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Morphological analysis of the retina in salt-loaded KK-Ay mice, obese and type 2 diabetic model

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Abstract: *Retinopathy, one of the microvascular complications in diabetes, can cause blindness. Salt-loading is known to exacerbate microvascular damage and may affect retinopathy. In this study, we investigated the effect of salt loading on early lesions of diabetic retinopathy. Male C57BL/6 and KK-Ay mice were salt-loaded with 1% sodium chloride (NaCl)-containing drinking water for 12 weeks. In addition, to determine the effects of high fat and high sucrose, a high fat/high sucrose diet (Quick Fat diet; QF) was also fed to the 1% NaCl-loaded group of mice of both strains. Retinal thickness was measured at an arbitrary location from the optic nerve disc, and thinning of the retina was observed in KK-Ay mice compared to C57BL/6 mice. Salt-loading caused retinal thinning in C57BL/6 mice, but further thinning was not found in salt-loaded KK-Ay mice. In KK-Ay mice, the effect of salt-loading may have been masked by the effects of obesity and diabetic status during this experimental period. There was also a small effect of QF on the retina, suggesting that dietary components other than salt loading may affect retinopathy.*

Keywords: *Diabetes, KK-Ay mice, Retinopathy*

INTRODUCTION

Retinopathy and cataract are ocular lesions that cause visual impairment and blindness [1-3], the most common causes of which are diabetes and hypertension [4]. Diabetic retinopathy (DR) is one of the complications of diabetes mellitus, and the prevalence of DR worldwide in 2010 was 35.4% (about 93 million people), of which 28 million were reported to have DR that may lead to vision loss [5]. Anti-VEGF agents, corticosteroids, and laser therapy have been used to control the progression of the DR [6], but no cure has yet been established. Therefore, analysis using animal models is very important for the detailed elucidation of the pathogenesis of DR and the development of new treatment methods. It is also necessary to understand the characteristics of model animals in consideration of extrapolation to humans.

Hyperglycemia due to diabetes is a major mechanism for the development of microvascular disorders such as retinopathy, kidney disease, diabetic cardiomyopathy, and peripheral neuropathy [7]. Furthermore, high salt intake has been shown in experimental settings and human studies to be associated with microvascular damage [8]. Salt intake impaired endothelial Ca^{2+} signaling, and reduced NO production and bioavailability in the endothelium [9, 10], causing microvascular damage. Furthermore, hypertension due to increased peripheral vascular resistance contributes to microvascular damage, creating a vicious cycle [8]. Thus, salt intake may exacerbate the microvascular disease.

High sucrose/high-fat diet has been reported to induce abnormalities in glucose/lipid metabolism, including insulin resistance [11, 12]. Blood glucose levels, liver weight, and plasma and liver lipids reportedly increased with high-fat diets in experimental animals [13, 14]. Furthermore, it has been experimentally confirmed that a high-fat diet causes microvascular damage [15, 16].

Various animal models of retinopathy have been established experimentally. Non-diabetic retinopathy models include transient hypertension-induced damage [17], optic nerve axon compression [18], and NMDA-induced damage [19]. On the other hand, diabetic retinopathy includes streptozotocin-induced models [20, 21], Otsuka Long-Evans Tokushima Fatty (OLETF) rats [22], and Zucker Diabetic Fatty (ZDF) rats [23]. In this study, we morphometrically investigated the effect of salt intake on DR, a microvascular disorder, in KK-Ay mice [24], an obese model of type 2 diabetes mellitus. We also examined the effect of adding high fat/high sucrose diet.

METHODS

Animals

Male C57BL/6 mice and KK-Ay mice (CLEA Japan, Tokyo, Japan) were used for the study (n=5). All animal procedures and the protocol complied with the guidelines for animal experimentation set by the Ethics Committee for Animal Use at Kyoto University. The mice were maintained at 23 ± 3°C on a 12 h/12 h light-dark cycle with ad libitum access to a normal diet (CE-2; CLEA Japan, Tokyo, Japan) and sterilized tap water until the beginning of the experiment.

Treatment of salt and high fat/high sucrose

Water or 1% sodium chloride (NaCl) solution was provided ad libitum for 8 weeks, from 7 to 15 weeks of age. Normal diet or high fat/high sucrose diet (Quick Fat diet: QF) were also provided for the same period. The group design is shown in Table 1 and below; group A) C57BL/6 mice with tap water and normal diet, group B) C57BL/6 mice with 1% NaCl and normal diet, group C) C57BL/6 mice with 1% NaCl and QF diet, group D) KK-Ay mice with tap water and normal diet, group E) KK-Ay mice with 1% NaCl and normal diet, group F) KK-Ay mice with 1% NaCl and QF diet.

Ocular histopathology

The animals were euthanized under isoflurane anesthesia, and the eyes were enucleated and fixed in 1% formalin/1.5% glutaraldehyde mix fixative solution. Paraffin-embedded eyes using standard techniques were sliced, and the sections were stained with hematoxylin-eosin (HE).

Measurement of retinal thickness

Retinal thickness at 250, 500, and 750 μm from the optic nerve disc was measured by the image processing software, Image J (NIH; https://imagej.nih.gov/ij/). Since the results were similar at all distances measured, only the results at 250 μm are presented in this paper. For each individual, the thickness from the internal limiting membrane (ILM) to the photoreceptor layer (PL) of the left and right eye was measured, and the average of the left and right values was used as the retinal thickness of the individual.

Statistical analysis

The results of retinal thickness were expressed as the mean ± standard deviation. Statistical analyses of differences between C57BL/6 mice and KK-Ay mice with tap water and normal diet (group A and D) were performed as follows: homogeneity of variance was tested with an F-test followed by Student's t-test for homoscedastic data. Statistical analysis of the differences between the groups of C57BL/6 mice or KK-Ay mice was performed as follows: homogeneity of variance was tested with a Bartlett test followed by Dunnett test or Steel test for homoscedastic data or heteroscedastic data, respectively. Differences were defined as significant at $P < 0.05$.

Table 1 Group composition

Group	Animal	Water	Feed	N
A	Male C57/BL6	Tap water	Normal diet (CE-2)	5
B		1% NaCl	Normal diet (CE-2)	5
C		1% NaCl	High fat/high sucrose diet (Quick Fat)	5
D	Male KK-Ay	Tap water	Normal diet (CE-2)	5
E		1% NaCl	Normal diet (CE-2)	5
F		1% NaCl	High fat/high sucrose diet (Quick Fat)	5

RESULTS AND DISCUSSIONS

Representative photographs of retinas with and without salt and QF loading in this experiment are shown in Fig. 1. Retinal thickness was statistically significantly reduced in KK-Ay mice fed normal water and diet compared to C57BL/6 mice (Fig. 2), which was considered reasonable since retinal thinning has already been reported in STZ and KK-Ay mice [25]. However, retinal thickening has been observed in Spontaneously Diabetic Torii (SDT) and SDT fatty rats, non-obese and obese type 2 diabetes models, respectively [26, 27]. On the other hand, retinas of non-obese diabetic (NOD) mice, a model of type 1 diabetes, are normal, but retinal thickening is observed when inflammation is induced by cytokines [28]. These findings suggest that there may be species or strain differences in the retinal changes in diabetes.

In C57BL/6 mice, retinal thickness was significantly reduced by NaCl loading but not by QF loading. In KK-Ay mice, on the other hand, NaCl loading had no effect, and QF loading caused a mild but significant decrease in retinal thickness (Fig. 2). Although both salt-loaded and high-fat diets induce microvascular damage, only salt-loading had an effect on retinal thickness in C57BL/6 mice, whereas diabetes mellitus was the main factor affecting retinal thickness in KK-Ay mice. The effects of salt-loading may be masked in KK-Ay mice by the effects of obesity and diabetic status during this experimental

period. There was also not a small effect of QF on the retina, suggesting that dietary components other than salt loading may affect retinopathy. However, it is considered to be controversial whether the thinning of the retina observed in KK-Ay mice fed QF diet is simply due to diabetes mellitus or not, since it has been reported that QF diet in SDT fatty rats causes ketosis and delays the onset of diabetes mellitus [29].

The changes in retinal thickness observed in this study were reductions in the inner and outer plexiform layer (IPL and OPL) which are synaptic areas of neurons, and PL, with the IPL being the most prominent reduction (Fig. 1). Since thinning of the OPL and PL is observed in the hereditary retinitis pigmentosa [30], the present results may partly reflect such manifestations. The detailed mechanism of retinal thinning observed in this study requires further investigation.

Immunostaining for glial fibrillary acidic protein (GFAP), vimentin, and vascular endothelial growth factor (VEGF) may help to clarify the changes in glial cells and retina.

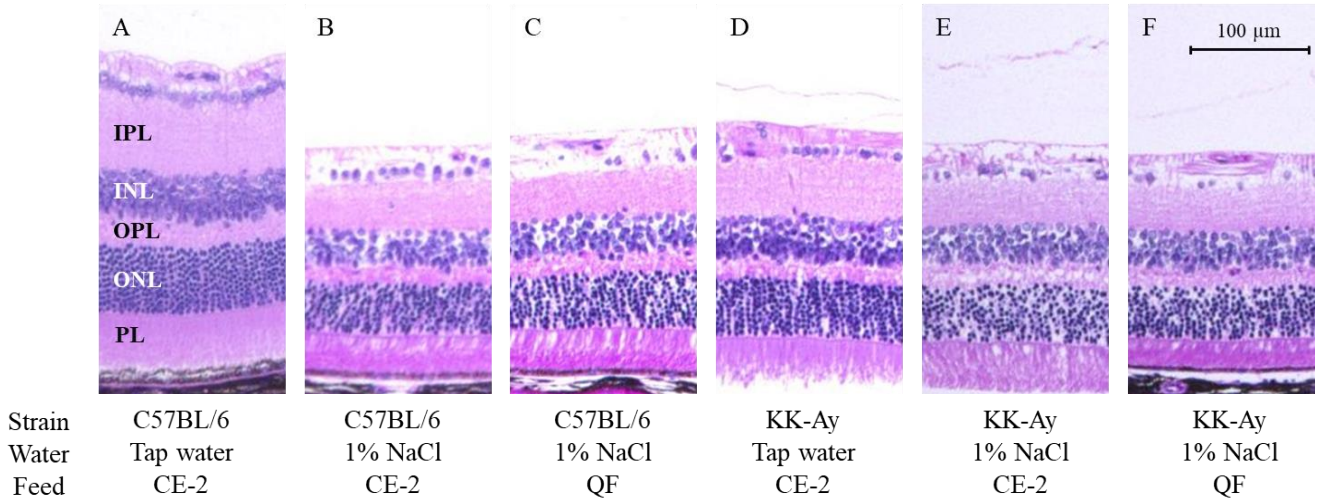

Fig. 1. Illustrative example of the retina. A to F are contrasted to the Group in Table 1. That is, A) C57BL/6 mice with tap water and normal diet, group B) C57BL/6 mice with 1% NaCl and normal diet, group C) C57BL/6 mice with 1% NaCl and QF diet, group D) KK-Ay mice with tap water and normal diet, group E) KK-Ay mice with 1% NaCl and normal diet, group F) KK-Ay mice with 1% NaCl and QF diet. IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, PL: photoreceptor layer, QF: Quick fat diet.

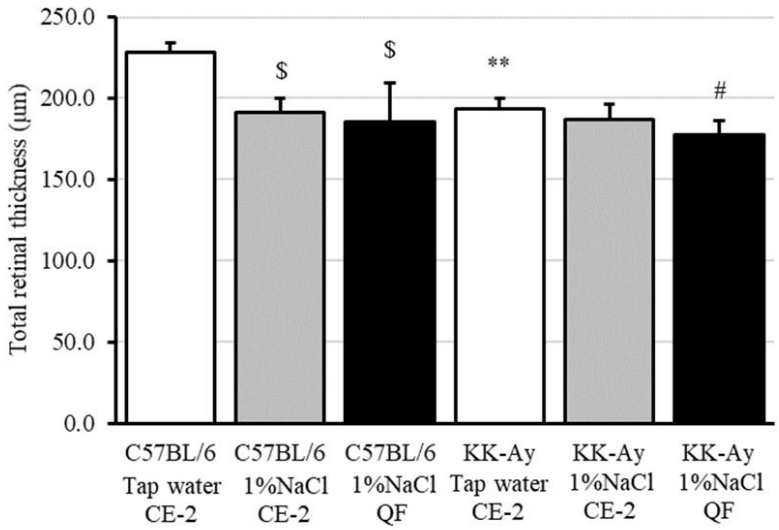

Fig. 2. The total retinal thickness at 250 µm from the optic nerve disc. Data represent means ± standard deviations (n = 5). **P <0.01 and $P <0.05; significantly different from C57BL/6 mice with tap water and normal diet. #P <0.05; significantly different from KK-Ay mice with tap water and normal diet.

CONCLUSION

In KK-Ay mice, retinal thinning was significantly influenced by diabetes, while salt intake and high-fat diet, which are aggravating factors for microvascular damage, had very weak effects. Although further studies are needed, analysis of the detailed mechanisms of retinal damage in these mice may

contribute to a detailed understanding of the pathogenesis of DR and the development of new treatment methods.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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