°C , 5% CO_2 , followed by NGF and CNTF addition. After treatment, the cell supernatant was collected for the IL-1 β and TNF- α content detection with ELISA. This experiment was repeated at least three times.

ROS detection

To detect the inhibitory effect of NGF and CNTF on extraocular muscle cells inflammatory response, after treatment, the ROS level in cells of different groups were measured by ROS detection kit following the instructions [14]. Briefly, the cells were isolated and cultured at 37°C , 5% CO_2 , followed by NGF and CNTF treatment. Subsequently, the cells were harvested for the ROS detection in flow cytometry.

miRNA prediction and luciferase activity assay

To predicate the miRNA which could regulate the expression of *ngf* and *cntf* genes, as well as the binding sites of miRNA with the target genes, the TargetScan software was used in this study. Based on the prediction results by bioinformatics software, the binding of miRNA with *ngf* and *cntf* genes was further confirmed by luciferase activity assay [15]. In short, the pMIR-REPORT luciferase reporter plasmids (Promega Corporation, Madison, WI, USA) used in this experiment was inserted with the wild-type (WT) and 3'-UTR mutant *ngf* and *cntf* genes. The *let 7-5p* mimic and the luciferase reporter plasmids were co-cultured into 293-T cells. After 24 h incubation in an incubator at 37°C , 5% CO_2 , the inflorescence intensity in each group were measured GloMax20/20 illuminometer (Promega Corporation). Data were showed as mean \pm SD. This experiment was repeated at least three times.

Western blotting

The relative expression of NGF and CNTF in the extraocular muscles of acute paralytic strabismus patients was further measured by western blot. This experiment was performed according to the protocols previous described with some modification [16]. Generally,

the extraocular muscle samples harvested from patients were used in the detection, the radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) containing protease inhibitor cocktail (Thermo Fisher) was recommended to lysis the cells for the protein extraction. After extraction, the BCA Protein Assay kit (Sigma, USA) was employed for the quantification of total protein concentration. Equal amounts of protein samples from different groups were separated on 12% sodium dodecyl sulfate-polyacrylamide denaturing gels and then transferred onto a polyvinylidene difluoride membrane (PVDF, Millipore, MA). After that, the PVDF membranes were blocked in 5% blocking buffer at room temperature for 2 hours to exclude non-specific binding. The primary antibodies against NGF and CNTF (Abcam) or GAPDH (Abcam) were incubated with PVDF membranes overnight at 4°C. Next, horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Finally, the chemiluminescence (ECL) detection kit (Thermo Fisher Scientific) was used for proteins expression visualized and quantification was performed using ImageJ software (BIO RAD) [17]. The experiment was in triplicate independently.

Statistical analysis

All the results were presented as means \pm SD in this research from three different repeats. SPSS 17.0 Software (IBM, Armonk, NY, USA) was recommended for the statistical analysis. Student t-test was used for the statistical comparisons of two groups, and one-way ANOVA was performed for statistical comparisons of more than three different groups. $p < 0.05$ was regarded as has statistically significant between groups.

Results and Discussion

Aberrant expression of *ngf* and *cntf* in extraocular muscle tissue

As two important types of neurotrophic factors, the *NGF* and

CNTF play a vital important role in the procession of peripheral nerve regeneration. However, the protection effect of *NGF* and *CNTF* on injured skeletal muscle was still unknown. Thus, in this experiment, the relative expression of *ngf* and *cntf* in extraocular muscle tissue was measured by RT-PCR and western blot, respectively. As the results showed in Figure 1A, the relative expression of in the superior oblique muscle of the paralysis was significantly weaker than that in the upper oblique muscle of the control group. The expression of *ngf* and *cntf* in the inferior oblique muscle of the paralysis was significantly enhanced compared with that in the control group ($p < 0.05$). The expression profiles of *ngf* and *cntf* in the superior and inferior oblique muscles of the control group showed no significant statistical differences.

NGF and CNTF promotes the proliferation of extraocular muscle cells

In the previous studies, we have confirmed the reduced-expression of *ngf* and *cntf* in the superior oblique muscle and the over expression in the inferior oblique muscle. Thus, we proposed a hypothesis; the content of *NGF* and *CNTF* may have closely relationship with the function of extraocular muscle. So, in this experiment, the effect of *NGF* and *CNTF* on muscle cell proliferation was determined by CCK-8 assay. As the results showed in Figure 2, both the *NGF* and *CNTF* showed promotion effect on the cell growth, there was a significant difference between the treatment group and the control group. This result revealed the protective effect of *NGF* and *CNTF* on muscle cell for the first time.

NGF and CNTF inhibit the ROS mediated apoptosis in extraocular muscle cells

It has been reported that during acute paralytic strabismus, there is usually an increased inflammatory response level in in extraocular muscle tissues, reflecting as the up-regulated level of IL-1 β , TNF- α and ROS [18]. Thus, whether the aberrant expression of *NGF* and *CNTF* in extraocular muscle tissue has relationship with the inflammatory

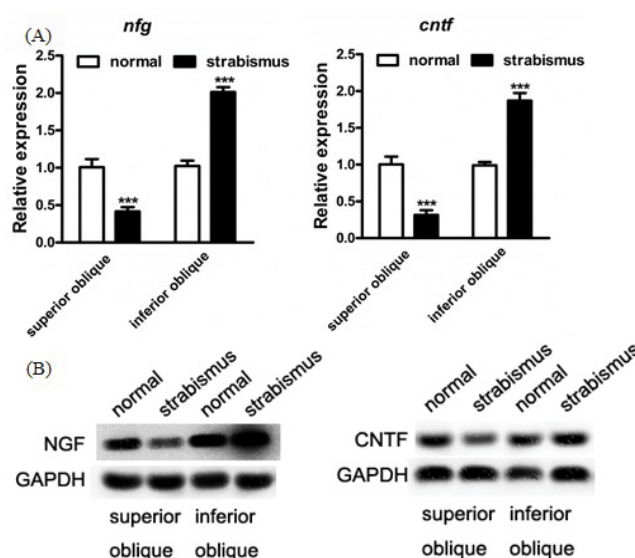


Figure 1: Non-neuronal protection of NGF and CNTF in extraocular muscle tissue. The relative expression of *ngf* and *cntf* in the superior and lower oblique in extraocular muscle tissue of acute paralytic strabismus patients was detected by RT-PCR (A) and western blot (B). ***means $p < 0.001$.

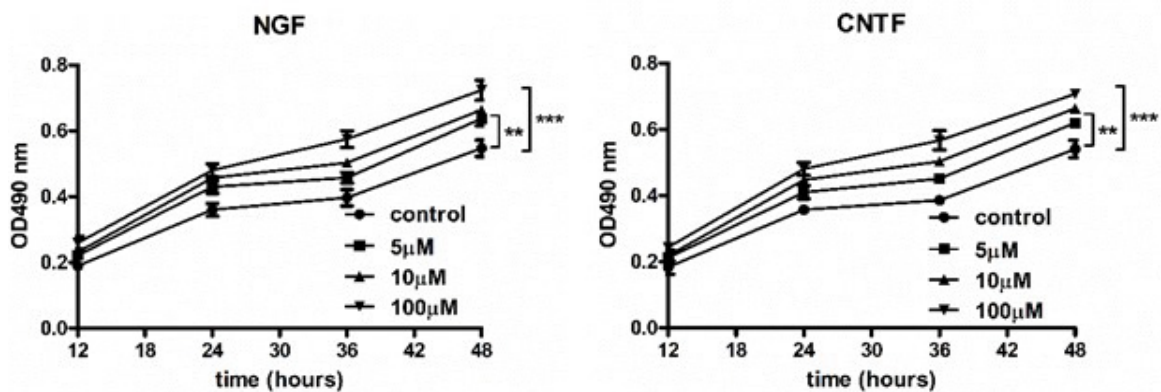


Figure 2 (a) At 77 K, 1a' s N₂ sorption isotherms; (b) At 273 K, 1a' s gas sorption isotherms; (c) At 296 K, 1a' s gas sorption isotherms; (d) The predicted IAST selectivity of 1a.

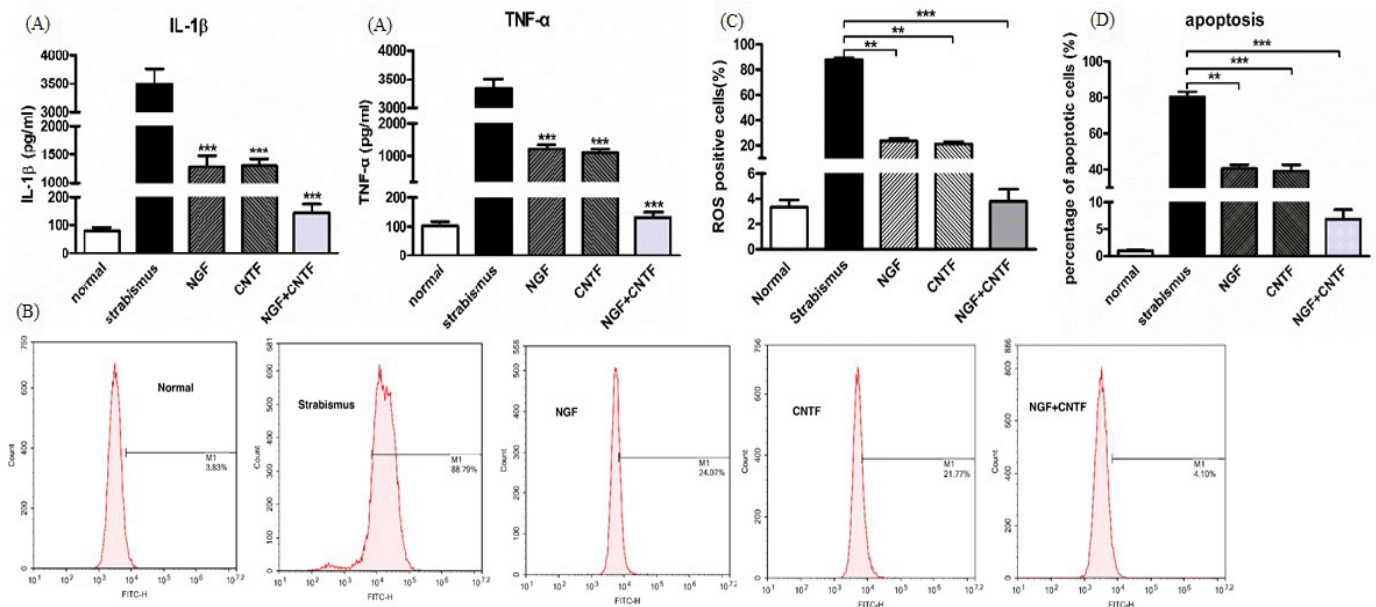


Figure 3: Reduced inflammatory response in extraocular muscle cells after NGF and CNTF. The extraocular muscle cells were isolated from patients' tissues and followed by NGF and CNTF treatment. (A) The IL-1 β and TNF- α content was measured by ELISA assay. (B) ROS level determined by DCFH-DA detection in flow cytometry. (C) Percentage of apoptotic extraocular muscle cells after NGF and CNTF treatment. (D) Percentage of apoptotic extraocular muscle cells after NGF and CNTF treatment was measured by in flow cytometry.

response level was detected in this experiment. In Figure 3A, the ELISA was performed to measure the IL-1 β and TNF- α content after treated with NGF and CNTF, results indicated that the significant inhibitory effect of NGF and CNTF on the IL-1 β and TNF- α release. Besides, the ROS detection also confirmed that the NGF and CNTF could inhibit the inflammatory response in extraocular muscle tissues through reducing ROS production (Figure 3B). The percentage of apoptotic cells were measured in flow cytometry and the results were calculated in Figure 3C.

Let 7-5p targets directly and negatively regulates *ngf* and *cntf* expression

As to the vital role of NGF and CNTF in the in extraocular muscle tissue during acute paralytic strabismus, we proposed a new question, how the *ngf* and *cntf* expression was regulated? To solve this

problem, we used the TargetScan software to predicate the protentional regulator and reveal the novel treatment target. In Figure 2A, *let 7-5p* was identified as one of the most potential target regulators of *ngf* and *cntf*, with the highest scores. Then, the expression of *let 7-5p* in the extraocular muscle samples were detected via RT-PCT (B) and the correlation between *let 7-5p* and *ngf* and *cntf* was further explored, as the results showed in Figure 2C, *let 7-5p* negatively regulates the expression of *ngf* and *cntf* expression in extraocular muscle tissue ($r = -0.9943$ and $r = -0.9948$, $p < 0.0001$). For the further confirmation of the directly binding of *let 7-5p* and *ngf* and *cntf*, the luciferase reporter assay was performed according to the protocols. The result in Figure 2D suggested that *let 7-5p* mimic significantly reduced the luciferase activity, and *let 7-5p* inhibitor obviously elevated the luciferase activity ($p < 0.005$). Above all, the *let 7-5p* was the up-stream of the *ngf* and *cntf* expression, which could negatively regulate *ngf* and *cntf* expression.

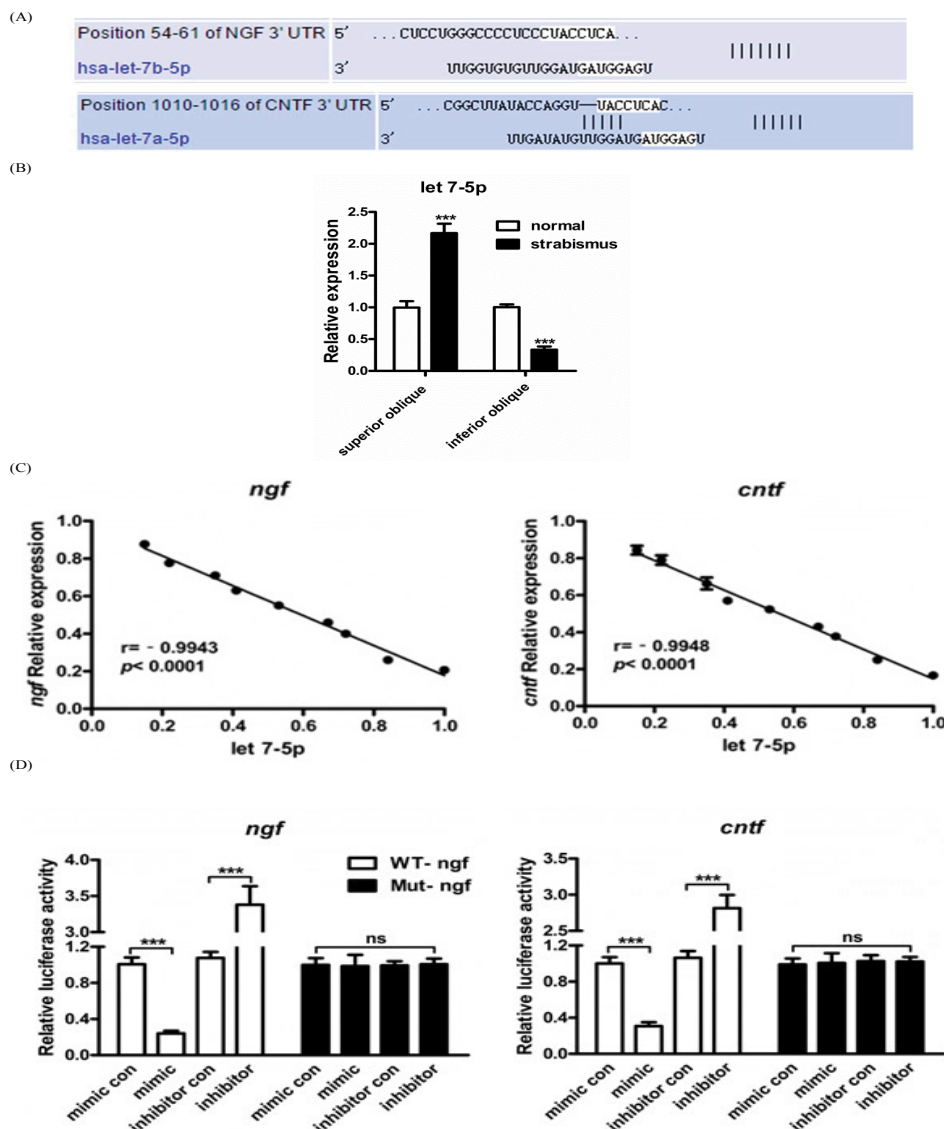


Figure 4: Let 7-5p negatively regulates *ngf* and *cntf* expression by directly binding. (A) The potential binding sites between let 7-5p and *ngf* and *cntf* mRNA 3'-UTR predicted by TargetScan software. The sequence of let 7-5p is shown allied with the *ngf* and *cntf* mRNA 3'-UTR sequence. (B) The expression of let 7-5p in the extraocular muscle samples detected by RT-PCT. (C) The negative correlation between let 7-5p expression and *ngf* and *cntf* expression level. (D) Direct binding between let 7-5p and *ngf* and *cntf* verified by luciferase activity assay.

Conclusion

Neurotrophic factors (NTFs) secrete peptides have been proved could promote axonal regeneration and play important roles in maintaining survival of neurons. Among these NTFs, the best studied are the NGF and CNTF [19]. In 2006, Li et al. have reported the different function of NGF and CNTF, the NGF could promote for the regeneration of sensory fibers, while the CNTF could significantly promote the regeneration of motor fibers. In addition to the synergetic effects of NGF and CNTF on survival and growth of sensory neurons, its protective effect on muscle tissues was still unknown. So, in this experiment, we firstly detect the expression of *ngf* and *cntf* in the upper oblique and oblique muscle of the paralysis via RT-PCR. We found there was a down-regulation of *ngf* and *cntf* expression level in superior oblique muscle, and an up-regulated level in inferior oblique,

which suggested that the content of NGF and CNTF may has a closely relationship with the function of extraocular muscle. Next, we further explore the promotion effect of NGF and CNTF on the extraocular muscle cells proliferation and the inhibition on the inflammatory response in muscle cells. Consistent with what we have envisaged, the results in Figure 2 and Figure 3 proved the extraocular muscle cell growth could be promoted by the addition of NGF and CNTF, and the inflammatory response level in cells was also be reduced by the NGF and CNTF, reflecting as the down-regulated level of IL-1 β and TNF- α release, as well as the ROS accumulation. And a new problem came followed, how the production of NGF and CNTF in the extraocular muscle tissues was regulated. To solve this problem, we searched related references and performed the following researches.

As a special kind of noncoding single-stranded RNA, the miRNAs

are widely exist in eukaryotes, which could bind to the 3'-UTR of the target genes mRNA and influence the genes expression by mRNA cleavage or translation regulation. Thus, in the further mechanism, we aimed to explore the up-stream miRNA, which regulates the *ngf* and *cntf* expression in extraocular muscle cells. For the potential miRNA predication, the TargetScan software was employed in our research, and the results revealed the *let 7-5p* won the highest rating, becoming the most likely regulator. For further verification, the *let 7-5p* expression level in isolated from acute paralytic strabismus patients was measured (Figure 4A) and the correlation between *let 7-5p* and *ngf* and *cntf* was calculated (Figure 4B). What surprises us is that there is a perfect negative correlation between *let 7-5p* expression and *ngf* and *cntf* expression level. Subsequently, the luciferase activity assay further confirmed the *let 7-5p* was the negative regulator of *ngf* and *cntf* in extraocular muscle cells.

Above all experiments, we not only proved the protective effect of *NGF* and *CNTF* on the treatment of acute paralytic strabismus by promoting the proliferation of extraocular muscle cells and inhibiting the IL-1 β and TNF- α release and ROS production, but also revealed the up-stream regulatory mechanism of *ngf* and *cntf* in extraocular muscle cells, proposed the important role of *let 7-5p* in the acute paralytic strabismus therapy. This research is the first time completely disclosure the protective effect of *NGF* and *CNTF* on extraocular muscle cells during acute paralytic strabismus, providing the new targets for the development of potential drugs in the further.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

Data Availability

The data used to support the findings of this study are included within the article.

Acknowledgements

The research did not receive any specific funding.

References

1. Clark, R.A. The role of extraocular muscle pulleys in incontinent non-paralytic strabismus. Middle East Afr J Ophthalmol, 2015. **22**:279-285.
2. Jeon, H., Jung, J.H., Yoon, J.A. & Choi, H. Strabismus is correlated with gross motor function in children with spastic cerebral palsy. Curr Eye Res, 2019.
3. Schiavi, C. Paralytic strabismus. Curr Opin Ophthalmol, 2000. **11**:318-323.
4. Flanders, M. Restrictive strabismus: Dignosis and management. Am Orthopt J, 2014. **64**: 54-63.
5. Kim, H., Li, Q., Hempstead, B.L. & Madri, J.A. Paracrine and autocrine functions of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in brain-derived endothelial cells. J Biol Chem, 2004. **279**:33538-33546.
6. Rocco, M.L., Soligo, M., Manni, L. & Aloe, L. Nerve growth factor: Early studies and recent clinical trials. Curr Neuropharmacol, 2018. **16**:1455-1465.
7. Cui, J., Zhou, B., Ross, S.A. & Zempleni, J. Nutrition, microRNAs, and human health. Adv Nutr, 2017. **8**:105-112.
8. Zendjabil, M., Favard, S., Tse, C., Abbou, O. & Hainque, B. The microRNAs as biomarkers: What prospects? C R Biol, 2017. **340**:114-131.
9. Gu, Q. Involvement of nerve growth factor in visual cortex plasticity. Rev Neurosci, 1995. **6**:329-351.
10. Khan-Malek, R. & Wang, Y. Statistical analysis of quantitative RT-PCR results. Methods Mol Biol, 2017. **1641**:281-296.
11. Gong, W., Qie, S., Huang, P. & Xi, J. Protective effect of mir-374a on chemical hypoxia-induced damage of PC12 cells In Vitro via the GADD45 α /JNK signaling pathway. Neurochem Res, 2018. **43**: 581-590.
12. Xia, Q., Zheng, Y., Jiang, W., Huang, Z., Wang, M., et al. Valproic acid induces autophagy by suppressing the Akt/mTOR pathway in human prostate cancer cells. Oncol Lett, 2016. **12**:1826-1832.
13. Holen, I., Lefley, D.V., Francis, S.E., Rennicks, S., Bradbury, S., et al. IL-1 drives breast cancer growth and bone metastasis in vivo. Oncotarget, 2016. **7**:75571-75584.
14. Xu, J., Lü, X.W., Huang, Y., Zhu, P.L. & Li, J. Synergism of simvastatin with losartan prevents angiotensin II-induced cardiomyocyte apoptosis in vitro. J Pharm Pharmacol, 2009. **61**:503-510.
15. Taggart, C.C., Cryan, S.A., Weldon, S., Gibbons, A., Greene, C.M., et al. Secretory leucoprotease inhibitor binds to NF-kappaB binding sites in monocytes and inhibits p65 binding. J Exp Med, 2005. **202**:1659-1668.
16. Kim, B. Western Blot Techniques. Methods Mol Biol, 2017. **1606**:133-139.
17. Wang, Y., Li, M., Dong, C., Ma, Y., Xiao, L., et al. Linc00152 knockdown inactivates the Akt/mTOR and Notch1 pathways to exert its anti-hemangioma effect. Life Sci, 2019. **223**:22-28.
18. Christiansen, S.P., Baker, R.S., Madhat, M. & Porter, J.D. Lengthening extraocular muscle with autologous muscle transplants. Strabismus, 1994. **2**:29-39.
19. Chen, J., Chu, Y.F., Chen, J.M. & Li, B.C. Synergistic effects of NGF, CNTF and GDNF on functional recovery following sciatic nerve injury in rats. Adv Med Sci, 2010. **55**:32-42.