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# The trophic effect of ciliary neurotrophic factor on injured masseter muscle in rat

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ABSTRACT

***Objective(s):*** Occlusal trauma is one of the most common forms of oral biting dysfunction. Long-term occlusal trauma could weaken the stomatognathic system, especially damage one's masticatory muscle. Through using the rat model, this study investigated the trophic effect of ciliary neurotrophic factor (CNTF) on injured masseter muscle.

***Materials and Methods:*** Male Wistar rats (n=36) were randomly divided into five experimental groups and one control group (6 rats per group). Animals in the experimental group were cemented modified crowns on their mandibular first molars to artificially induce occlusal trauma in 1, 3, 7, 14, and 28 days. Control group was sham-treated with forced mouth-opening for about 5 min, while no crowns were placed. After 28 days of treatment, all rats were euthanized and their masseter muscle was collected. Through immunofluorescence and real-time quantitative PCR, the expression of desmin, CNTF, and CNTFRα was investigated in rat masseter muscle. The microstructure of masseter muscle was observed by transmission electron microscope.

***Results:*** The expression of desmin showed a time-dependent decrease on traumatic and non-traumatic sides masseter, until reached the nadir at the 14th day, then restored to its normal level at the 28th day; however, the expression of CNTF and CNTFRα on the traumatic and non-traumatic sides increased from day 7, reached the peak at the 14th day, and returned to normal level on the 28th day.

***Conclusion:*** CNTF, as an important neurotrophic factor, was tightly associated to the restoring of rat injured masseter muscle, which provides new target and treatment method for clinical application.

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## Introduction

Occlusal trauma is one of the most common forms of mouth bite dysfunction (1). It is characterized with severe uncoordinated uncoordinated movements and always concentrated in one area; occlusal trauma may lead to the contraction of masticatory muscles, joint asymmetry, and other abn ormalities (2). The aim of our study was to investigate the trophic effect of ciliary neurotrophic factor (CNTF) on injured masseter muscle in rat model.

CNTF is a pluripotent neurotrophic factor originally isolated from chick embryo ciliary neurons (3). Currently, CNTF is the only known factor which shows direct trophic effects on muscle and nerve system, and may have therapeutic effects on motor neuron diseases, nerve damage, and muscular atrophy (4). It has been reported that CNTF is widely distributed in muscle and maintains its normal morphology and function, while participates in repairing injured nerve and muscle. CNTF may play a direct role in the physiological process of muscle contraction, and has been known as an important regulator of muscle strength during growth (5, 6). Subcutaneous injection of CNTF slows down the atrophy of muscle fiber and functional degradation. In addition, CNTF could regulate skeletal muscle's ability of taking up glucose and fatty acid by activating AMPK and PI3-kinase/Akt signaling pathways, which play an important role in energy metabolism in skeletal muscle (6-8). Studies have confirmed that CNTF plays a vital role in skeletal muscle regeneration (9). However, related reports on whether CNTF has trophic effect on injured masseter muscle restore is not yet available.

As a specific receptor of CNTF, CNTFRα, which was previously thought to be restricted in neural tissue, has been recently found to be expressed in skeletal muscle at high level (6). CNTFRα binds to cytomembrane, and will be cleaved by the membrane phospholipid. Moreover, CNTFRα knockout mice

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showed severe loss of function in motor system, and died immediately after birth (10). In this study, CNTFRα was investigated to explore the mechanism of CNTF.

Muscle cells contain several intermediate filament (IF) proteins, such as desmin, vimentin, nestin, synemin, syncoilin, lamins, and cytokeratins (11). Desmin is the major muscle-specific IF protein, which plays an important role in maintaining the normal structure of muscle cells (12, 13). Furthermore, their main function is to anchor intracellular structures (14). Studies have shown that desmin deficiency is a sensitive indicator of skeletal muscle microdamage (15, 16). In this study, the expression of desmin was used as an indicator to reflect the extent of masseter muscle injury.

CNTF has correlations with masticatory muscle and muscular movement pattern; meanwhile, occlusal trauma would alter bilateral masticatory muscle and muscular movement pattern. Further research on these problems would lead to a better understanding of the trophic effect of CNTF on injured masseter muscle, and suggest new targets and treatment methods in clinical practice. The goal of the study is to investigated the trophic effect of ciliary neurotrophic factor (CNTF) on injured masseter muscle in rat.

## Materials and Methods and

### *Animal preparation*

Male Wistar rats (n=36) from Shandong University Animal Center, China, with an average weight of 200-250 g were applied. Rats were housed at 25-28 ˚C with a 12 hr light/dark cycle and were given food and water on time. All efforts were made to minimize animal's suffering and to reduce the numbers of animals used.

### *Grouping and modeling*

Rats were divided into five experimental groups and one control group (6 rats in each group) with a random number table. The cemented modified crowns method made from Co–Cr alloy on the mandibular first molars were applied for the experimental groups rats to induce occlusal trauma, in 1, 3, 7, 14, and 28 days (Figure 1). The thickness of the crowns was 1 mm, which was designed to cover the 4/5 surfaces as described by Ye Cao *et al* (17). Control group was sham-treated with forced mouth-opening for about 5 min, but no crowns were applied.

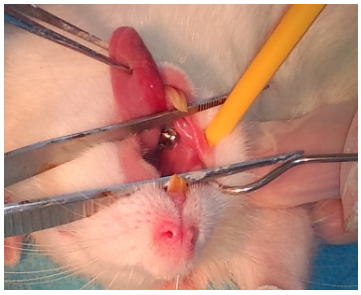

**Figure 1.** Cemented modified crown on the mandibular first molar

### *Detection method*

*Transmission electron microscope*

The microstructure of injured masseter muscle was observed under transmission electron microscope (TEM) (JEM-2100F, JEOL, Japan).

*Immunofluorescence*

The morphology of masseter muscle was confirmed through detecting the marker protein desmin by immunofluorescence staining method. Specimens were washed with Phosphate buffered saline (PBS) three times and fixed with 4% paraformaldehyde. Cell membranes were permeabilized with ice cold 0.3% Triton X-100 for 10 min and then incubated in blocking solution containing PBS and 0.5% (m/v) bovine serum albumin (BSA) for 1 hr. Cells were incubated with desmin antibodies (1:600, Santa Cruz, America), after which incubated with fluorescein (FITC)–conjugated goat anti-rabbit secondary antibody (Zhongshan, Beijing, China) for 30 min. Then, sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI), (Zhongshan, Beijing, China) and visualized under immunofluorescence microscope (Olympus CX-RFL-2, Tokyo, Japan).

*Real-time quantitative PCR*

Total RNA was extracted from masseter tissues using TRIzol (Takara, Dalian, China) based on the manufacturer's protocol. SYBR Prime Script™ RT reagent kit (Takara, Dalian, China) was used to synthesize the first strand cDNA by reverse transcription. The target mRNA levels in specimens were analyzed by quantitative real-time PCR through SYBR Green I dye (Takara, Dalian, China). Sequences of primers used for PCR are listed in Table 1.

The amplifications were conducted three times under the LightCycler 480 QPCR system (Roche Diagnostics Ltd., Bern, Switzerland). Every gene was normalized against the corresponding GAPDH levels and other sections genes expression was fold change ($2^{-\Delta\Delta Ct}$) using the control group as calibrator.

### *Statistical analysis*

Normally distributed variables were expressed as means±SD. Unpaired Student's t-test or ANOVA were used to compare differences between groups. The differences were statistically significant at $P<0.05$ using SPSS statistical software package version 17.0.

## Results

### *Microstructure of the masseter under transmission electron microscope*

The changes of the masseter muscle tissue at different time points were observed under TEM. Shrinked nuclear membrane and uneven chromatin appeared at the 3rd day on traumatic side of the experimental groups (Figure 2 (B)). Large irregular

**Table 1.** Sequences of primers used for real-time quantitative PCR

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| CNTF | TCGTTCAGACCTGACTGCTCTTATG | GGTACGGTAAGCCTGGAGGTTC |
| CNTFRα | CTTGGAGAGCATCTGGTGGT | TTGGGGTAAGGTTGGAACGG |
| Desmin | GGACATCCGTGCTCAGTATGAG | TATTGGCTGCCTGAGTCAAGTC |
| β-actin | CATTGCTGACAGGATGCAGAAG | GAGCCACCAATCCACACAGAGT |

morphology of mitochondria, mitochondrial degeneration, nuclear membrane depression, and obvious chromatin condensation into coarse lumps were observed at the 7th day on traumatic side of the experimental groups; damages also appeared on the non-traumatic side which were not severe (Figure 2 (C and D)). Disappearance of the bright band, shortening of the dark band, broadening of the Z line, shortening of the sarcomere, margination of the chromatin with crescent-shape, condensed nuclear, disappearance of the cell junctions were also observed at the 14th day on traumatic side of the experimental groups (Figure 2 (E)). Chromatin margination can be found at the 14th day on non-traumatic side of the experimental groups (Figure 2 (F)). 28 days group showed abnormal performance (Figure 2 (G)).

***Characterizations of desmin in immunofluorescence staining experiment***

Under fluorescence microscope, red ray in muscle cell was positively stained by desmin antibody and represents the change of desmin. As displayed in Figure 3 (A, B, C, E, G, and H), a time-dependent decrease of desmin expression on traumatic side masseter was detected, reached the nadir at the 14th day, and then restored to its normal level at the 28th day. Decreased and irregular expression of desmin at 7th and 14th days on non-traumatic side masseter also appeared (Figure 3 (D and F)).

***Expression of CNTF and CNTFRα***

Compared with the normal masseter muscle, the presence of tan particles around or in the neuron indicates the positive expression of CNTF, brown granules in masseter muscle represent the positive expression of CNTFRα. The expression of CNTF and CNTFRα the traumatic and non-traumatic sides increased at the 7th day, reached the peak at the 14th day, and returned to normal level at the 28th day (Figure 4 and 5).

***Mean Integral optical density (IOD) of desmin, CNTF, and CNTFRα***

We found a time-dependent decrease of desmin expression on traumatic side masseter, until reached the nadir at the 14th day, then restored to its normal level at the 28th day; the expression of desmin at the 7th and 14th days on group's non-traumatic side masseter was reduced, either. The expression of CNTF and CNTFRα on the traumatic and non-traumatic sides increased at the 7th day, reached the peak at the 14th day, and returned to normal level at the 28th day (Figure 6).

The results showed that: the mean Integral optical density (IOD) at the 3rd day on traumatic side began to decline, reached the lowest point at the 14th day, and got to normal level at the 28th day. 3rd, 7th, and 14th days in traumatic side and 7th and 14th days in non-traumatic side were significantly different from the control group ($P$<0.05); at the 3rd, 7th, and 14th days, the expression of desmin was significantly different on traumatic side from that of non-traumatic side ($P$<0.05). The mean IOD of CNTF and CNTFRα on the traumatic and non-traumatic sides increased at the 7th day, reached the peak at the 14th day, and returned to normal level at the 28th day; The traumatic and non-traumatic sides at the 7th and the 14th days in experimental groups were significantly different from the control group ($P$<0.05); the expression of CNTF on traumatic side at the 7th and 14th days in experimental groups were significantly different from non-traumatic side ($P$<0.05).

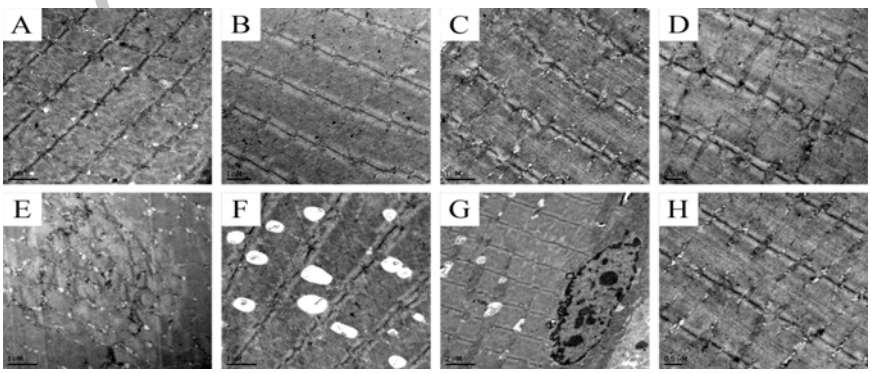


**Figure 2.** The microstructure of masseter. A: the traumatic side at the first day, B: the traumatic side on day 3, C: the traumatic side on day 7, D: the non-traumatic side on day 7, E: the traumatic side on day 14, F: the non-traumatic side on day 14, G: the traumatic side on day 28, and H: the control group

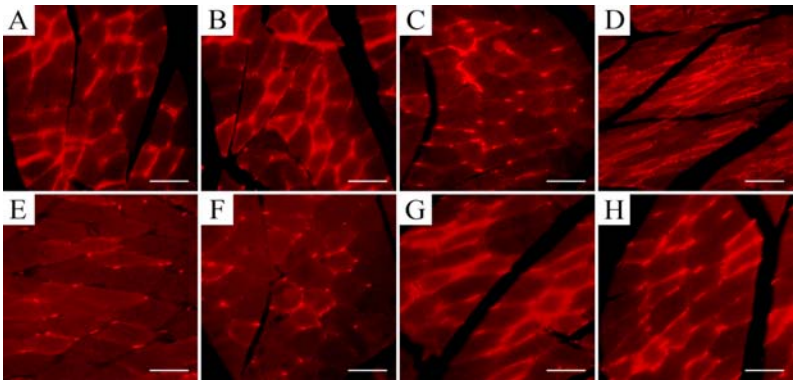


**Figure 3.** The expression of desmin. A: the traumatic side on first day, B: the traumatic side on day 3, C: the traumatic side on day 7, D: the non-traumatic side on day 7, E: the traumatic side on day 14, F: the non-traumatic side on day 14, G: the traumatic side on day 28, and H: the control group. Bar=100 μm

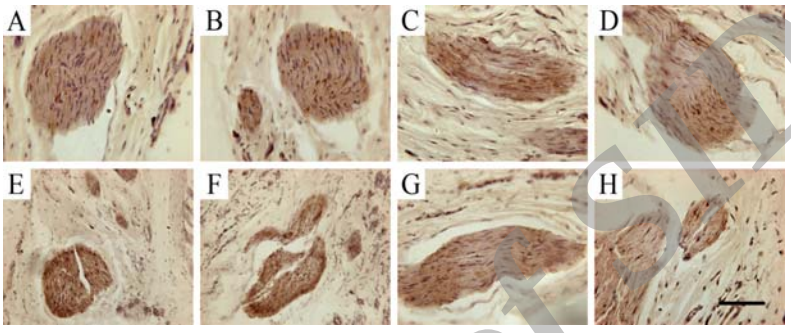


**Figure 4.** The expression of CNTF. A: the traumatic side on the first day, B: the traumatic side on day 3, C: the traumatic side on day 7, D: the non-traumatic side on day 7, E: the traumatic side on day 14, F: the non-traumatic side on day 14, G: the traumatic side on day 28, and H: the control group. Bar=100 μm

### *Real-time quantitative PCR*

We showed a time-dependent increase of desmin expression in traumatic side masseter; its maximum of expression observed on day 14, then restored to its normal level on day 28. It is also showed that the expression of desmin increased on day 7 and 14 on non-trauma side masseter. The expression of CNTF and CNTFRα on the traumatic and non-traumatic sides increased on day 7, reached the peak on day 14, and returned to normal level on day 28 (Figure 7).

The average expression of desmin mRNA increased from day 3 and reached the peak on day 14 then restored to the average level on day 28. Traumatic side, at the 3$^{rd}$, the 7$^{th}$, and the 14$^{th}$days and the non-traumatic side, at the 7$^{th}$ and the 14$^{th}$ days were significantly different from the control group ($P$<0.01). The mRNA expression of desmin on traumatic side on days 3, 7, and 14 was significantly different from non-traumatic side ($P$<0.05). The mean mRNA of CNTF and CNTF"=R increased from day 7, then reached peak on day 14, and finally returned to almost the normal level at the 28$^{th}$ day. The traumatic side on days 7 and 14 and the non-traumatic side on day 14 were significantly different from those in control group ($P$<0.01); the mRNA expression of CNTF on traumatic side at the 7$^{th}$ and the 14$^{th}$ days was significantly different from non-traumatic side ($P$<0.05).

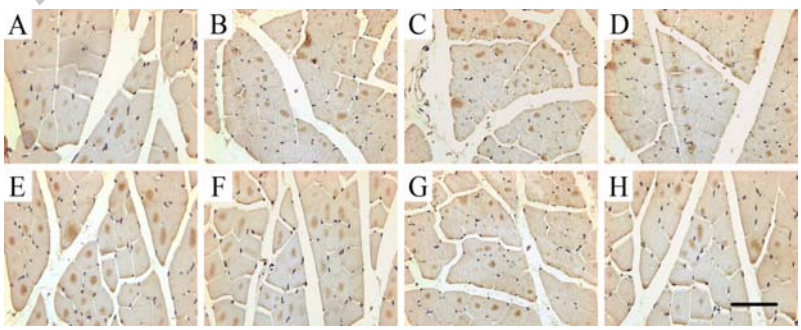


**Figure 5.** The expression of CNTFRα. A: the traumatic side on the first day, B: the traumatic side on day 3, C: the traumatic side on day 7, D: the non-trauma side on day 7, E: the traumatic side on day 14, F: the non-traumatic side on day 14, G: the traumatic side on day 28, and H: the control group. Bar=100 μm

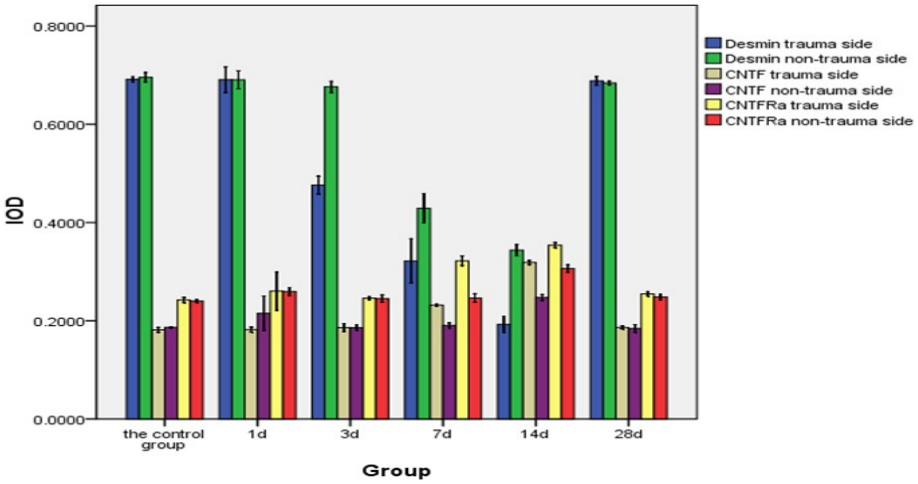


**Figure 6.** The mean Integral optical density (IOD) of desmin, CNTF, and CNTFRα

## Discussion

It has been reported that premature contact spots of occlusion can cause rat occlusal trauma (18). In this study, we applied the method of cemented modified crowns on the mandibular first molars of experimental rats to establish animal model. We observed the damage of masseter muscle under TEM, for obtaining more intuitive and visible images. Furthermore, the validity of the results was confirmed by conducting real-time RT-PCR. Both of the occlusal trauma and the non-traumatic sides were observed at the first, 3rd, 7th, 14th, and 28th days, to acquire the ever-changing results. We found that the aforementioned method of inducing traumatic occlusion is feasible, and there are many molecules which could repair such minor injuries, such as CNTF which was the focus of our experiment.

We take the expression of desmin as the evaluation index of damaged masseter muscle in this study. The reason is that mice with desmin deficiency can lead to skeletal muscle disease (19-21); desmin knocked out can cause abnormal formation of costameres and embryo with a variety of postnatal skeletal muscle myopathies (22). In the present study, the expression of desmin showed a time-dependent decrease in traumatic side masseter, until the nadir on day 14, and then restored to its normal level on day 28. The result showed that the damage of masseter in traumatic side begins on day 3 after the operation, but similar symptoms in non-traumatic side appeared on day 7; both sides muscle damages were most severe at the 14th day, and almost restored to normal level at the 28th day. The expression of desmin mRNA also confirmed the phenomenon. Because of the Temporomandibular joint (TMJ) as a linkage joint, once one side is damaged, the other side will be affected later; therefore, when the trauma side muscle was injured, the other side will be also affected. We could see that the change trend of desmin is associated with the microstructure of masseter muscle under TEM. According to our results, using desmin as an indicatorto describe the degree of masseter muscle damage is feasible.

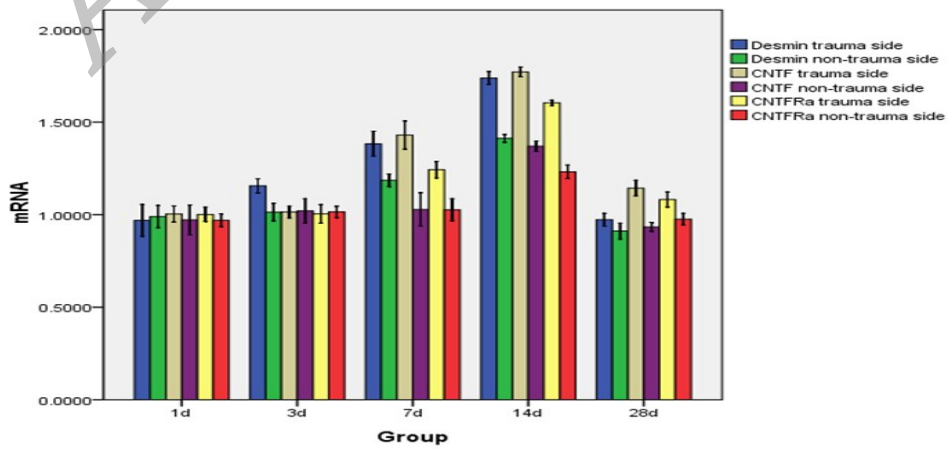


**Figure 7.** The mean mRNA expression of desmin, CNTF, and CNTFRα

Occlusal trauma resulted in noxious stimulation of the nerve endings in the periphery of masseter muscle, and passing the signals to the central nervous system (CNS) via activating neurochemicals. After receiving the signals, various instructions of CNS issues, such as CNTF's secreting and gathering occurred to resist tissue injury. Moreover, CNTF could activate JAK/STAT pathway, and STAT protein to form a dimeric protein compound, which could be transported into the nucleus to bind to DNA, initiate transcription, and thus result in damage resistance. CNTF can also regulate glucose and fatty acid uptake in skeletal muscle via AMPK and PI3-kinase/Akt signaling pathways (7, 23) and prevent prostaglandin E2 (PGE2) and creatine kinase (CK) from releasing, which play a vital role in energy metabolism in skeletal muscle (24). Studies have shown that CNTF could re-regulate skeletal myoblast's differentiation through p44/p42 MAPK signaling pathway, in order to promote myoblasts differentiation into multipotent progenitor cells (25); multipotent myogenic progenitor cells could increase MYF5 and MyoD expression via more than 20 pathways and differentiate into new phenotypes, mainly neurons, glial cells, smooth muscle cells, and fat cells.

In this experiment, the expression of CNTF and CNTFRα were synchronized, while, the expression of desmin, which was the mark to measure the degree of masseter muscle injury, was reversed. Based on our results CNTF may participate in restoring injured masseter muscle. CNTF has a broad spectrum of nutritious effect mainly through regulation of several enzymes activity and recovering of damage, which could not only promote the expression of proteins and some key molecules, but also accelerate axonal transport, and the repairing processes (6). Moreover, CNTF could inhibit apoptosis via multiple pathways, such as activating of STAT proteins in the nucleus, inducing specific gene expression, reducing the synthesis of Caspase3, and inhibiting the function of activated Caspase3 (7).

According to the results of real-time quantitative PCR, expression of CNTF and desmin were closely related. However, the change of desmin was earlier than CNTF and CNTFRα, which indicates that the injury of masseter muscle was anterior than the restoring effect of CNTF. CNTF keeps normal morphology and function of skeletal muscle via its direct nutritious effect on muscle, so plays a vital physiological effect on muscle contraction (2), and is involved in the repairing process after the nerve and muscle damage.

The expression of CNTF and the masseter muscle damage were closely related; it appears in the injured masseter muscle and participates in the repair of the injury of the masseter muscle in rat.

## Conclusion

CNTF is involved in the process of reconstruction of the damaged masseter muscle which is caused by occlusal trauma in rats. This experiment studied the trophic effect of CNTF on masseter injury in rat, while the specific mechanism still need to be further studied.

## Acknowledgment

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## Conflict of interest

The authors confirm that there is no conflict of interest to disclose.

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