Original Article

Expression of Sirt1 and Sirt2 in the Injured Optic Retina of Calorie-Restricted Rats

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Abstract

Purpose: To investigate retinal ganglion cell (RGC) survival after optic nerve injury in calorie-restricted (CR) rats, and analyze the potential role of Sirtuins.

Methods: Ten-month old male Sprague-Dawley rats (n=14) were divided into calorie restricted (CR) and ad libitum (AL) groups. In the CR group (n=7), the rats were denied access to food every other day. Animals in the AL group (n=7) had had free access to food. PN-ON grafting was carried out on the right eye of all subjects after 5 months of feeding. Three weeks postoperatively, retina samples were collected, half of which were fixed in 4% paraformaldehyde (PFA) and subjected to standard immunofluorescence staining for TUJ-1. The remaining samples were subjected to total RNA analysis and RT-PCR for Sirt1 and 2 expression.

Results: Comparing the amount of TUJ-1 staining between the groups, the mean density and the total number of RGCs showed no significant difference. RT-PCR results indicated that mRNA expression of Sirtuin2 in the CR group was significantly lower than that in the AL group, whereas no statisti cally significant difference was observed between the two groups regarding the mRNA expression of Sirt1.

Conclusion: Calorie restriction had no impact on the survival of injured RGCs. The down-regulated mRNA expression of Sirt2 in the CR group may indicate an improved capacity for regeneration among these animals, but more work is needed to explore this possibility.(***Eye Science 2011; 26:221-224***)

Keywords: calorie restriction; sirtuins; retinal ganglion cell

The repair capacity of injured retinae decline with age. Calorie restriction (CR) has been commonly utilized in an attempt to prevent aging. The longevity gene family Sirtuins serve as a vital factor in mediating the anti-aging effect of CR. In this study, we investigated the survival capacity of retinal ganglion cells (RGCs) and the changes in Sirtuins expression in CR rat models with retinal injuries, attempting to analyze whether Sirtuins exert an anti-aging effect upon the retina of CR rat models.

Materials and methods

Reagent and instrument

Mouse anti-rat TUJ-1 monoclonal antibody (BabCO, USA); CY3 secondary antibody (Jackson Immuno, USA); reverse transcription kit (TAKARA, Japan); total RNA extract kit Nucleo Spin RNAII (MN, Germany); forward and reverse primers (Shang hai Invitrogen, China); DNA 50 bp ladder (Invitrogen, USA); Leica fluorescence microscope (Leica, Germany); gel-imaging system (Biorad, USA); 3130 PCR device (ABI, USA); Platinum Taq (Invitrogen, USA).

Animal grouping and feeding

Fourteen SD rats aged 10 months (Shanghai Slac Animal Laboratory, China), weighing (300±10)g on average, were randomly divided into CR and ad libitum (AL) groups. In CR group (n=7), the rats were denied access to food every other day. Those animals in the other group (n=7) had AL food ac-

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cess. All animals were fed for five months.

Surgical procedure

The rats underwent PN-ON surgery in their right eyes as below: complete anesthesia was induced using Ketamine and Xylazine at a ratio of 1∶1. Upper eyelid, upper cornea and extraocular muscle were cut open, exposing the optic nerve. The optic nerve, located approximately 2 cm behind the eyeball, was cut off. A 1.5 cm-autologous nerve was grafted at the near-end of the optic nerve cut, and the distal end of the peroneal nerve was fixed on the skull. Aureomycin was used to prevent infection. The treated animals were kept and fed for another three weeks after suture.

Perfusion and sampling of experimental animals

The rats were sacrificed under complete anesthesia by using chloral hydras. The thoracic cavity was opened, the pericardium cut open, and a tube inserted into the left ventricle, and the right atrium ear cut open. Physiological saline was used for perfusion until the effluent fluid was clear and bright. The eyeballs were taken out, the retinae were peeled under a dissecting microscope, and then immersed in lysate for total RNA-extract analysis. The fixed retinae were further perfused with 4% paraformaldehyde; the obtained retinae were fixed in 4% paraformaldehyde for one hour.

Immunofluorescence analysis

Retina samples were placed into a 24-well plate for immunoreaction, closed by a goat-serum working solution at room temperature for 30 minutes, and then removed. Mouse anti-rat TUJ-1 (1∶400) was then added and they were incubated overnight in a swing bed at 4℃; thoroughly washed by PBS three times (five minutes for each); supplemented by a CY3 secondary antibody (1∶400); incubated in a swing bed for one hour; section sealed and then observed, numbered and photographed under a fluorescence microscope.

RT-PCR analysis

RT-PCR was employed for total RNA extracts in retinal tissues, 150 mg RNA was used for cDNA synthesis. Sirtuins 1 and 2 were amplified by PCR, 18s was used as an internal reference. The sequence of primers is shown in Table 1, below. PCR products were subjected to 2% agarose gel electrophoresis, gel-imaging system photograph, and gray value analysis by Quantity One.

Table 1 The sequences of primers

Genes	Forward primer (5′–3′)	Reverse primer (5′–3′)
Sirt1	CCAGATCCTCAAGCCATGT	TTGGATTCCTGCAACCTG
Sirt2	TACCCAGAGGCCATCTTTGA	TGATGTGTGAAGGTGCCGT
18S rRNA	ATTCCGATAACGAACGAGAC	GGCATCACAGACCTGTTATTG

Statistical analysis

SPSS 11.0 software was used for data analysis. An independent sample *t*-test was utilized for immunofluorescence cell counting. AVONA was employed to analyze RT-PCR gray values. $P<0.05$ was considered as statistically significant.

Results

Immunofluorescence analysis

TUJ-1 counting in two groups indicated that the average density of living RGCs was 175.145±34.153 mm^{-2} in the AL group and 168.989±46.495 mm^{-2} in the CR group. In addition, the total amount of living RGCs achieved 11682.138 ±2277.763/retina in the AL group, and 11271.491 ±3101.206/retina in the CR group. No significant difference was noted between two groups in terms of the total amount and the average density of RGCs, as shown in Figure 1, below.

RT-PCR

The expression of Sirt1 and Sirt2 were compared with that of internal reference. The results revealed that Sirt1 expression level had no significant difference from internal controls, and Sirt2 expression was evidently lower than internal controls, as indicated in Table 2, below.

Discussion

Heretofore, CR has been experimentally proven to be uniquely effective in delaying aging. Two methods are commonly applied. One is to feed animals in a limited quantity every day; the other is to feed animals every other day. It has been validated that these two methods can effectively extend life expectancy, and also enhance immune resistance to disease. In particular, the intermittent fasting method

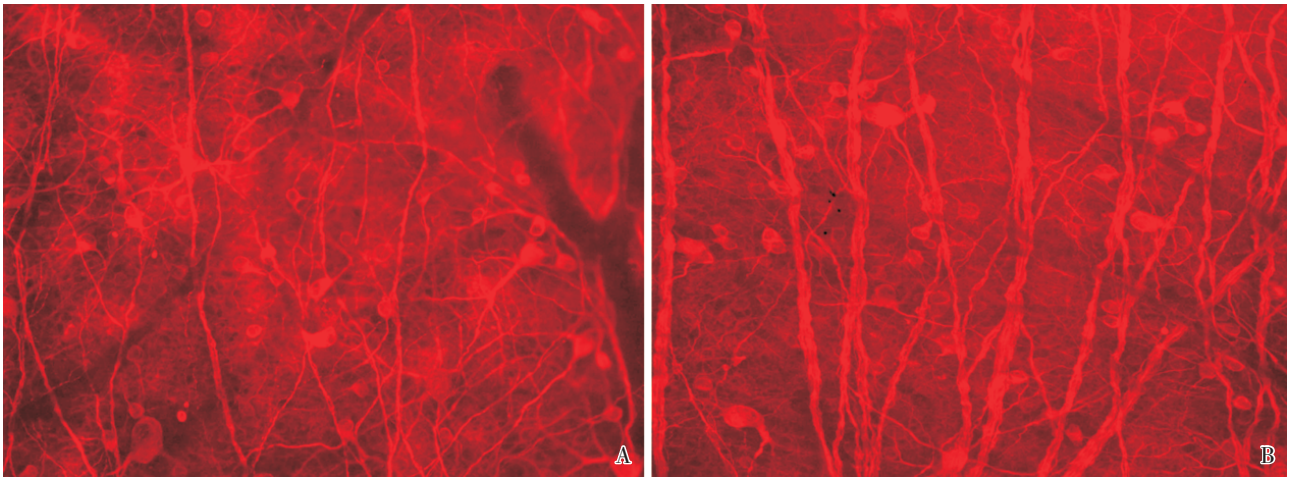

Figure 1 The survival conditions of RGCs by using immunofluorescence staining between AL and CR groups (× 200)

Table 2 The expression levels of Sirt1 and Sirt2 between the AL and CR groups

	Sirt1	Sirt2
AL	0.215±0.099	1.41±0.079
CR	0.213±0.095	1.15±0.153*

Note: * represents $P<0.05$

exerts a pronounced protective effect upon the optic nerve[1]; therefore, we adopted fasting on alternate days in this study.

The possible anti-aging effect of CR has been widely debated. Some scholars have suggested that the number of RGCs in CR-treated rats is higher than in normal controls[2]. For those rats with acute damage in RGCs induced by a steep elevation in intraocular pressure (IOP), the survival rate of RGCs in CR rats was significantly higher than that in their normal counterparts[3]. However, few studies focusing upon the protective effect of CR on retinae and optic nerves have been conducted. In addition, disparate results have been obtained by early investigations[4]. In the current study, the protective effect of CR was insignificant merely based upon the amount of RGCs alive, which might be related to the late age at which CR was administered. However, the evaluation of the protective effect on the optic nerve merely based on the number of surviving RGCs is not comprehensive. Immunohistochemical findings revealed that the rats in the CR group showed relatively bigger RGCs and thicker fibers, which requires stereoscopy investigation to further validate.

The Sirtuins family consists of an atypical category of NAD^+-dependent deacetylase, including seven family members (Sirt 1–7). Sirt1 and Sirt2 exert the most evident effect upon regulating neuro-degeneration. Sirt1 mainly gets involved in the deacetylation of histones H3, H4, transcription factor NF-kB, p53 and FoxO[5,6]. In addition, Sirt1 can also regulate genetic stability, cellular metabolism and apoptosis, etc. The up-regulation of Sirt1 is able to stimulate the survival of neuronal soma.

In this study, the changes in the level of Sirt1 mRNA were not evident. In combination with morphological observations, we found that CR exerted a limited effect upon the survival of RGCs, which differs from the findings reported by Shindler et al. Shindler utilized Srt501, an agonist of Sirt1, in rats optic neuritis and revealed that Sirt1 can prolong the survival time of RGCs. In the current investigation, dietary restriction was initially administered to middle-aged rat models (10 months old), whose RGCs were inclined to aging. CR is incapable of reversing aging activities, and is merely able to postpone the process of aging. Therefore, CR neither gives rise to a high expression of Sirt1 mRNA in retinae nor enhances the survival of RGCs during a short observation period.

This study also noted that the expression of Sirt2 mRNA was significantly down-regulated. Previous studies have indicated that neurons primarily expressed Sirt2 throughout the cell body in the neurites and growth cones[7], which is possibly associated with

axon growth. The application of an inhibitor of Sirt2 is capable of postponing the axon degeneration in Parkinson's disease models[8]. α-tublin, as a microtubules protein, serves as one among several major substrates of Sirt2. Acetylated α-tublin is primarily located in axon, which plays a vital role in extend ing axon[9]. Therefore, the down-regulation of Sirt2mRNA indicates that further studies should be performed to investigate axon regeneration.

Collectively, we identify no parallel relationship between CR and the survival and regeneration of RGCs. CR exerts a protective effect on injured RGCs mainly in the pattern of neuron regeneration. Nevertheless, morphological studies and further detection of alternative regeneration factors (GAP-43) should be conducted to validate these results.

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