# Involvement of Beclin-1 in axonal protection by short-term hyperglycemia against TNF-induced optic nerve damage

KANA SASE[1], YASUSHI KITAOKA[1,2], CHIHIRO TSUKAHARA[1,2] and HITOSHI TAKAGI[1]

[1]Department of Ophthalmology, St. Marianna University School of Medicine; [2]Department of Molecular Neuroscience, St. Marianna University Graduate School of Medicine, Kawasaki, Kanagawa 216-8511, Japan

Received April 16, 2018; Accepted September 24, 2018

DOI: 10.3892/mmr.2018.9568

**Abstract.** Beclin-1 serves a pivotal role in autophagosome formation. A previous study demonstrated that streptozotocin-induced hyperglycemia (HG) ameliorates axonal loss induced by tumor necrosis factor (TNF) with upregulation of autophagy in rats. The aim of present study was to examine whether Beclin-1 is involved in this autophagy machinery. Immunoblot analysis of optic nerves demonstrated that HG upregulated Beclin-1 protein expression when compared with normoglycemia (NG). Intravitreal administration of TNF did not alter the optic nerve Beclin-1 expression in NG nor in HG. Beclin-1 immunoreactivity was revealed to be mainly in astrocytes in optic nerves; however, it was also observed in the neurofilaments of the HG group. Morphometric analysis revealed that HG appeared to have substantial ameliorative effects on axon loss and this ameliorative effect was partially prevented by Beclin-1 small interfering RNA. These results indicated that Beclin-1 may exist in neurons and glia in optic nerves and increased Beclin-1 expression may be at least partially associated with axonal protection by HG.

## Introduction

Conflicting relationship has been demonstrated between glaucoma and diabetes mellitus (DM). For example, some previous studies showed that DM increases a risk for development of glaucoma (1,2). Conversely, other studies suggested that DM prevented glaucoma occurrence (3,4). Moreover, a recent study showed that DM is associated with glaucoma, but this association disappeared after adjustment for triglyceride levels (5). In the histological level, previous studies reported that short-term hyperglycemia (HG) preserves retinal structure in a transient high intraocular pressure-induced ischemic rat model (6) and a common carotid artery occlusion rat model (7). Other study demonstrated that HG condition prevents axon loss in an ocular hypertension rat model (8). Furthermore, a recent study has shown that subconjunctival applied glucose partially preserves retinal ganglion cell (RGC) somata in the transient high intraocular pressure-induced ischemic rat model and transiently increases contrast sensitivity in human subjects with severe primary open-angle glaucoma (9).

Our previous study found that short-term HG ameliorates tumor necrosis factor (TNF)-induced axon loss (10). Since a close relationship between TNF and glaucoma has been implicated (11-16), this TNF-mediated axon loss model may be helpful to clarify the molecular events by which axons are degenerated in RGCs (17). In optic nerves, the short-term HG enhances autophagy machinery (10). Autophagy plays central roles in the pathophysiology of several human diseases (18) and its impaired condition has been linked to neurodegenerative diseases (19-21). Among the autophagy-related (Atg) genes, microtubule-associated protein light chain 3 (LC3)/Atg8 is known as a maker for autophagosomes (22). Beclin-1/Atg6 constitutes Beclin-1 complex which is necessary for autophagic function (23). Up-regulation of Beclin-1 was shown in RGCs after optic nerve transection in rats (24). In addition, increased Beclin-1 protein levels were shown in retinal samples in the rat hypertensive glaucoma model (25) and a monkey hypertensive glaucoma model (26). However, its expression and localization in optic nerve have not yet to be demonstrated. In the present study, we tested whether Beclin-1 is involved in the ameliorative effect of short-term HG against axon loss caused by TNF.

## Materials and methods

*Animals*. The present study used 8-week-old male Wistar rats and was approved by Ethics Committee of the Institute of Experimental Animals of St. Marianna University School of Medicine. The rats were maintained in the controlled rooms (23±1˚C; humidity at 55±5%; light on 06:00 to 18:00).

*Streptozotocin-induced hyperglycemic (HG) rat model*. Single i.p. administration of physiological saline solution (PSS) or 60 mg/kg streptozotocin (STZ; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was carried out for the normoglycemic (NG) rats or the hyperglycemic (HG) rats, respectively. The plasma glucose levels were measured using a glucometer

*Correspondence to:* Dr Yasushi Kitaoka, Department of Ophthalmology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan
E-mail: kitaoka@marianna-u.ac.jp

*Key words:* autophagy, tumor necrosis factor, Beclin-1, optic nerve, hyperglycemia, streptozotocin

(Johnson & Johnson, Tokyo, Japan) 4 days after intraperitoneal injection. We only included the individuals as the HG group when plasma glucose exceeded 250 mg/dl. The plasma glucose levels of HG groups were 441.5±71.3, 435.0±32.3, and 432.1±73.3 mg/dl, immunoblot analysis, immunohistochemical analysis, and morphometric analysis studies, respectively.

*Intravitreal injection*. Intravitreal administration of 10 ng TNF (2 $\mu$l) was carried out into the right eye of rats under anesthetization with a combination of ketamine and xylazine. The left eye was received an intravitreal administration of phosphate-buffered saline (PBS). These intravitreal administrations were carried out 4 days following i.p. injection of PSS or STZ. In the HG group, a simultaneous intravitreal administration of 50 pmol Beclin-1 siRNA (Cell Signaling Technology, Inc., Danvers, MA, USA) with TNF was carried out into the right eyes.

*Immunoblot analysis*. Thirty-six rats (NG: 18 rats; HG: 18 rats) were euthanatized 1 week after intravitreal administrations for immunoblot analysis. Four mm optic nerves from immediately behind the eye ball were homogenized in protein extraction buffer. Since the optic nerve pieces were small, each sample included two optic nerves. Equal amount of proteins (3 $\mu$g) determined by the Bradford assay was applied and loaded. Then, samples were transferred to PVDF membranes. After blocking, membranes were exposed with the primary antibodies: Anti-Beclin-1 antibody for overnight (1:200; Medical & Biological Laboratories, Co., Ltd., Nagoya, Japan) or anti-β-actin antibody for 2 h (1:500; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). After three times washing, membranes were reacted with the secondary antibodies: Rabbit IgG or mouse IgG. Immunoblots were evaluated with the Amersham ECL detection system (GE Healthcare Life Sciences, Little Chalfont, UK).

*Immunohistochemical analysis*. Six rats were used for immunohistochemical analysis. One week following intravitreal administration, optic nerve samples were immersed in 10% neutral-buffered formalin. Paraffinized cross sections were made in 2 $\mu$m thick and incubated with 1% bovine serum. The primary antibodies were anti-Beclin-1 antibody (1:100; Medical & Biological Laboratories, Co., Ltd.), glial fibrillary acidic protein (GFAP, a marker of astrocytes; 1:200; Agilent Technologies, Inc., Santa Clara, CA, USA), and neurofilament-L (a marker of neurons; 1:100; Agilent Technologies, Inc.). The secondary antibodies were FITC-labeled and rhodamine-labeled IGG. The slides were mounted in 4',6-diamidino-2-phenylindole-including medium (Vector Laboratories, Ltd., Peterborough, UK).

*Morphometric analysis*. Fourteen rats (NG: 4 rats; HG:10 rats) were euthanatized 2 weeks after intravitreal administration for axon morphometric analysis (10,27). Optic nerve samples were immersed in Karnovsky's solution for overnight. After embedded in plastic blocks, cross thin sections were made beginning 1 mm from the eye ball. The sections were stained with 1% paraphenylen-diamine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Five images (center and periphery in quadrant per optic nerve) were acquired and quantified using an image-processing software (Aphelion, ADCIS, Hérouville Saint-Clair, France). The average of axon number in each optic nerve was expressed as the number per mm$^2$.

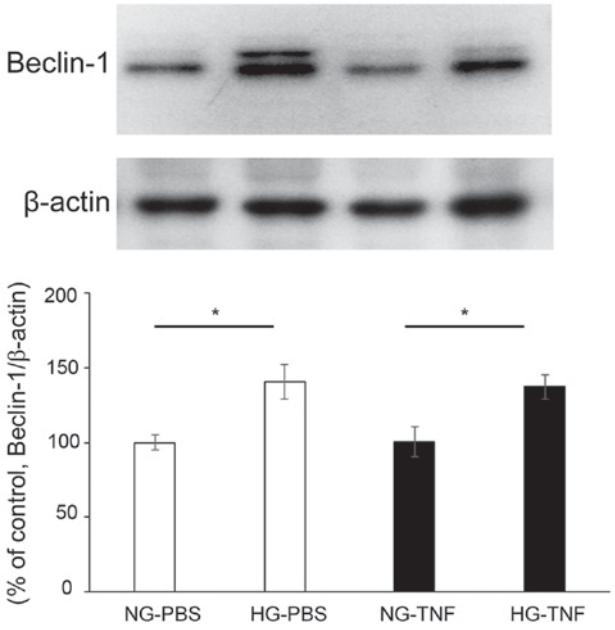


Figure 1. Beclin-1 protein expression in the optic nerves 1 week following intravitreal administration. Normalization was conducted using β-actin levels. Values are expressed as a percentage of the control and are presented as the mean ± standard error of the mean (n=8-9). *P<0.05, as indicated. HG, hyperglycemia; TNF, tumor necrosis factor; NG, normoglycemia.

*Statistical analysis*. Data are presented as mean ± standard error of the mean. Differences among groups were analyzed using one-way analysis of variance with Dunnett's post hoc test. JMP v12.0.1 software (SAS Institute, Inc., Cary, NC, USA) was used for statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Effects of TNF and HG on Beclin-1 levels in Optic Nerve*. The current study found that HG condition markedly increases Beclin-1 protein levels in optic nerves (Fig. 1). These significant increments were seen in both PBS-treated and TNF-treated eyes (Fig. 1). However, intravitreal administration of TNF did not alter the Beclin-1 expression in both NG and HG conditions.

*Localization of Beclin-1 in optic nerve*. In cross sections, immunohistochemical study revealed abundant colocalization of Beclin-1 and GFAP in the optic nerves in NG group (Fig. 2A-C). Although immunoreactivity pattern of Beclin-1 is different from that of neurofilament (Fig. 2D-F), some immunoreactivities of Beclin-1 were apparently colocalized with those of neurofilament in the HG group (Fig. 2G-L). These findings suggest that Beclin-1 is present mainly in glial cells, but partially in neurofilament, and that the expression of Beclin-1 may be upregulated by HG.

*Effect of HG and Beclin-1 siRNA on Axonal Loss induced by TNF*. Consistent with our previous findings (10), the current study

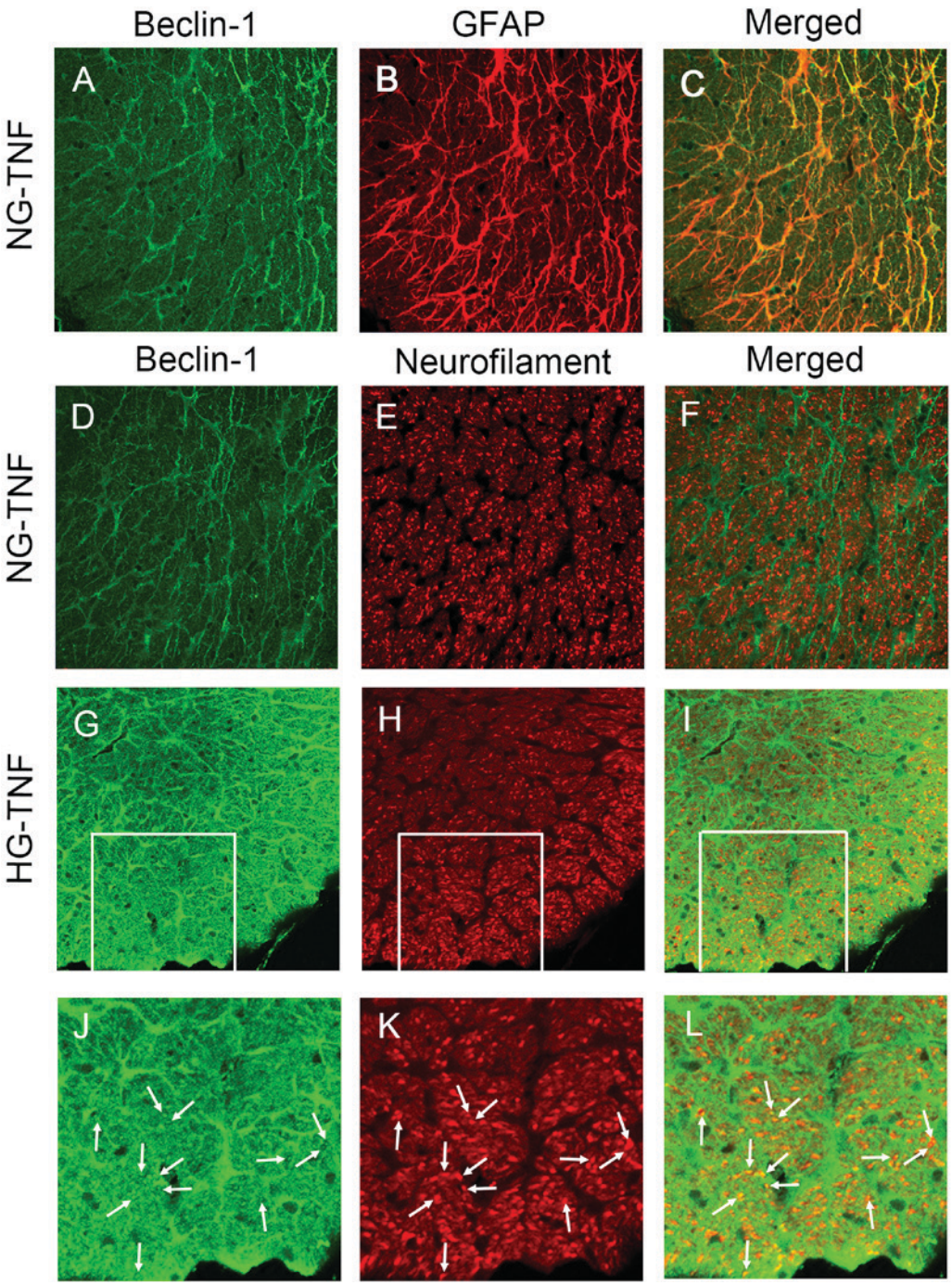


Figure 2. Immunohistochemical analysis of optic nerve cross sections. Substantial colocalization of Beclin-1 and GFAP was seen in optic nerves in the (A-C) NG-TNF group. Double-staining for Beclin-1 and neurofilaments revealed different staining patterns in the (D-F) NG-TNF group. (G-I) Double-staining for Beclin-1 and neurofilaments in the HG-TNF group. (J-L) High-magnification images of the inset area in the HG group. Beclin-1 immuno-positive dots were colocalized with neurofilament-positive dots (arrows). (A-I) Magnification, x40; and (J-L) Magnification, x80. HG, hyperglycemia; TNF, tumor necrosis factor; NG, normoglycemia; GFAP, glial fibrillary acidic protein.

showed that STZ-induced HG condition appeared a significant ameliorative effect on axonal loss caused by TNF (Fig. 3B, C, E). Since we found a significant upregulation of Beclin-1 protein level in the HG group, we examined whether Beclin-1 siRNA alters this protective effect. Noticeable degenerative changes were seen in the HG-TNF with Beclin-1 siRNA treatment group (Fig. 3D). Although no significant difference in the axon number was seen in between the HG-TNF group and the HG-TNF with Beclin-1 siRNA treatment group, no significant difference was also seen in between the NG-TNF group and the HG-TNF with Beclin-1 siRNA treatment group (Fig. 3E). These observations suggested that the protective effect of HG was only partially suppressed by Beclin-1 siRNA.

## Discussion

Opposite autophagic status was demonstrated in skeletal muscle between in the glucose-infusion HG rat and the STZ-induced HG rat (28). In STZ-induced HG rats, low insulin level prevented the m-TOR signaling, thereby leading

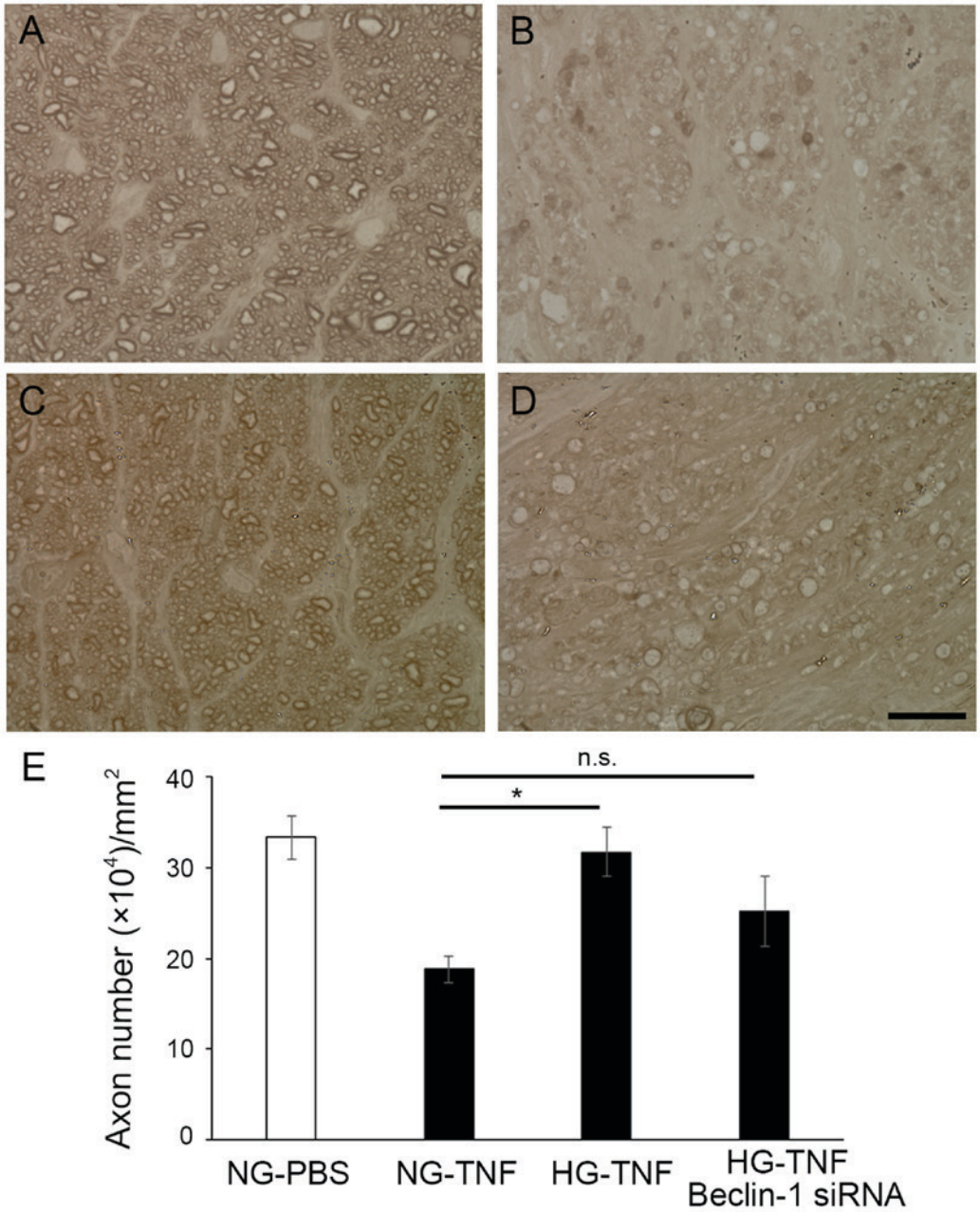


Figure 3. Beclin-1 siRNA only partially suppresses the axonal-protective effects of short-term HG. Histological results of cross sectioned optic nerves in the (A) NG-PBS, (B) NG-TNF, (C) HG-TNF, or (D) HG-TNF + 50 pmol Beclin-1 siRNA groups. Scale bar=10 $\mu$m; magnification, x100. (E) Comparison of axon numbers as shown by computer-assisted image analysis (Aphelion imaging software). Five different areas of 1,446.5 $\mu m^2$ each (totaling 7,232.5 $\mu m^2$) from each eye were used for analysis (n=4-5 per group). *P<0.05, as indicated. HG, hyperglycemia; TNF, tumor necrosis factor; NG, normoglycemia; siRNA, small interfering RNA; n.s., not significant.

to enhancement of autophagy (28). Consistent with this finding, our previous study found enhanced autophagy in optic nerve in the STZ-induced HG rats (10). In addition, a recent study demonstrated enhanced autophagy in hippocampus with ischemia in the STZ-induced HG rats (29). In the current study, remarkable increase in Beclin-1 expression was found in the optic nerve in the STZ-induced HG rats. It has been shown that Beclin-1 plays an essential role for the formation of autophagosomes in a HT22 hippocampal cells (30). That study also demonstrated that Beclin-1 is necessary for upregulation of LC3-II (30). Moreover, some recent studies demonstrated increased Beclin-1 protein levels in the hippocampus in STZ-induced HG rats (31) and in the retina in STZ-induced HG mice (32). Therefore, upregulation of Beclin-1 can be observed in several types of neuronal tissue as well as optic nerve under STZ-induced HG condition. Thus, we next examined the localization of Beclin-1 in optic nerve.

Although LC3 immunoreactivity exists in nerve fiber in optic nerve (10), Beclin-1 immunoreactivity is present mainly in glia in optic nerve. Consistently, a previous study indicated that Beclin-1 expression was found in the primary astrocytes (33). On the other hand, because it was reported that Beclin-1 presents RGC bodies (24), and we observed partial colocalization of Beclin-1 and neurofilaments, it is likely that Beclin-1 also exists in neurons. In addition, it was shown that Beclin-1 mainly expressed in neuronal cells and scarcely

expressed in GFAP-positive astrocytes in mouse cerebral cortex slices (34). It was also shown that the colocalization of Beclin-1 and neuronal cells was observed in rat brain slices (35). Therefore, the distribution of Beclin-1 may vary depending on the type of neurons, but it may be present both in neurons and glia. It was assumed that intravitreal administration of siRNA downregulates the protein level of optic nerve which exists in neurofilaments (27). Our current morphometric analysis showed that the intravitreal administration of Beclin-1 siRNA failed to abolish the protective effect of HG. It is reasonable to speculate that intravitreal administration of Beclin-1 siRNA may affect the Beclin-1 expression in RGC bodies and their axons but not glial cells in optic nerve. Because it is difficult to distinguish the change in Beclin-1 protein level between intraaxon and glia in optic nerve after intravitreal injection of siRNA, this method (i.e., local knockdown) may have a limitation to address the role of protein which exists both in axon and glia. Nonetheless, since no significant difference in the axon number was seen in between the NG-TNF group and the HG-TNF with Beclin-1 siRNA treatment group, one hypothesis posits that Beclin-1 inside axons can play some roles for protective effect of HG. A protective role of Beclin-1 has been shown in a mouse neurodegeneration model (36). Further studies will be necessary to elucidate the detail role of Beclin-1 in axonal degeneration.

In conclusion, our findings suggest that Beclin-1 exists both in neurons and glia in optic nerve and enhanced Beclin-1 may be at least partially associated with axonal protection by HG induction.

**Acknowledgements**

The authors would like to thank Ms. Yukari Hara (Department of Ophthalmology, St. Marianna University School of Medicine, Kanagawa, Japan) and Ms. Chizuko Sasaki (Institute for Ultrastructural Morphology, St. Marianna University School of Medicine, Kanagawa, Japan) for their helpful assistance.

**Funding**

The present study was supported by Grants-in-Aid in Japan (grant nos. 15K10908 and 17K11469).

**Availability of data and materials**

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

**Authors' contributions**

KS, YK and CT performed experiments. KS, YK, CT and HT conceived and designed the research, and analyzed the data. KS, YK and HT wrote and revised the article. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of the Institute of Experimental Animals of St. Marianna University Graduate School of Medicine (approval no: 1610004).

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**

1. Zhao D, Cho J, Kim MH, Friedman DS and Guallar E: Diabetes, fasting glucose, and the risk of glaucoma: A meta-analysis. Ophthalmology 122: 72-78, 2015.
2. Zhou M, Wang W, Huang W and Zhang X: Diabetes mellitus as a risk factor for open-angle glaucoma: A systematic review and meta-analysis. PLoS One 9: e102972, 2014.
3. Gordon MO, Beiser JA, Brandt JD, Heuer DK, Higginbotham EJ, Johnson CA, Keltner JL, Miller JP, Parrish RK II, Wilson MR and Kass MA: The Ocular Hypertension Treatment Study: Baseline factors that predict the onset of primary open-angle glaucoma. Arch Ophthalmol 120: 714-720; discussion 829-830, 2002.
4. Akkaya S, Can E and Öztürk F: Comparison of optic nerve head topographic parameters in patients with primary open-angle glaucoma with and without diabetes mellitus. J Glaucoma 25: 49-53, 2016.
5. Ko F, Boland MV, Gupta P, Gadkaree SK, Vitale S, Guallar E, Zhao D and Friedman DS: Diabetes, triglyceride levels, and other risk factors for glaucoma in the national health and nutrition examination survey 2005-2008. Invest Ophthalmol Vis Sci 57: 2152-2157, 2016.
6. Casson RJ, Chidlow G, Wood JP and Osborne NN: The effect of hyperglycemia on experimental retinal ischemia. Arch Ophthalmol 122: 361-366, 2004.
7. Holman MC, Chidlow G, Wood JP and Casson RJ: The effect of hyperglycemia on hypoperfusion-induced injury. Invest Ophthalmol Vis Sci 51: 2197-2207, 2010.
8. Ebneter A, Chidlow G, Wood JP and Casson RJ: Protection of retinal ganglion cells and the optic nerve during short-term hyperglycemia in experimental glaucoma. Arch Ophthalmol 129: 1337-1344, 2011.
9. Shibeeb O, Chidlow G, Han G, Wood JP and Casson RJ: Effect of subconjunctival glucose on retinal ganglion cell survival in experimental retinal ischaemia and contrast sensitivity in human glaucoma. Clin Exp Ophthalmol 44: 24-32, 2016.
10. Sase K, Kitaoka Y, Munemasa Y, Kojima K and Takagi H: Axonal protection by short-term hyperglycemia with involvement of autophagy in TNF-induced optic nerve degeneration. Front Cell Neurosci 9: 425, 2015.
11. Yan X, Tezel G, Wax MB and Edward DP: Matrix metalloproteinases and tumor necrosis factor alpha in glaucomatous optic nerve head. Arch Ophthalmol 118: 666-673, 2000.
12. Yuan L and Neufeld AH: Tumor necrosis factor-alpha: A potentially neurodestructive cytokine produced by glia in the human glaucomatous optic nerve head. Glia 32: 42-50, 2000.
13. Tezel G and Wax MB: Increased production of tumor necrosis factor-alpha by glial cells exposed to simulated ischemia or elevated hydrostatic pressure induced apoptosis in cocultured retinal ganglion cells. J Neurosci 20: 8693-8700, 2000.
14. Tezel G, Li LY, Patil RV and Wax MB: TNF-alpha and TNF-alpha receptor-1 in the retina of normal and glaucomatous eyes. Invest Ophthalmol Vis Sci 42: 1787-1794, 2001.
15. Kang JH, Wiggs JL and Pasquale LR: A nested case control study of plasma ICAM-1, E-selectin and TNF receptor 2 levels, and incident primary open-angle glaucoma. Invest Ophthalmol Vis Sci 54: 1797-1804, 2013.
16. Sawada H, Fukuchi T, Tanaka T and Abe H: Tumor necrosis factor-alpha concentrations in the aqueous humor of patients with glaucoma. Invest Ophthalmol Vis Sci 51: 903-906, 2010.
17. Kitaoka Y, Kitaoka Y, Kwong JM, Ross-Cisneros FN, Wang J, Tsai RK, Sadun AA and Lam TT: TNF-alpha-induced optic nerve degeneration and nuclear factor-kappaB p65. Invest Ophthalmol Vis Sci 47: 1448-1457, 2006.
18. Mizushima N, Levine B, Cuervo AM and Klionsky DJ: Autophagy fights disease through cellular self-digestion. Nature 451: 1069-1075, 2008.

19. Frake RA, Ricketts T, Menzies FM and Rubinsztein DC: Autophagy and neurodegeneration. J Clin Invest 125: 65-74, 2015.
20. Menzies FM, Fleming A and Rubinsztein DC: Compromised autophagy and neurodegenerative diseases. Nat Rev Neurosci 16: 345-357, 2015.
21. Puorro G, Marsili A, Sapone F, Pane C, De Rosa A, Peluso S, De Michele G, Filla A and Saccà F: Peripheral markers of autophagy in polyglutamine diseases. Neurol Sci 39: 149-152, 2018.
22. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y and Yoshimori T: LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 19: 5720-5728, 2000.
23. Wirawan E, Lippens S, Vanden Berghe T, Romagnoli A, Fimia GM, Piacentini M and Vandenabeele P: Beclin1: A role in membrane dynamics and beyond. Autophagy 8: 6-17, 2012.
24. Kim SH, Munemasa Y, Kwong JM, Ahn JH, Mareninov S, Gordon LK, Caprioli J and Piri N: Activation of autophagy in retinal ganglion cells. J Neurosci Res 86: 2943-2951, 2008.
25. Park HY, Kim JH and Park CK: Activation of autophagy induces retinal ganglion cell death in a chronic hypertensive glaucoma model. Cell Death Dis 3: e290, 2012.
26. Deng S, Wang M, Yan Z, Tian Z, Chen H, Yang X and Zhuo Y: Autophagy in retinal ganglion cells in a rhesus monkey chronic hypertensive glaucoma model. PLoS One 8: e77100, 2013.
27. Kitaoka Y, Munemasa Y, Hayashi Y, Kuribayashi J, Koseki N, Kojima K, Kumai T and Ueno S: Axonal protection by 17β-estradiol through thioredoxin-1 in tumor necrosis factor-induced optic neuropathy. Endocrinology 152: 2775-2785, 2011.
28. Lv P, Huang J, Yang J, Deng Y, Xu J, Zhang X, Li W, Zhang H and Yang Y: Autophagy in muscle of glucose-infusion hyperglycemia rats and streptozotocin-induced hyperglycemia rats via selective activation of m-TOR or FoxO3. PLoS One 9: e87254, 2014.
29. Xia L, Lei Z, Shi Z, Guo D, Su H, Ruan Y and Xu ZC: Enhanced autophagy signaling in diabetic rats with ischemia-induced seizures. Brain Res 1643: 18-26, 2016.
30. Fekadu J and Rami A: Beclin-1 deficiency alters autophagosome formation, lysosome biogenesis and enhances neuronal vulnerability of HT22 hippocampal cells. Mol Neurobiol 53: 5500-5509, 2016.
31. Ma LY, Lv YL, Huo K, Liu J, Shang SH, Fei YL, Li YB, Zhao BY, Wei M, Deng YN and Qu QM: Autophagy-lysosome dysfunction is involved in Aβ deposition in STZ-induced diabetic rats. Behav Brain Res 320: 484-493, 2017.
32. Piano I, Novelli E, Della Santina L, Strettoi E, Cervetto L and Gargini C: Involvement of autophagic pathway in the progression of retinal degeneration in a mouse model of diabetes. Front Cell Neurosci 10: 42, 2016.
33. Pereira GJ, Tressoldi N, Hirata H, Bincoletto C and Smaili SS: Autophagy as a neuroprotective mechanism against 3-nitropropionic acid-induced murine astrocyte cell death. Neurochem Res 38: 2418-2426, 2013.
34. Wang L, Xu XB, You WW, Lin XX, Li CT, Qian HR, Zhang LH and Yang Y: The cytoplasmic nuclear shuttling of Beclin 1 in neurons with Alzheimer's disease-like injury. Neurosci Lett 661: 63-70, 2017.
35. Qiao L, Fu J, Xue X, Shi Y, Yao L, Huang W, Li J, Zhang D, Liu N, Tong X, *et al*: Neuronalinjury and roles of apoptosis and autophagy in a neonatal rat model of hypoxia-ischemia-induced periventricular leukomalacia. Mol Med Rep 17: 5940-5949, 2018.
36. Pickford F, Masliah E, Britschgi M, Lucin K, Narasimhan R, Jaeger PA, Small S, Spencer B, Rockenstein E, Levine B and Wyss-Coray T: The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. J Clin Invest 118: 2190-2199, 2008.