• Basic Research •

Protective effects of ciliary neurotrophic factor on the retinal ganglion cells by injure of hydrogen peroxide

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Abstract

• AIM: To explore the effect of ciliary neurotrophic factor (CNTF) on retinal ganglion cell (RGC)-5 induced by hydrogen peroxide (H_2O_2).

• METHODS: After cell adherence, RGC-5 culture medium was changed to contain different concentrations of H_2O_2 from 50 to 150 μmol/L at four time points (0.5, 1, 1.5 and 2h) to select the concentration and time point for H_2O_2 induced model. Two different ways of interventions for injured RGC-5 cells respectively were CNTF as an addition in the culture medium or recombinant lentiviral plasmid carrying CNTF gene transfecting bone mesenchymal stem cells (BMSCs) for co-culture with RGC-5.

• RESULTS: Compared to the control group, H_2O_2 led to RGC-5 death closely associated with concentrations and action time of H_2O_2 and we chose 125 μmol/L and 2h to establish the H_2O_2-induced model. While CNTF inhibited the loss of RGC-5 cells obviously with a dose-dependent survival rate. Nevertheless two administration routes had different survival rate yet higher rate in recombinant lentiviral plasmid group but there were no statistically significant differences.

• CONCLUSION: Both the two administration routes of CNTF have effects on RGC-5 cells induced by H_2O_2. If their own advantages were combined, there may be a better administration route.

• KEYWORDS: retinal ganglion cells; ciliary neurotrophic factor; hydrogen peroxide; neuroprotection; recombinant lentiviral vector

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INTRODUCTION

Glaucoma is a multifactorial eye disease worldwide leading to blindness characterized by retinal ganglion cells (RGCs) degeneration[1-3]. Visual formation involves pallium pillow of brain, eyeball and visual pathway and RGCs are the unique link to transport visual information between the two organs[4-7]. Furthermore, RGC axons play an important role in transporting nutrients within cycles through cell body and terminal[8]. As RGCs is axonal damage and steady loss, glaucomatous optic neuropathy progresses with gradual vision loss and visual field deficits[9-10].

Several risk factors of glaucoma are recognized including age, family history, elevated intraocular pressure (IOP) and myopia[11-12]. Among them abnormally elevated IOP is considered as a vital risk factor of visual function deficits[12-13]. Clinical therapies focus on decreasing IOP to normal tension even the lower with eye drops or operations. Nevertheless these managements are not enough to prevent RGCs death[14]. In addition, eye drops have limitations such as poor compliance due to frequency and low bioavailability due to various barriers while outflow pathway blockade relapses several years after surgery[15]. Given this, gene therapy rises gradually in sorts of experiments.

Several nerve growth factors (NGFs) are confirmed to benefit to cultured RGCs such as brain derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) after injury[16-18]. However these peptides with short half-life have immediate effects[19]. If recombinant viral vector is conducted, survival rate of injured RGCs *in vitro* and *in vivo* increases in a considerable time owning to sustained NGFs especially CNTF[20-22]. Recent advances elucidate CNTF stimulates gene expression, cell proliferation and neural stem cell differentiation as well as provokes neuroprotection and axons regeneration[23-26]. And CNTF is reported to elicit biological effects for under several pathological conditions[25,27-28].

Mechanisms in relation to RGC destruction in glaucoma are currently deemed to incompetent axon transport of neurotrophic factors, reactive oxygen species (ROS), excitoxicity and chronic intermittent ischemia[29-30]. Among above factors, the interplay between ischemia and ROS for the formation of vicious circle induces RGCs death[29,31-32]. Besides ROS as a direct damage element of glaucomatous neurodegeneration, it also may participate in activating signal pathway to RGCs death as a

second messager[33-34]. On the base of the pathology theory, we adopted hydrogen peroxide (H_2O_2) modeling on RGC-5 cell and focused on CNTF effect on injury model.

MATERIALS AND METHODS

Retinal Ganglion Cell-5 Identification by Immunofluorescence RGC-5 cells were cultured by dulbecco's modified eagle medium (DMEM) medium supplemented with 10 mL of 10% fetal bovine serum, 1 mL of 10^5 U/L penicillin and 1 mL of 100 mg/L streptomycin in each 100 mL medium. Cells were located in 5% CO_2 incubator at 37℃ every 2d to change solution. The cell line RGC-5 at logarithmic phase was selected in the culture flask with 5% bovine serum albumin and 0.1% Triton X-100 in 0.01 mol/L phosphate buffered saline (BSAT) instead of medium at 4℃ over night. Then mixture of 5% BSAT solution and first antibody, Brn3a (Cell Signaling Technology. Inc., Boston, USA), with a proportion of 1:100 was added into flask without medium also at 4℃ over night. The following day poured away mixture, washed flask for five minutes by six times with 1×phosphate buffered saline and 0.1% Triton X-100 (PBST) and then added mixture of 5% BSAT solution and second antibody, Cy3 (Cell Signaling Technology. Inc., Boston, USA), with a proportion of 1:500 at indoor temperature for 2h.

Foundation of Lentiviral Expression Vector Carrying Ciliary Neurotrophic Factor Gene The target gene synthesis with upstream primer and downstream primer (Shanghai GeneChem Company. Ltd., Shanghai, China) was amplified for purified CNTF gene segments in polymerase chain reaction (PCR). Recombinant vector contained CNTF gene and plasmid GV287 (Shanghai GeneChem Company. Ltd., Shanghai, China) (Figure 1). The forward primer sequences of CNTF was 5′-GAG GAT CCC CGG GTA CCG GTC GCC ACC ATG GCT TTC ACA GAG CAT TC-3′ and the reverse sequence was 5′-TCC TTG TAG TCC ATA CCC ATT TTC TTG TTG TTA GC-3′. The gene sequencing was conducted on the modified gene (pGC-FU-CNTF). After exact sequenced identification CNTF protein expression in 293T cells was assayed by Western blot. Correctly constructed recombinant plasmid of CNTF transfected bone mesenchymal stem cells (BMSCs) to detect virus titre by limited dilution. The viral titer of pGC-FU-CNTF was 1×10^8 TU/mL. Working solution with 10 μL of the virus suspension was to transfect BMSCs.

Hydrogen Peroxide Injury Model of Retinal Ganglion Cell-5 RGC-5 cells were seeded on 60-well plates in 5% CO_2 incubator at 37℃ for 24h to follow-on (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) experiment if inoculated cells were growth well. Experimental groups was set up by H_2O_2 concentration as follow: 0 (control group), 50, 75, 100, 125 and 150 μmol/L. Cell proliferation was performed at four time points, respectively, 0.5, 1, 1.5 and 2h. An appropriate concentration was chosen for model on basis of MTT results.

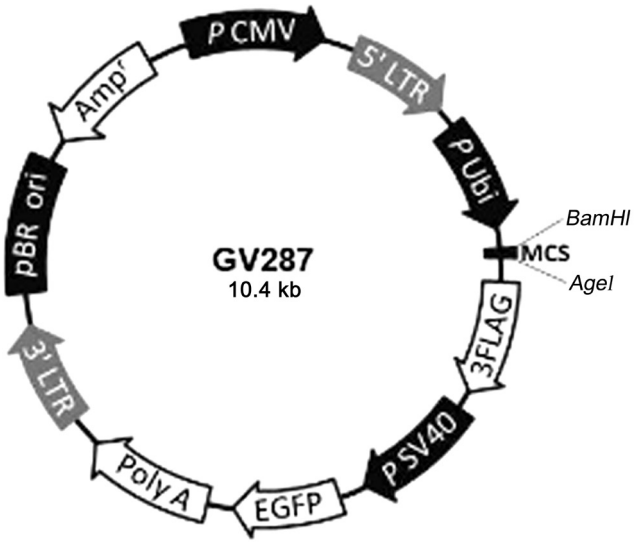

Figure 1 Plasmid GV287 diagram The circular plasmid containing AgeI cleavage site loaded with exogenous CNTF gene to construct recombinant plasmid.

Real-time Polymerase Chain Reaction While RGC-5 cells cocultured with BMSCs carrying recombinant plasmid, CNTF gene expression and neuroprotective agents such as glial cell line-derived neurotrophic factor (GDNF) and BDNF gene expression, measured by Real-time PCR at 3 and 7d versus control group without recombinant plasmid. Total RNA was extracted from cultured cells with TRIzol reagent (Shanghai Pufei Biotech Co., Ltd., Shanghai, China) according to the kit instructions. cDNA synthesis was implanted by reverse transcription by virtue of RNA PCR protocol (Promega Biotech Co., Ltd., Beijing, China). Glyceraldehyde phosphate dehydrogenase (GAPDH) was acted as a normalizing control. The several primers were as follows: CNTF: forward: 5′-CCA CCT ACA TCC CCA ATA CC-3′; reverse: 5′-GCT GAC ACT TAT GGA GAC CTT G-3′; BDNF: forward: 5′-GGT TAT TTC ATA CTT CGG TTG C-3′; reverse: 5′-CCC ATT CAC GCT CTC CAG-3′; GDNF: forward: 5′-CGA TAT TGT AGC GGT TCC TGT-3′; reverse: 5′-CCT TGT CAC TTG TTA GCC TTC T-3′; GAPDH: forward: 5′-TTC AAC GGC ACA GTC AAG G-3′; reverse: 5′-CTC AGC ACC AGC ATC ACC T-3′ (Genechem Co., Ltd., Shanghai, China). The PCR reaction system was performed in a volume of 25 μL using the SYBR Master Mix (Toyobo Co., Ltd., Shanghai, China) on the MX3000p real-time PCR instrument (Agilent Technologies Inc., California, USA). In SYBR Green PCRs the melting curve was in the 60℃-95℃ range to evaluate amplification products. Relative quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method.

Detection of Cell Viability by Application of Ciliary Neurotrophic Factor by MTT Assay To explore CNTF efficacy to H_2O_2 injury model of RGC-5, each groups were measured by MTT assay. Experimental groups including: pGC-FU-CNTF group, 50 ng/mL CNTF group, 100 ng/mL CNTF

group, 150 ng/mL CNTF group, 200 ng/mL CNTF group and 250 ng/mL CNTF group acted on RGC-5 damage model while control group was without any treatment. Two effective approach to carry drugs, direct administration of CNTF and synthesis by pGC-FU-CNTF, had different administration times such as one day and seven days, respectively. The reason of different times was that pGC-FU-CNTF required for synthesis and secretion while the former one did not. After administration of CNTF, H_2O_2 intervened for same time then RGC-5 cell viability was measured by MTT assay.

Statistical Analysis Statistics were described by mean±standard deviation (SD). The differences between groups were analyzed by one-way ANOVA. *P* values less than 0.05 were considered statistically significant differences.

RESULTS

Culture and Identification of Retinal Ganglion Cell-5 RGC-5 cells showed logarithmic growth and the cell revealed long spindle in shape. Anti-Brn3a protein (1:100) for immunohistochemical identification of high-purity cultured cells fluoresced red under a inverted phase contrast microscope (Figure 2).

Hydrogen Peroxide Induced Retinal Ganglion Cell-5 Model Along with H_2O_2 concentration increasing from 50 to 150 μmol/L, RGC-5 cell viability showed a decreasing trend generally, and then had an obvious decrease of the cell viability at 150 μmol/L. In view of H_2O_2 was an acknowledged and effective modeling method and we done the curve with H_2O_2 in order to find a proper concentration and time point for our study. We ultimately decided to choose 125 μmol/L and 2h as the condition to establish model accounted for 23.20% cell death (Figure 3).

Efficacy of pGC-FU-CNTF Transfers into Bone Mesenchymal Stem Cells Immunofluoresent assay was conducted to detect CNTF gene transferring efficacy in BMSCs. It was obvious that BMSCs with lentiviral vector carrying CNTF gene glow green fluorescent owing to green fluorescent protein junctional of recombinant plasmid (Figure 4A). RGC-5 cells were elongated spindle shape against the wall under a fluorescent microscope.

On the other hand, CNTF mRNA expression and production increased over time rather than control group with RGC-5 only. The mRNA expression levels of CNTF on day 7 (817.35±177.40) were higher than those in day 3 (8.30±2.64, $P<0.05$) and control group (1.01±0.18, $P<0.05$; Figure 4B).

Since BMSCs enable to secret various growth factors, as carriers they trigger upregulation of BDNF, GDNF expression and so on. Compared with control group, there was significant upregulation of BDNF gene expression in BMSCs additional groups at 7d (332.29±146.23, $P<0.05$) while GDNF gene expression was not significant in any experimental groups (Figure 4C, 4D). And elevated levels of BDNF expression was in accord with prolonged time.

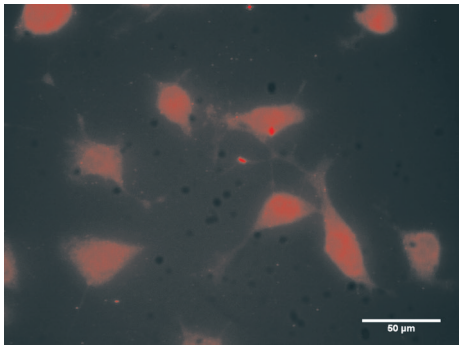

Figure 2 RGC-5 identification by immunofluorescence The detection of Brn3a showed strong red fluorescence staining revealing cell shape (×400).

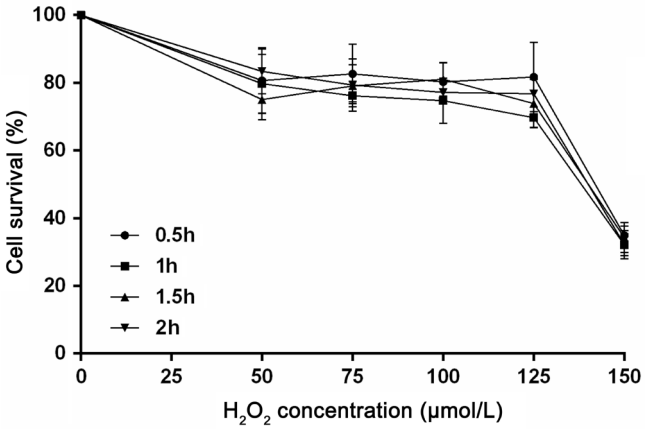

Figure 3 A cell viability assay of RGC-5 cells subject to different concentrations of H_2O_2 in several times by MTT Cell survival rate was in inverse proportion of H_2O_2 concentrations and intervened time. Longitudinal changes in H_2O_2-induced RGC-5 cell death at 0.5, 1, 1.5 and 2h increased along with concentrations.

Ciliary Neurotrophic Factor Effect of Two Ways Against Hydrogen Peroxide Induced Retinal Ganglion Cell-5 Model Two ways of CNTF as follow one was addition of CNTF in the culture medium directly and the other one was recombinant plasmid carrying CNTF gene while they were adopted with its own advantages such as certain dosage and continuous pharmic infusion. As shown in Figure 3, 125 μmol/L H_2O_2 alone was toxic to the cells causing at most 30.24% cell death while the effects of CNTF differentiated to some extent on resistance to oxidative damage. In the first day the survival rate of RGC-5 cells with additional CNTF had no significant difference among every CNTF concentration groups. When RGC-5 cells cultured in second day, the low level of CNTF groups (50 and 100 ng/mL) had statistic difference with high concentration groups (150, 200 and 250 ng/mL) in cell viability. However there were no difference in every high concentration groups. And in third day the results were same as the previous day. Only 50 ng/mL group had difference with the groups of the other concentrations in fourth day. While analyses from all groups revealed no difference five days later (Figure 5). Therefore we chose 150 ng/mL to intervene for three days and

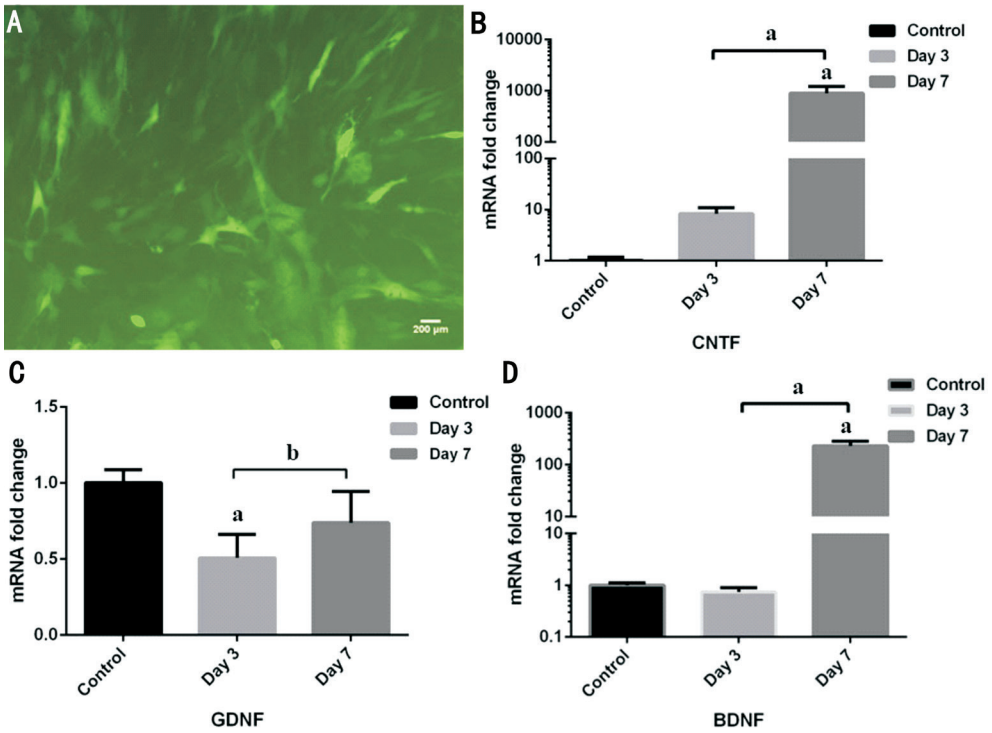

Figure 4 Transfection efficiency of recombined lentivirus in BMSCs A: Representative confocal image of BMSCs immuolabelled with green fluorescence protein for its gene was transferred into BMSCs by lentivirus vector (×100). B-D: Real-time PCR confirmed mRNA expression levels of three neurotrophic factors in day 3, day 7 groups after recombined lentivirus transfection and control group. B: CNTF gene expression in day 7 group had statistically significant differences versus the other two groups; C: GDNF gene expression in day 3 group was below control level with significant differences but no significant differences was found between intervened groups; D: The increased BDNF gene expression in day 7 group had statistically significant differences versus the other two groups. [a]P<0.05, [b]P>0.05 (one-way ANOVA followed by post hoc comparisons).

this method of administration would be compared with the approach by recombinant lentivirus.

Both 150 ng/mL CNTF and recombinant plasmid made a difference on resistance to oxidative damage and on protection of RGCs. As comparison with the optimal concentration group of 24.57% cell death, recombinant plasmid group reduced cell death to 19.23% with no statistically significant differences (P>0.05; Figure 6).

DISCUSSION

RGCs, as a precious medium to visual formation, reduce in glaucoma and there is no available remission as far. In many researches, NGF are confirmed to protective factors for injured RGCs as well as our study demonstrated that CNTF had a positive effect on RGC-5 cell death induced by H_2O_2. Taken together, the acknowledged mechanisms of RGCs death are as follows: 1) neurotrophic factors deficiency due to invalid axonal transport; 2) glutamic toxicity; 3) free radical concentration; 4) nitric oxide toxicity; 5) apoptosis[35]. H_2O_2, one member of ROS as same as free radical, was used to make model in the study to cause RGC-5 loss by oxidative stress. The negative correlation between H_2O_2 concentration and RGC-5 survival lay in the irreversible cell injure model. H_2O_2 is danger to RGC-5 and is pathogenic microenvironment to

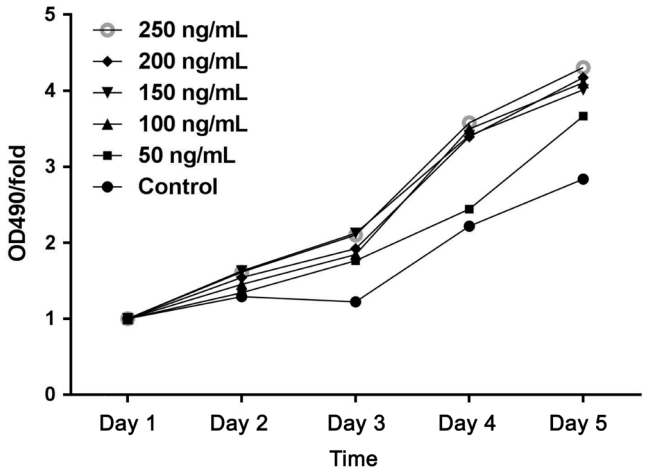

Figure 5 Dose- and time-dependent changes in the numbers of RGCs represented by OD490 value in the other groups versus day 1 Whether with CNTF addition or not OD490/fold value increased linearly in all groups with cultivation time.

disease. Complicated and various etiological factors always are unlikely indicated in detail, what we can do is to improve microenvironment as far as possible. So then, the aid of protective agents can yet be regarded as a novel option. For RGC-5 involved with vision, it is critical to make full use of CNTF.

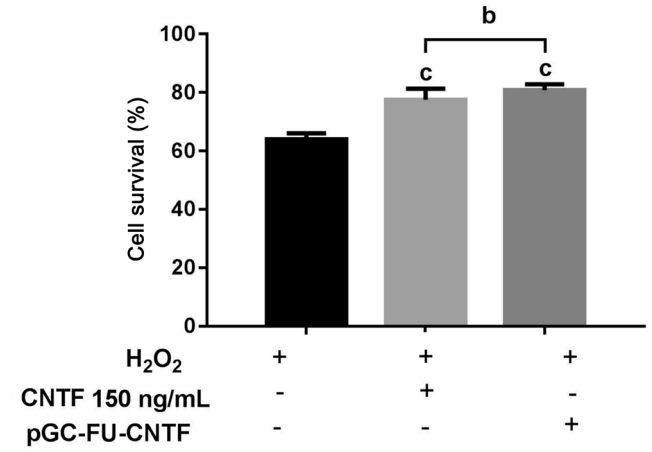

Figure 6 RGC-5 cell survival rate in different treatments for H_2O_2 injure H_2O_2 injure only had significant differences with two experiment groups-CNTF 150 ng/mL given in the culture media directly and by recombinant plasmid. However there was no significant differences between the experiment groups. $^bP>0.05$ (one-way ANOVA followed by post hoc comparisons); $^cP<0.001$ *vs* control.

In the course of exploring appropriate H_2O_2 concentration to intervene, gradually increasing concentration brought about a rise in the number of dead cells but the correlation applied in the range of 50 to 150 μmol/L. Once H_2O_2 concentration is above 150 μmol/L, the mortality rate of RGC-5 obviously increased with rare survival cells. Even if the relative survival rate was about 33.36% in 150 μmol/L group at each time, maybe the surrounding microenvironment abounding in ROS was too terrible to exist. At the beginning, RGC-5 was in outset of cell degeneration and overmuch ROS finally induced irreversible cell damage. Hence early detection and diagnosis of RGC related opthalmopathy (*e.g.* optic neuropathy) are worth preventing hypopsia and blindness. Eventually we chose 125 μmol/L H_2O_2 for 2h to establish model. Because there are very low cell survival rate when the concentration of H_2O_2 reaches 150 μmol/L and it is available to assure cellular activity in the follow-up study.

The advantage of direct intervention was known dose while recombinant lentiviral plasmid provided continuous CNTF. If *in vitro* and *in vivo* experiments selected a proper CNTF, an implantable drug delivery system in eyes can conduct in the above dose. A positive correlation was confirmed between a rise of CNTF concentration and RGC-5 survival rate. CNTF function in neuron *via* heterotrimeric complex that is ciliary neurotrophic factor receptor-α (CNTFRα) the only one part anchored at surface membrane, gp130 and leukemia inhibitory factor-β (LIFRβ)[36]. CNTF binds CNTFRα at the first step then does the heterodimer of gp130 and LIFRβ[37-39]. Whereas RGCs are unable to secrete proteins, the choice of BMSCs as medium was because not only it had the stable biological characteristics but also could secrete CNTF itself. Based on the influence of CNTF on cell model we could select the optimal concentration which was a hint for *in vivo* experiments by intravitreal injection or implanted intraocular secretion apparatus.

In general, CNTF is an effective neurotrophin for RGCs through sorts of signal pathways acting on recipient cells. Special process about how CNTF plays a pivotal role is still an open question.

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Conflicts of Interest: Wang WJ, None; **Jin W,** None; **Yang AH,** None; **Chen Z,** None; **Xing YQ,** None.

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