Hyperglycemia Suppresses Age-Related Increases in Corneal Peripheral Sensory Nerves in Wistar Bon Kobori (WBN/Kob) Rats

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PURPOSE. Nerve fiber density in the cornea is an alternative marker for diabetic peripheral neuropathy combined with intraepidermal nerve fiber density (IENFD). Recent studies investigated corneal nerves using rodent models of diabetes. Male Wistar Bon Kobori (WBN/Kob) rats spontaneously develop long-lasting diabetes and human-like diabetic peripheral neuropathy with vascular lesions. This study investigated corneal nerve fiber density and IENFD in diabetic male WBN/Kob rats as morphological markers of diabetic peripheral neuropathy.

METHODS. Male WBN/Kob rats exhibit abnormal glucose tolerance and diabetes at approximately 30 weeks of age, which progresses until approximately 90 weeks of age. Male WBN/Kob rats aged 36 and 90 weeks were therefore used for histological investigations and compared with age-matched nondiabetic female rats.

RESULTS. Terminal epithelial nerve density and subbasal nerve plexus density in the central cornea were significantly greater in nondiabetic female rats aged 90 weeks when compared with nondiabetic female rats aged 36 weeks. However, terminal epithelial nerve density and subbasal nerve plexus density did not increase with age in diabetic male WBN/Kob rats, instead lowering by up to 40%, relative to measurements in nondiabetic female rats aged 90 weeks. However, this difference was not statistically significant. IENFD was significantly lower in diabetic male rats aged 90 weeks than in male rats aged 36 weeks, but did not differ between diabetic male rats and nondiabetic female rats aged 90 weeks.

CONCLUSIONS. In WBN/Kob rats, hyperglycemia suppresses an age-related increase in peripheral sensory corneal nerve density; therefore, corneal sensory nerves may be important morphological markers of diabetic peripheral sensory neuropathy.

Keywords: cornea, diabetes, corneal nerve, confocal microscopy, rat

Peripheral neuropathy constitutes a major complication of diabetes mellitus. A widely used method for the morphological analysis of diabetic peripheral neuropathy comprises sural nerve biopsy. However, because of the high invasiveness of this method, skin biopsy for assessment of intradermal nerve fiber density (IENFD) is the recommended standard method. Moreover, corneal confocal microscopy has become popular for the evaluation of small corneal nerve fibers and represents an alternative method for assessment of peripheral neuropathy.

Animal models of diabetes are used to analyze diabetic peripheral neuropathy. Using animal models, corneal nerves have been examined, along with intradermal and sural nerves, to evaluate morphological characteristics of diabetic complications. In these studies, most experiments used rats and mice with streptozotocin (STZ)-induced diabetes. Notably, the results have been contradictory, such that a subset of studies demonstrated considerable reduction in fiber density, whereas others have reported no abnormalities. Recently, we reported that, following long-term (35 weeks) hyperglycemia, mice with STZ-induced and alloxan-induced type 1 diabetes exhibited significant reductions in corneal sensory nerve fiber density. Our findings suggested that extended exposure to hyperglycemia could cause corneal lesions in mice with STZ-induced or alloxan-induced type 1 diabetes.

STZ-induced diabetic rats and mice show rapid and overt hyperglycemia, slow nerve conduction, and a mild degree of axonal atrophy. However, peripheral nerves in STZ-induced diabetic mice generally do not show clear demyelination, degeneration, or fiber loss, whereas these findings are commonly observed in diabetic patients. Male WBN/Kob rats develop long-lasting hyperglycemia, glucosuria, hypoinsulinemia, and severe diabetic peripheral motor neuropathy, including axonal atrophy, segmental demyelination, and reduced motor nerve conduction velocity. Motor nerve conduction velocity and peripheral motor neuropathy are detectable at approximately 12 months old and deteriorate with the duration of hyperglycemia. In addition, an endoneurial microangiopathic change usually found in human diabetic neuropathic patients is also seen. However, the animals develop chronic pancreatitis, which is not the underlying basis of type 1 or type 2 diabetes, and also manifest motor rather than sensory abnormalities. Despite this, insulin treatment can decrease peripheral motor neuropathy without motor nerve conduction velocity in diabetic Wistar Bon Kobori (WBN/Kob) rats, and the neuronal changes observed in them may be induced by hyperglycemia and...
Thus, male WBN/Kob rats may be useful in the evaluation of hyperglycemia-related morphological changes in peripheral nerve changes. However, no previous analysis has been done on corneal and intradermal nerves of WBN/Kob rats, and morphological changes to the sensory nerve terminal have not yet been elucidated.

This study evaluated corneal nerve fiber density and IENFD in male diabetic WBN/Kob rats, relative to those of nondiabetic age-matched female WBN/Kob rats under short and long-lasting diabetic conditions to establish the usefulness of this model to study diabetic sensory neuropathy.

**METHODS**

**Animals and Housing Conditions**

The Committee for Animal Experiments of Setsumon University approved all experiments, which followed the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the Guide for the Care and Use of Laboratory Animals, and the regulations of Setsumon University. Female and male WBN/Kob rats were supplied by Japan SLC, Inc. (Hamamatsu, Japan). Animals were housed and fed as described in a prior report.

**Experimental Design**

Male WBN/Kob rats exhibit diabetic symptoms from approximately 30 weeks of age (initial diabetic stage), and their diabetic condition progresses until approximately 90 weeks of age (long-lasting diabetic stage). In the present study, WBN/Kob rats were separated into four groups (n = 7 per group): (1) 36-week male rats, (2) 36-week female rats, (3) 90-week male rats, (4) 90-week female rats. As the mother strain of WBN/Kob rats was not available, we used female rats as age-matched and nondiabetic controls.

**Blood Glucose and Urinary Glucose**

Blood glucose and urinary glucose were measured at 13, 23, and 36 weeks of age in rats killed at 36 weeks of age. For rats killed at 90 weeks of age, the same measurements were taken at 45, 57, 69, 81, and 90 weeks of age. Tail vein blood samples and urine samples were obtained and assessed as in a prior report.

**Histological and Morphometrical Analysis on Intraepidermal Nerve**

Histological and morphometrical analysis was performed as in a prior report. Briefly, the rats were killed by exsanguination, and foot pads were collected from the plantar surface of the hind paw. The sections were fixed, sectioned (80-μm sections), stained with a pan-neuronal marker (rabbit polyclonal anti-protein gene product 9.5 (PGP9.5) antibody [dilution, 1:200, Z5116; Dako, Santa Clara, CA, USA]), and visualized with Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) using the method described in a prior report. Negative control staining was also performed as in the prior report. The IENFD was measured as in the prior report.

**Histological and Morphometrical Analyses on the Corneal Nerve**

Histological and morphometrical analyses were performed as in a prior report. Briefly, whole eyes were collected and fixed; corneas were excised and prepared by staining with a rabbit polyclonal anti-PGP9.5 antibody (dilution: 1:200; Dako), and a mouse monoclonal anti-tubulin β3 antibody (dilution: 1:1000, 801201; BioLegend, San Diego, CA, USA) was then visualized with Alexa Fluor 488-conjugated and 568-conjugated secondary antibodies. Quantification was also as performed in the prior report. We used two different antibodies to label the peripheral nerves. As the PGP9.5 antibody also showed nonspecific labeling of the corneal epithelium, it was used to accurately mark the position of terminal epithelial nerves (TENs) and the subbasal nerve plexus (SBNP). Tubulin β3 was used for analysis of the terminal epithelial nerve density (TEND) and SBND because it did not show nonspecific labeling.

**Statistical Analysis**

Statistical analysis was performed as in a prior report. Differences with $P < 0.05$ were regarded as statistically significant. Analyses were performed with JMP Academic Suite 11.2 Pro software (SAS Institute, Tokyo, Japan).

**RESULTS**

**Glucosuria and Glycemia**

Among seven male rats killed at the age of 36 weeks, two and four rats showed glucosuria at the ages of 23 and 36 weeks, respectively. Mean blood glucose levels at the ages of 23 and 36 weeks were 152.9 and 167.6 mg/dL, respectively, and tended to increase when compared with those at 13 weeks of age (91.1 mg/dL; Figs. 1, 2). Among seven male rats killed at the age of 90 weeks, three and four rats showed glucosuria at the ages of 45 and 57 weeks, respectively; all seven rats showed...
glucosuria at the age of 69 weeks, which continued until the age of 90 weeks. The mean blood glucose level was 236 mg/dL at the age of 45 weeks; it exceeded 400 mg/dL after the age of 69 weeks (Figs. 1, 2). All seven female rats were normoglycemic (72 to 108 mg/dL) and showed no glucosuria during the experimental period (Figs. 1, 2).

**Corneal Nerve of Nondiabetic Female Rats Aged 36 Weeks**

Figure 3a shows that fine TENs from the SBNP innervated corneal epithelial cells. Figure 4a shows whorl-like structures created by central corneal subbasal nerve bundles in the subbasal zone, whereas subbasal nerve bundles of the peripheral cornea were radially arranged toward the limbus of the cornea (Fig. 5a). Connections among these subbasal bundles formed the SBNP. Moreover, large nerves were separated into several branches in the corneal stroma, which then connected to the SBNP.

**Age-Related Change of Corneal Nerve in Nondiabetic Female Rats**

In the central cornea, TEND and subbasal nerve plexus density (SBNPD) were significantly greater in female rats aged 90 weeks when compared with female rats aged 36 weeks (Figs. 6a, 6b). The TENs and SBNP exhibited diffuse dispersion in the central cornea in female rats aged 36 weeks (Figs. 3a, 4a). However, in female rats aged 90 weeks, the TENs showed uniform and dense distribution (Fig. 3b), and a clear and dense whorl-like structure of SBNP was observed at this point (Fig. 4b). Furthermore, in female rats aged 90 weeks, nerve fibers of SBNP in the peripheral cornea showed a dense and radial distribution (Fig. 5b), and SBNPD increased with age (Fig. 6d). TEND in the peripheral cornea also slightly increased with age (Fig. 6c).

**Change in Corneal Nerve of Diabetic Male Rats**

The TENs of male rats showed sparse distribution at the age of 36 weeks (Fig. 3c) and became sparser at the age of 90 weeks (Fig. 3d). However, TEND did not significantly differ between the two groups of rats (Figs. 6a, 6c). In addition, the central corneal SBNP of male rats showed sparse whorl-like structures at the age of 36 weeks (Fig. 4c); peripheral corneal SBNP also showed sparse and radially arranged structures (Fig. 5c). Furthermore, the SBNP of male rats remained sparse at the age of 90 weeks (Figs. 4d, 5d, 6b, 6d) and showed little change from the age of 36 weeks. Hyperglycemic duration and severity differed between individual male 90-week-old rats, but was not associated with changes in TEND and SBNPD.

**FIGURE 3.** Representative confocal microscopy images of tubulin β3-positive nerve fibers in TENs of the corneal epithelium in the central cornea of the four groups: (a) female rat aged 36 weeks, (b) female rat aged 90 weeks, (c) male rat aged 36 weeks, (d) male rat aged 90 weeks.
Comparison Between Diabetic Male and Nondiabetic Female Rats

When comparing between male and female rats aged 90 weeks, central and peripheral corneal TEND and SBNPD were markedly lower in male diabetic rats, but this difference was not statistically significant because of variation (Figs. 6a, 6b, 6d). Furthermore, when comparing the temporal changes between the ages of 36 and 90 weeks in both sexes, age-related increases in TEND and SBNPD were suppressed by the progression of diabetes in the male rats. The TEND and SBNPD of male rats aged 90 weeks were markedly reduced by up to 40% when compared with those parameters in female rats aged 90 weeks (Figs. 6a, 6b). The SBNPD of the peripheral cornea in the male rats aged 90 weeks was reduced by up to 40% when compared with that in the female rats aged 90 weeks (Fig. 6d).

Intraepidermal Nerve

IENFD was significantly lower in the male rats at the age of 90 weeks than in the male rats at the age of 36 weeks; however, IENFD in the diabetic male rats and nondiabetic female rats did not significantly differ at the age of 90 weeks (Fig. 7).

DISCUSSION

When comparing male diabetic WBN/Kob rats between 36 and 90 weeks of age, we found that SBNPD was nearly identical, which initially suggested that SBNPD was not affected by diabetes in this strain of rats. However, the SBNPD of the cornea increased with age in nondiabetic female WBN/Kob rats, suggesting that hyperglycemia in male WBN/Kob rats suppressed age-related increases in the corneal nerve. These findings are generally consistent with the results of diabetic patients and diabetic rodent models in previous studies.6–12,31 These studies show that the SBNPD of the cornea either remains consistent or is slightly lowered with age in normoglycemic humans and mice, and long-term hyperglycemia significantly lowers the SBNPD.6-12,31 Thus it is highly probable that hyperglycemia affects the corneal nerve in diabetic patients and diabetic rodent models.

Some studies have shown that STZ-induced type 1 diabetic rats do not demonstrate obvious changes in TEND or SBNPD.12,13 However, we have recently reported that long-lasting hyperglycemia is necessary to cause a reduction in SBNP and TENs small nerve fiber density in STZ-induced and alloxan-induced diabetic mice.22 The results of our previous study suggest that long-lasting hyperglycemia, as well as low insulin levels caused by chemically induced pancreatic islet...
destruction, might cause a reduction of corneal small nerve fibers in TENs and SBNP. Male WBN/Kob rats with pancreatic islet destruction (because of chronic pancreatitis) also exhibit low insulin and diabetes. Thus, both long-lasting hyperglycemia and low insulin in male WBN/Kob rats may cause a reduction in the number of corneal nerve fibers in a manner similar to that in STZ-induced and alloxan-induced diabetic mice. The hyperglycemia in the male diabetic WBN/Kob rats in this study was much milder when compared with the constant high blood glucose levels of more than 600 mg/dl in STZ-induced and alloxan-induced diabetic models. This may have caused the large individual variance in the neurological lesions in the male diabetic WBN/Kob rats, which could explain the lack of a statistically significant difference between diabetic males and nondiabetic females in this study.

Our findings clarified that central corneal TENs and SBNP in nondiabetic female WBN/Kob rats were significantly and remarkably increased with age. Corneal nerve fibers appear to develop slowly in the WBN/Kob strain; it takes many months for the density of corneal nerves to mature in these rats, whereas the intraepidermal nerve fibers in the skin reach maximal density much earlier. Dvorscak and Marfurt reported that SBNP increases with age in F344 rats. In contrast, SBNP corneal nerve fibers are lost in mice, and do not change in Sprague Dawley (SD) rats. The exact mechanism of the age-related increase in the density of the rat SBNP remains unknown, but it is likely that differences exist between species or strain regarding the maturity of corneal nerves. During corneal aging, several small-diameter subbasal nerves can arise from large-diameter subbasal nerves. In response to various stimuli, the corneal epithelial cells release neurotrophic factors, which promote neurite extension and corneal nerve survival. Thus, neurotrophic stimuli may promote proliferation of corneal nerves in the WBN/Kob strain of rats for a longer duration when compared with other rat strains.

IENFD reductions have also been detected in rodent models of type 1 and type 2 diabetes as well as in type 1 and 2 diabetes patients. We found that the IENFD of long-lasting diabetic male WBN/Kob rats were significantly lower at the age of 90 weeks than at the age of 36 weeks. However, the IENFD did not significantly differ between diabetic male rats and nondiabetic female rats at the age of 90 weeks. Diabetic male WBN/Kob rats exhibited mild sensory neuropathy, whereas their manifestation of diabetic peripheral motor neuropathy was severe. These observations suggest that the rigorous evaluation of intraepidermal nerve fiber in WBN/Kob rats might be difficult if diabetes slightly affects the intraepidermal nerve fiber, which constitutes the sensory terminal small nerve.

![Representative confocal microscopy images of tubulin β3-positive nerve fibers in the SBNP of the peripheral cornea of the four groups: (a) female rat aged 36 weeks, (b) female rats aged 90 weeks, (c) male rat aged 36 weeks, (d) male rat aged 90 weeks.](image-url)
FIGURE 6. TEND and SBNPD in the central and peripheral cornea of the four groups: (a) TEND in the central cornea, (b) SBNPD in the central cornea, (c) TEND in the peripheral cornea, (d) SBNPD in the peripheral cornea. Data are expressed as the mean ± SD. *P < 0.05.

FIGURE 7. (A) Representative sections of intraepidermal nerve fiber stained for PGP9.5: (a) female rat aged 36 weeks, (b) female rat aged 90 weeks, (c) male rat aged 36 weeks, (d) male rat aged 90 weeks. (B) IENFD in the hind paw. Data are expressed as the mean ± SD. * P < 0.05.
The present study suggests that hyperglycemia suppresses age-related increases in corneal peripheral sensory nerves in WBN/Kob rats. Therefore, diabetic male WBN/Kob rats could be used as models to evaluate diabetic peripheral sensory neuropathy by analyzing the TEND and SBNPD in the cornea.

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**References**


