· Original article ·

Protective effects of ciliary neurotrophic factor on retinal neurons in rats with early diabetes

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Received:2012-09-11 Accepted:2012-10-22

Abstract

• AIM: To evaluate the protective effects of ciliary neurotrophic factor (CNTF) against retinal neurons damage in a rat model of diabetes mellitus(DM).

• METHODS: Forty Sprague Dawley rats (weighing 250g-280g) received an intraperitoneal injection of streptozotocin (60mg/kg). The rats were then randomly divided into two groups: the treatment (DM+CNTF) or control (DM+BSS) group. The CNTF (0.5μg/μL) or balanced salt solution (2μL) was injected into the vitreus of the rats. Apoptosis of retinal ganglion cells (RGCs) was measured and retinal ultrastructure was assessed 4 and 12 weeks after establishing the model and performing the injections.

• RESULTS: No difference in blood glucose or body weight was noted between the two groups. There were significantly fewer apoptotic RGCs in DM+CNTF group (8.56±1.22, $P<0.05$). From week 4, the neurons in the two groups of rats had degenerative changes (*i. e.*, shrunken membrane disk spaces, swollen photoreceptor nuclei, and chromatin condensation) were noted on transmission electron microscopy. In DM+CNTF group, these abnormalities were improved by week 12.

• CONCLUSION: CNTF has no significant effects on blood glucose levels or body weight in either group. CNTF shows protective effects on RGCs and photoreceptors based on apoptosis and transmission electron microscopy. Additional studies to establish the optimal time of administration and dosage of neurotrophic factors to achieve the greatest protective effects on retinal nerve tissue are warranted.

• KEYWORDS: ciliary neurotrophic factor; diabetic retinopathy; neuropathology; rat; apoptosis
DOI:10.3969/j. issn. 1672-5123. 2012. 11. 01

Citation: Liang ZY, Zhang MN. Protective effects of ciliary neurotrophic factor on retinal neurons in rats with early diabetes. *Guoji Yanke Zazhi*(*Int Eye Sci*) 2012;12(11):2045-2049

INTRODUCTION

Ciliary neurotrophic factor (CNTF) is an acidic protein with a molecular weight of 22-26kDa. This factor was named because it was initially found in the ciliary body of chickens and could maintain the survival of the parasympathetic ganglion of chicken. CNTF has a variety of biological activities. Notably, NCTF can promote the survival of a variety of nerve cells[1]. Currently, research involving drug treatments for diabetic retinopathy (DR) primarily focuses on growth factors involved in the formation of new blood vessels. With an increased understanding of the role of the nervous tissue of the retina in DR, the protective effects of neurotrophic factors on these nervous tissues are also becoming the focus of research efforts. In the present study, a streptozotocin-induced rat model of diabetes was established and rats were treated with exogenous recombinant rat CNTF. Protective effects of CNTF on the nervous tissue of the retina at an early stage of diabetes were observed, which may provide an experimental basis for exploring drugs with protective effects on DR.

MATERIALS AND METHODS

Materials A closed population of experimental Sprague Dawley rats ($n=40$) weighing 250g-280g were provided and raised by the Experimental Animal Center of PLA General Hospital. Streptozotocin (Sigma, USA) was used to establish the experimental rat model of diabetes by selectively destroying pancreatic β cells. The rats were administered an intraperitoneal injection of STZ at a dose of 60mg/kg in the right, lower abdomen. Model construction was considered successful if the blood glucose levels were >16.7mmol/L 48 hours after drug administration and if polydipsia and polyuria were noted[2]. The rats were randomly divided into either a treatment (DM+CNTF, $n=20$) or control group (DM+BSS, $n=20$). Blood glucose and body mass were measured at specific time points for the remainder of the 12-week study period.

Methods

Administration of CNTF or BSS After model construction, DM rats were anesthetized with an intraperitoneal injection of 0.8mL/kg sumianxin injection solution (a combination of haloperidol, xylidinothiaxoline and dihydrotestosterone etorphine, PLA University of Agriculture and Animal Husbandry and Veterinary Research Institute). The pupil of the right eye of each rat was dilated using tropicamide compound eye drop (Beijing Double-Crane Pharmaceutical Company). Using a surgical microscope, a 5μL microinjector was pierced into the eyeball wall 0.5mm posterior to the corneal limbus in the superior temporal quadrant. The microinjector was then directed

Table 1 Changes in body mass and blood glucose levels in rats included in DM+BSS and DM+CNTF groups

Time	DM+BSS ($n=20$)		DM+CNTF ($n=20$)	
	Body mass(g)	Blood glucose(mmol/L)	Body mass (g)	Blood glucose (mmol/L)
0 week	258.32±18.42	4.32±1.23	262.58±11.91	4.44±1.22
4 weeks	278.20±25.57	27.66±3.41	284.68±21.35	28.44±2.60
8 weeks	346.94±24.17	26.62±2.46	351.52±23.89	27.34±1.28
12 weeks	370.23±20.43	26.98±2.30	360.25±26.73	27.92±1.72

into the vitreous cavity with its tip pointing toward the optic nerve. After slowly injecting 0.5μg/μL CNTF (made by dissolving recombinant CNTF freeze-dried powder [Peprotech Asia Company] dissolved balanced salt solution [0.5μg/μL]) into the vitreous cavity in DM+CNTF group, the microinjector remained in the vitreous cavity for 30 seconds before its slow withdraw. Chlortetracycline eye ointment was applied at the conjunctival sac. Rats in DM + BSS group underwent the identical procedure except only 2μL BSS solution was injected. The two groups of rats received intravitreal injections once every 4 weeks for 12 weeks.

Measurement of Apoptosis Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was used to detect the number of apoptotic retinal ganglion cells (RGCs). Four rats (7 eyes) were randomly selected from DM+CNTF and DM + BSS groups 4 weeks and 12 weeks after model construction. Five slices of the retina were cut consecutively from the optic disc toward the ora serrata. Three independent visual fields were randomly selected from each slice (areas measured 0.2mm×0.2mm). Apoptotic cells were identified based on the brown staining of the nucleus. Fluorescent TUNEL staining was performed and fluorescence - labeled apoptotic RGCs in the ganglion cell layer were observed under a fluorescence microscope.

Ultrastructure of the Retina One eye was randomly selected from DM+CNTF and DM+BSS groups 4 weeks and 12 weeks after model construction and was fixed in glutaraldehyde. The eye was incised 0.5mm posterior to the corneal limbus and the retina was peeled off, placed in phosphate buffer, fixed with 10g/L osmium tetroxide, and washed with phosphate buffer. Gradient alcohol dehydration was subsequently performed and the samples were embedded in epoxy resin. Semi-thin sections with a thickness of 1μm were cut and positioned under the light microscope. Ultra-thin sections were then made and stained with urany acetate and lead citrate. The sections were examined using a transmission electron microscope (JEM - 1230 Japanese Electronics Company).

Statistical Analysis SPSS13.0 statistical software was used for data analysis. The normality of data was tested and t-tests were performed. Experimental data were summarized as mean ± standard deviation and $P < 0.05$ was considered statistically significant.

RESULTS

Body Mass and Blood Glucose The body mass of rats in both DM + CNTF and DM + BSS groups decreased with advancing age, but there was no significant difference between the two groups ($P > 0.05$). After model construction, the average blood glucose level of DM+CNTF group and DM+BSS group were above the blood glucose criteria for the models (>16.7mmol/L, $n=40$). As summarized in Table 1, there was no significant difference in blood glucose levels between the two groups ($P > 0.05$). No endophthalmitis occurred following the intravitreal injections in DM + BSS and DM + CNTF groups. Typical physical signs of diabetes including polydipsia, polyuria, polyphagia, pruritis, hair loss, weight loss, and reduced physical activity gradually developed in the diabetic rats in two groups as the disease progressed. Different degrees of cataracts developed in two groups of rats approximately 9 weeks after establishing the model.

Apoptosis of Ganglion Cells Apoptosis of RGCs with tawny brown staining after TUNEL was noted in the retinas from rats in both DM + CNTF and DM + BSS groups 4 weeks after establishing the model, but there was no significant difference between the two groups ($P > 0.05$). Apoptosis of RGCs increased in DM + BSS group by week 12. Apoptotic RGCs with tawny brown staining showed a band-like distribution in the ganglion cell layer (Figures 1A and B), which was significantly different from the apoptotic RGCs noted in DM+CNTF group ($n=7$, Figure 2). Fluorescence-labeled RGCs in both groups showed positive expressions under fluorescent microscope at 4 weeks. The positive yellow - green signals were significantly at 12 weeks compared with those signals noted after 4 weeks (Figures 3A and B). Positive apoptotic RGCs in the two groups had similar distributions and, in some areas of the DM+CNTF group, expression of positive signals was less.

Transmission Electron Microscopy No significant changes in the structure of the retina were noted in DM+BSS group at week 4. Chromatin in the photoreceptor cells was homogenous and there were a large number of mitochondria in the oval body, which were arranged neatly along the periphery. The outer segment disk membrane was arranged regularly, but the local gap was widened slightly and local dissolution was seen. No significant changes were observed between DM + CNTF group and DM+BSS group. At week 12, the membranous disc gap of the outer segment was significantly expanded in DM+BSS group and loosening, fracture, and dissolution occurred (Figure 4). Chromatin aggregation and condensation of the photoreceptor cells were observed and cell deformation and dissolution occurred (Figure 5). Structural abnormalities of the ganglion cells were noted in addition to mitochondrial

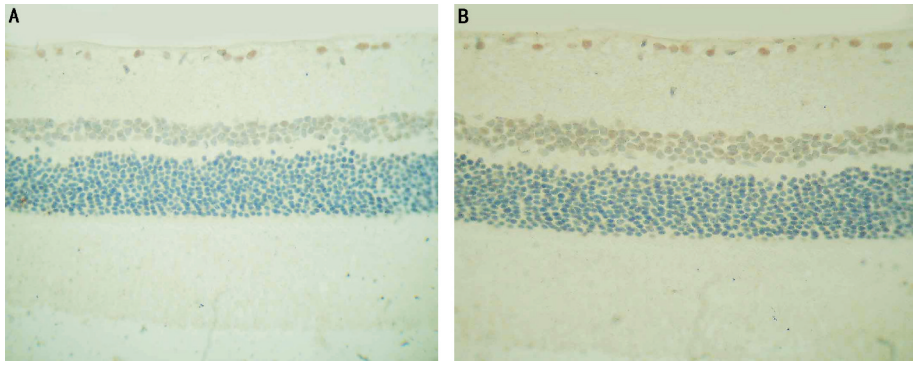

Figure 1 TUNEL staining on retinal cells at week 12 A: DM+CNTF group; B: DM+BSS group (original magnification×400).

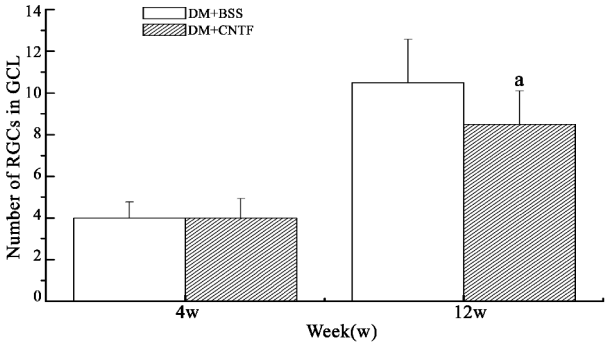

Figure 2 Apoptosis of RGCs measured in rats in DM+BSS and DM+CNTF groups at week 4 and 12 of the study $^{a}P<0.05$ ***vs*** **DM+BSS group.**

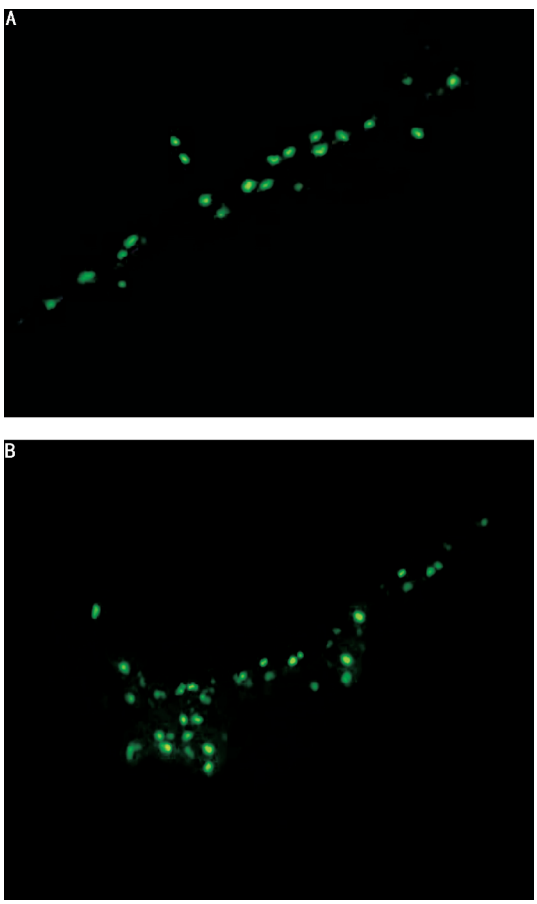

Figure 3 Apoptotic RGCs fluorescently stained by TUNEL at week 12 A: DM+CNTF group; B: DM+BSS group (original magnification ×400).

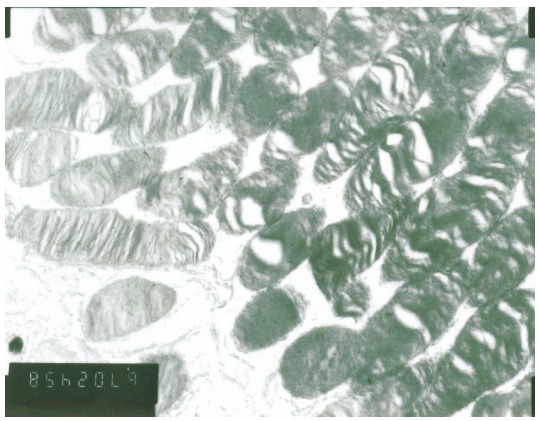

Figure 4 By week 12, the membranous disc gap of the outer segment was significantly expanded in DM + BSS group. Loosening, fracture, and dissolution also occurred (transmission electron microscopy, original magnification ×8000).

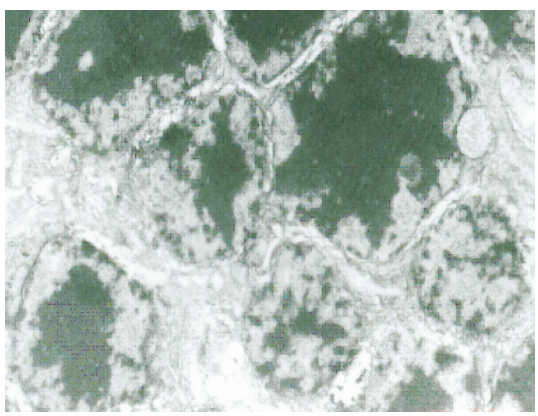

Figure 5 By week 12, chromatin aggregation and condensation of the photoreceptor cells were observed and cell deformation and dissolution occurred (transmission electron microscopy, original magnification ×8000).

swelling. The pathological changes in DM+CNTF group and DM + BSS group were similar, but the changes in the photoreceptor cells and outer segment disk membrane in DM+CNTF group were less significant (Figures 6, 7).

DISCUSSION

Currently, there is an increased understanding of the protective role of neurotrophic factor in DR. Further, there is growing evidence that the incidence of DR is related to interactions

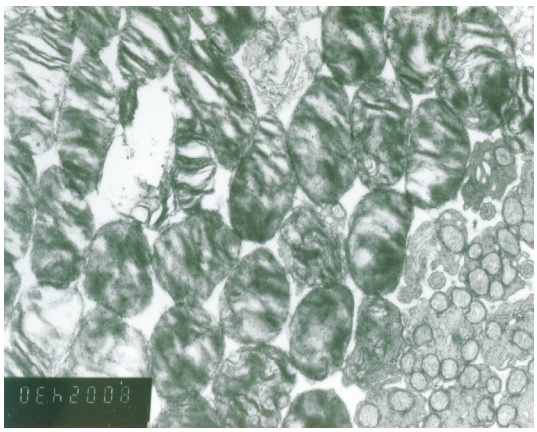

Figure 6 The membranous disc of the outer segment of the retina in DM + CNTF group at week 12 of the study (transmission electron microscopy, original magnification × 8000).

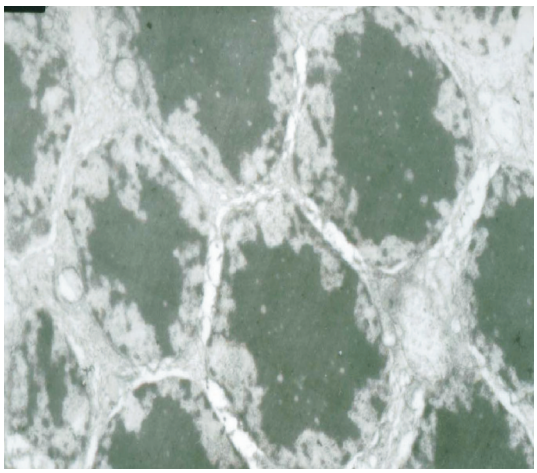

Figure 7 Appearance of the photoreceptor cells in DM+CNTF group at week 12 of the study (transmission electron microscopy, original magnification ×8000).

among neurotrophic factors. One study found that brain-derived neurotrophic factors (*i. e.*, basic fibroblast growth factor, and neurotrophin-4) may prevent the degeneration of retinal photoreceptor cells, enhance photoreceptor cell repair after injury, promote the development of RGCs, and stimulate the axonal regeneration of ganglion cells[3]. In a study on DR, Seki *et al*[4] found that exogenous BDNF could prevent degeneration of amacrine cells and promote their survival. CNTF is a neurotrophic factor that does not belong to the neurotrophic factor (NGF) family[1]. It has a variety of biological activity *in vivo* and mainly acts on the nervous system. For example, it has extensive survival-promoting effects on a variety of central and peripheral neurons.

CNTF can cause weight loss in mice[5] and can significantly reduce fasting and postprandial blood glucose levels in diabetic mice, improve oral glucose tolerance test results, and alleviate hyperinsulinemia[6]. In the present study, the body mass of the rats in the two groups showed a tendency to decrease slowly over the study period as the rats aged, but there was no significant difference in the body mass or blood glucose levels between the two study groups. These results suggested that CNTF does not cause any significant effects on blood glucose levels or body weight in diabetic rats in this study. This finding is not consistent with previous reports. It is possible that the concentration of CNTF injected into the vitreous cavity in this study was low and that this low dose of CNTF did not cause systemic effects. From another perspective, the results indicated the stability of model construction of the diabetic rat model and the safety of this treatment method.

In 1999, Cho *et al*[7] studied the implantation of a segment of peripheral nerve after intraorbital optic nerve transaction. That study showed that in the CNTF treatment group, RGCs had regenerated axons extending into the peripheral nerves. Further, the number of axons was about 4 times higher than the number measured in the control group. LaVail *et al*[8] found that intravitreal injection of CNTF slowed the degeneration of photoreceptors in RD (retinal degeneration) and nr (nervous) mice with Q344ter mutation. Those results suggested that CNTF can protect photoreceptors of animal models with the same or similar genes as those responsible for human inherited retinal degeneration.

In the present study, two groups of TUNEL-labeled ganglion cells were significantly different at 12 weeks, suggesting that CNTF had a protective effect on ganglion cells of diabetic rats. Transmission electron microscopy revealed that at 12 weeks, the membranous disc gap of the outer segment of the retina was significantly expanded in DM+BSS group and that loosening, fracture, and dissolution occurred. Chromatin aggregation and condensation of the photoreceptor cells were observed and cell deformation and dissolution were also noted. The pathological changes in DM+CNTF group and DM+BSS group were similar, but the changes in the photoreceptor cells and outer segment disk membrane in DM+CNTF group were less significant than that in DM+BSS group. Together, this data indicated that the photoreceptor cells of rats in the treatment group received some protection from the CNTF.

In recent years, some researchers have studied the effects of CNTF in delaying the loss of photoreceptor cells and preserving the survival of ganglion cells. For example, Peterson *et al*[9] injected a CNTF analogue, axokine, into the vitreous cavity. This caused phosphorylation of STAT3 and MAPK and delayed up-regulation of STAT3 and STAT1 proteins in the retina. Most activated STAT3 are concentrated in the nucleus of retinal Müller cells, ganglion cells, and astrocytes. Although CNTFRα mRNA and protein are mainly concentrated in the retinal neurons, STAT3 signal transmitted by CNTF can be observed in both glial cells and neurons. Wahlin *et al*[10] reported that after intravitreal injection of CNTF in C57BL/6J mice, the immunoreactivity of kinase (pERK), and the immunoreactivity of c-fos in Müller cells and ganglion cells rapidly increased; however, photoreceptors were not stained for either pERK or c-fos. These findings indicated that CNTF affects photoreceptors through indirectly activating Müller cells or other non-photoreceptor cells.

In summary, CNTF appears to protect retinal ganglion cells and photoreceptor cells in diabetic rats. The rats were treated

with recombinant rat CNTF, which may result in different or reduced effects compared to other forms of CNTF. Therefore, further studies are required to study the optimal time of administration and dosage of neurotrophic factors in order to achieve the greatest protective effects on retinal nerve tissue.

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睫状神经营养因子对糖尿病早期大鼠视网膜神经组织的保护作用

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摘要

目的: 研究睫状神经营养因子(ciliary neurontrophic factor, CNTF)对糖尿病早期大鼠视网膜神经组织的保护作用。

方法: 选择40只健康成年雄性SD大鼠(250g-280g)一次性腹腔注射链脲佐菌素(Streptozotin ,STZ) 60mg/kg诱发糖尿病模型(DM),将DM大鼠随机分组为DM+CNTF组和DM+BSS组。DM+CNTF组给予玻璃体腔内注射CNTF(1.0μg/2μL),DM+BSS组注射BSS液(2μL)。分别观测0、4 、8、12wk两组大鼠体质量和血糖变化,于4wk和12wk进行原位细胞调亡(TUNEL法)的检测及视网膜神经组织超微结构的观察。

结果: DM+CNTF组大鼠的血糖和体质量与DM+BSS组比较无显著性差异($P>0.05$)。12wk时TUNEL检测DM+CNTF组大鼠神经节细胞凋亡与DM+BSS组相比减少($P<0.05$)。透射电镜下观察发现从4wk起两组大鼠视网膜神经组织出现细胞凋亡的改变,经CNTF治疗细胞凋亡改变有所减轻,表现为外节膜盘间隙减小,感光细胞水肿减轻及核染色质浓集减轻等。

结论: CNTF对DM+CNTF组和DM+BSS组大鼠的体重及血糖无明显影响。CNTF治疗组结果显示对本实验糖尿病大鼠视网膜神经节细胞及感光细胞有一定保护作用。

关键词: 睫状神经营养因子;糖尿病视网膜病变;神经病变;大鼠;细胞凋亡