

ORIGINAL ARTICLE

Nobiletin ameliorates streptozotocin induced diabetic retinopathy in experimental rats

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ABSTRACT

Prolonged hyperglycemia in diabetes mellitus results in severe vascular complications leading to decrease in longevity of affected individuals. Diabetic retinopathy is a major cause of blindness in diabetes. Nobiletin is a polymethoxyflavone present in high concentration in citrus fruits. Here we have evaluated the treatment with nobiletin on the amelioration of diabetic retinopathy in streptozotocin-induced diabetic rats. Diabetes was induced by a single intraperitoneal administration of STZ (50 mg/kg). Animals with blood glucose levels >350mg/dL after 48 hours of STZ injection were subjected to further treatments. Diabetic rats were treated daily with nobiletin (10 mg/kg and 25 mg/kg) for four weeks after four weeks of induction of diabetes. At the end of eight weeks, blood retinal barrier permeability was quantified in animals treated with nobiletin and compared with that of vehicle control. Histopathological analysis of the retinal sections was done using H&E stain and the outer limiting membrane to inner limiting membrane distance was measured. Further, outer nuclear membrane thickness was compared between the treatment groups. The study suggested that nobiletin reduced blood retinal barrier permeability and improved the thickness of retinal layers. Thus, treatment with nobiletin can be used as an approach to ameliorate diabetic retinopathy.

Keywords: *diabetes mellitus, nobiletin, diabetic retinopathy*

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Introduction

Diabetic retinopathy (DR) is a slow-progressing, multifactorial microvascular complication of diabetes and is the major cause of blindness affecting 80% of diabetic individuals worldwide. The incidence of DR at the time of the diagnosis of diabetes is much lower with type I diabetes mellitus (0.4%) than type II diabetes mellitus (7.6%).¹ Prolonged hyperglycemia in diabetes leads to the activation of alternative pathways for excessive circulating glucose, which on long term, affect the ocular vasculature leading to retinal vascular defects and neuroretinal dysfunction and degeneration. The exact mechanism by which hyperglycemia causes blindness remains incompletely understood. Probable mechanisms of ocular vasculature disruption include the intraocular formation of reactive oxygen species that lead to pathological and biochemical changes like protein glycation² and protein kinase-C activation which may lead to enhanced permeability of retinal vasculature, endothelial growth factors (VEGF) and cellular signalling by vascular basement membrane thickening.^{3,4} Matrix metalloproteinases (MMPs), specifically MMP-2 and MMP-9 that are involved in modulation of extracellular matrix (ECM) have shown

to play a role in the retinal neovascularization seen in the later stages of diabetic retinopathy.^{5,6}

MMPs is a family of over 25 zinc-dependent proteinases which degrade different components of the extracellular matrix (ECM) and regulate many normal and pathological processes.⁷ Several studies have indicated that the gelatinases, specifically MMP-2 and MMP-9, are increased in the epiretinal neovascular membranes of patients with proliferative diabetic retinopathy⁸ as well as in retinas in an animal model of retinal neovascularization.⁹ MMP-2 and MMP-9 have also been shown to have significantly increased levels of activation in vitreous samples from individuals with proliferative diabetic retinopathy.¹⁰ Recent research has shown that MMPs have a dual role in the development of diabetic retinopathy; in the early stages of the disease (pre-neovascularization), MMP-2 and MMP-9 facilitate the apoptosis of retinal capillary cells, possibly via damaging the mitochondria, and in the later phase, they help in neovascularization.¹¹

Several natural flavonoids have shown to possess MMP inhibitory activity. Nobiletin, a polymethoxy flavone present in high concentrations in peels of citrus fruits, is reported for its many potential health benefits.¹² The present study aimed at evaluating the benefits of long term administration of

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nobiletin in STZ induced diabetic retinopathy. Since nobiletin is a potential molecule with MMP inhibitory effects seen in cancer cell lines,¹² treatment with nobiletin could be beneficial in amelioration of diabetic retinopathy in experimental animals.

Materials and methods

Materials and chemicals

Streptozotocin (STZ) was purchased from Sigma Aldrich (St. Louis MO, USA). Nobiletin was purchased from Baoji Hongyuan Biotechnology Co. Ltd. (Baoji City, China). A gift sample of Minocycline was received from US Vitamins (Mumbai, India). All the biochemical diagnostic kits were procured from Erba Diagnostics (Mumbai, India). All the other reagents and chemicals used for the study were of analytical grade.

Preparation of drug solution

STZ was dissolved in ice cold citrate buffer (pH 4.5) just before use. Nobiletin and minocycline (MINO) were suspended in 0.5% CMC (carboxymethyl cellulose) solution before use.

Animals, induction of diabetes and study protocol

Male Wistar rats (weighing 190–230 gms and 10–12 wks of age), purchased from Haffkine Institute (Lower Parel, Mumbai), were employed in the study. Animals were housed in clean environment and maintained at a temperature of $25 \pm 1^\circ\text{C}$, RH 45–55% under a 12 hr light/ dark cycle and had free access to food and water ad libitum.

The study animals were fasted for 12 hrs and diabetes was induced using a single dose of STZ (50 mg/kg, ip). Blood sugar levels (BSL) were checked for individual animals 48 hrs after STZ injection and the animals with BSL >350 mg/dL were considered diabetic and used for further study. The BSL was further monitored weekly throughout the eight week study period.

Animals were randomly divided into five groups of six animals each. Four weeks after the induction of diabetes, oral daily treatment of nobiletin was carried out for further four weeks. Group I served as normal control (NORMO) with non-diabetic normal animals, Group II served as vehicle control and received 0.5% CMC solution (1 mL/kg); Group III and IV were the treatment groups and received nobiletin (NOB) at a dose of 10 mg/kg (NOB10) and 25 mg/kg (NOB25) respectively; Group V received minocycline (MINO) at a dose of 50 mg/kg.

Blood retinal barrier permeability

At the end of eight weeks period, the blood retinal barrier (BRB) permeability was measured using an Evans blue technique.¹³ Briefly, rats were anesthetized with pentobarbital sodium (45 mg/kg). After cannulating left femoral artery and vein, Evans blue (45 mg/kg) was injected into the femoral vein. Two minutes after the injection of Evans blue, 0.2 ml blood was drawn to obtain the initial Evans blue plasma concentration. Subsequently, at 15-minute intervals, 0.1 mL blood was drawn up to 2 hours after injection to obtain the time-averaged Evans blue plasma concentration. The blood samples were centrifuged at 12000 rpm for 30 min and the plasma was diluted 1/10000 in formamide. Absorbance of Evans blue was measured by spectrophotometry at 620 nm. The concentration of dye in the plasma was calculated from a standard curve of Evans blue in formamide. Subsequently, the chest cavity was opened, and rats were perfused via the left ventricle at 37°C with 60 mL citrate buffered paraformaldehyde (1% w/v) over a 2 min interval to clear Evans blue from the circulation. The retina from the eye was collected, dried for 2 h and weighed. The Evans blue dye was extracted by incubating each retina in 120 mL of formamide for 18 h at 70°C . The extract was centrifuged at 14000 g for 12 min at 25°C . The absorbance of 80 mL of the supernatant was measured by spectrophotometry. The BRB permeability was calculated as follows:

$$\frac{[\text{Evans blue } (\mu\text{g})/\text{retina dry weight (g)}]}{[\text{Time-averaged Evans blue concentration } (\mu\text{g})/\text{plasma } (\mu\text{L}) \times \text{circulation time (h)}]}$$

Histology and measurement of retinal thickness

After the BRB measurements, the animals were sacrificed with deep anaesthesia. The eyeballs were removed and fixed in 10% formaldehyde solution. Histopathological analysis was done after staining with hematoxylin and eosin (H&E) for light microscopy by taking serial sections (5 μM) that passed through the optic nerve head and cutting marker. Retinal thickness of different layers was measured at a $400\times$ magnification, including: (1) outer limiting membrane to inner limiting membrane (OLM-ILM) (2), outer nuclear layer (ONL), and (3) inner nuclear layer (INL). Two measurements were taken on each section, at the two reference lines which were 1 mm away from the optic nerve on both superior and inferior sides.

Statistics

All data are expressed as mean \pm SD. Statistical analysis was performed using GraphPad Prism (version 4.0, Graph Pad Inc., San Diego, USA) software. For multiple comparisons, one-way analysis of variance (ANOVA) was used. In case ANOVA showed significant differences, post-hoc analysis was performed with Tukey's test or Dunnett test, $p < 0.05$ was considered statistically significant.

Results

Body weight and blood glucose levels

The body weights and BSL of the animals was monitored weekly during the experiment. The weekly body weight data of animals indicated that there was a decrease in the body weights of animals in the first four weeks after STZ injection followed by an increase towards the end of eight week. Vehicle control group showed lesser recovery of body weights compared to treatment groups (Table 1). The BSL of the treatment group at the end of eight weeks showed no significant decrease compared to vehicle control group (Table 1).

Group	8 weeks BSL (mg/dL)	8 weeks body weight (g)
NORMO	102.18 \pm 11.58**	230.33 \pm 8.50**
DB + 0.5% CMC	553.98 \pm 37.72	187.33 \pm 10.97
NOB10	542.65 \pm 52.29	182.83 \pm 14.57
NOB25	521.78 \pm 48.56	181.33 \pm 15.73
DB + MINO	553.83 \pm 27.01	177.67 \pm 9.89

Table 1. Body weight and BSL of animals after 4 wk treatment with NOB. The values are given as mean \pm S.D.; ** $P < 0.01$ when compared with vehicle treated diabetic group. NORMO (Normal Group), DB + 0.5% CMC (Diabetic group treated with 0.5% CMC), NOB10 (Diabetic group treated with Nobiletin 10mg/kg), NOB25 (Diabetic group treated with nobiletin 25mg/kg), DB + MINO (Diabetic group treated with standard minocycline).

Assessment of BRB permeability

Evans blue was used to quantitate the degree of vascular permeability of the retina. One eye from each of the animal was used for the study. At the end of eight week study, the vascular permeability of the retina was determined and it was observed that there was an increase in the retinal vascular permeability in the STZ animals compared to the normal control. Treatment with NOB25 improved the retinal vasculature as seen in reduction of BRB permeability (Figure 1).

Histological analysis of retina

The retinal sections were taken and morphometric analysis was done using H&E stain. It was observed that the

retinal thickness was compromised in STZ diabetic animals compared to normal control. Primarily, the reduction in thickness occurred in the ONL in diabetic rats up to 2 months (Figure 2). For INL in diabetes, there was no significant difference in 2 months diabetes compared with normal controls. Treatment NOB25 significantly attenuated ONL thickness when compared with vehicle treated diabetic group.

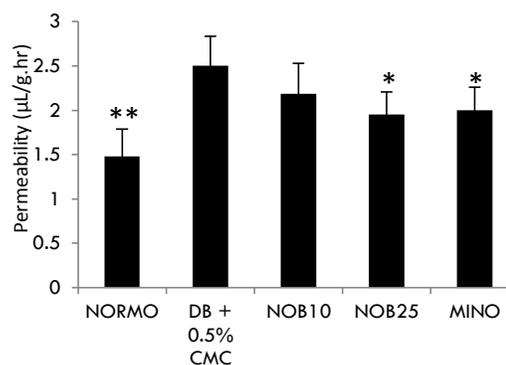


Figure 1. Effect of four week treatment with NOB10 and NOB25 on retinal permeability. * $p < 0.05$, ** $p < 0.01$ vs vehicle treated diabetic rats. (n = 6 for all groups)

Discussion

Diabetic retinopathy is a severe complication of diabetes characterized by excessive permeability of retinal blood vasculature that when left untreated leads to blindness. The present study aimed at evaluating the effect of nobiletin on STZ induced diabetic retinopathy. Comparison of eight week BSL of the treatment groups with vehicle control indicated that four week treatment with nobiletin at a dose of 10 mg/kg (NOB10) and 25 mg/kg (NOB25) did not have any significant effect on reducing the BSL. Thus, it can be stated that nobiletin attenuates diabetic retinopathy by other than glucose lowering mechanism.

The blood retinal barrier (BRB) is localized at the endothelial cells of the retinal capillary vessels and at the epithelial cells of the retinal pigment epithelium. The presence of transport processes and the existence of tight junctions between the endothelial and the epithelial cells that form the BRB prevent the entrance of toxic molecules into the retina and the escape of important ions from the retina. Thus, BRB has an important role in the homeostasis of the retina. Breakdown of the BRB is an early feature of diabetic retinopathy and results in vascular leakage and the development of retinal edema.¹⁴⁻¹⁸ STZ induced diabetes caused increased permeation of BRB as seen in experimental animals of vehicle control group. It was observed that BRB permeability was improved in

animals on treatment with NOB25. Treatment with NOB10 did not have any significant effect on BRB permeability. Histological analysis of the retinal sections stained with H&E indicated that treatment with NOB 25 improved the retinal thickness as seen with increase in thickness of the ONL and the distance between the OLM and ILM.

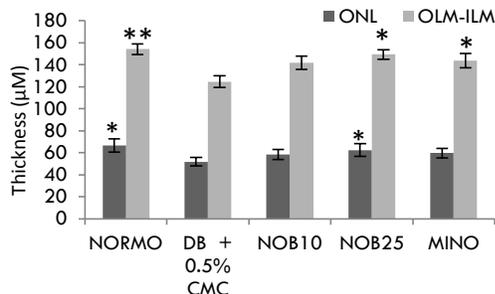


Figure 2. Effect of four week treatment with NOB10 and NOB25 on retinal thickness. * $p < 0.05$, ** $p < 0.01$ vs vehicle treated diabetic rats. (n = 6 for all groups). OLM -ILM (Outer Limiting Membrane to Inner Limiting Membrane), ONL (Outer Nuclear Layer).

Several research studies suggest the role of matrix metalloproteinases (MMPs) in the development of diabetic retinopathy.¹⁹ It is clearly understood that latent MMPs are activated in the retina and its capillary cells on prolonged hyperglycemia in diabetes mellitus and over activation of MMPs, specifically MMP-2 and -9 induces apoptosis of retinal capillary cells leading to retinal damage. MMPs have an important role in maintaining the integrity of the blood-retinal barrier (BRB). Increased retinal MMPs in diabetes facilitate the increase in vascular permeability via proteolytic degradation of the tight junction protein occludin and disruption of the overall tight junction complex.²⁰ Pharmacological inhibition of MMPs is shown to prevent retinal and choroidal neovascularisation²¹ and inhibit MMP-9-mediated retinal vascular permeability and inflammation.²² In another study, administration of a synthetic MMP inhibitor prevented the induction of proliferative vitreoretinopathy.²³ MMP-2 and MMP-9 inhibition in presence of COX inhibitor has also shown to prevent retinal abnormalities.²⁴ We hypothesized that nobiletin, a flavonoid from citrus fruits, could be beneficial in the treatment of diabetic retinopathy. Nobiletin possesses MMP-2 and MMP-9 inhibitory activity. In our previous study, we reported decreased serum MMP-2 and MMP-9 levels by nobiletin treatment in diabetic rats.²⁵ In the present study, treatment with nobiletin in STZ induced DR showed improvement in the condition. Thus, nobiletin can ameliorate diabetic retinopathy on long term administration. This activity of nobiletin can be attributed to its MMP inhibitory property.

In conclusion, present study showed efficacy of nobiletin and indicated that nobiletin can be a potential molecule in the treatment of diabetic retinopathy.

Conflict of interest

None

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