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RESEARCH ARTICLE

Vaccinium myrtillus extract prevents or delays the onset of diabetes–induced blood–retinal barrier breakdown

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Abstract

Many dietary supplements have been sold through advertising their large number of beneficial effects. The aim of this study was to determine whether bilberries (*Vaccinium myrtillus*) help to prevent diabetes-induced retinal vascular dysfunction *in vivo*. *V. myrtillus* extract (VME; 100 mg/kg) was orally administered to streptozotocin-induced diabetic rats for 6 weeks. All diabetic rats exhibited hyperglycemia, and VME did not affect the blood glucose levels and body weight during the experiments. In the fluorescein-dextran angiography, the fluorescein leakage was significantly reduced in diabetic rats treated with VME. VME treatment also decreased markers of diabetic retinopathy, such as retinal vascular endothelial growth factor (VEGF) expression and degradation of zonula occludens-1, occludin and claudin-5 in diabetic rats. In conclusion, VME may prevent or delay the onset of early diabetic retinopathy. These findings have important implications for prevention of diabetic retinopathy using a dietary bilberry supplement.

Keywords

Anthocyanins, blood–retinal barrier, bilberry, diabetic retinopathy, vascular endothelial growth factor

History

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Introduction

Diabetic retinopathy is a common complication of diabetes mellitus. Increased vascular permeability caused by the breakdown of the blood–retinal barrier (BRB) results in diabetic macular edema, which is a major cause of vision loss in diabetic patients (Moss et al., 1998). The endothelium is a single layer of cells that composes the inner lining of blood vessels. Adjacent endothelial cells are firmly sealed by tight junction protein complexes (Mehta & Malik, 2006). These complexes contribute to the barrier function and consist of transmembrane proteins, such as occludin, claudins and junctional adhesion molecules (Harhaj & Antonetti, 2004). The endothelial cell barrier can be used as a therapeutic target for vascular permeability-associated diabetic retinopathy (Barber, 2003).

Vascular endothelial growth factor (VEGF), an angiogenic factor and a major vasopermeability factor, is a key mediator of BRB breakdown in diabetic retinopathy (Shweiki et al., 1992). In streptozotocin (STZ)-induced diabetic rats, intravitreal injection of VEGF induces retinal vascular leakage, but blockade of VEGF abolishes retinal vascular leakage (Qaum et al., 2001). VEGF can cause hyperpermeability by inducing phosphorylation of tight junction proteins, such as occludin and zonula occludens-1 (ZO-1) (Antonetti et al., 1999).

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Recently, considerable attention has focused on dietary constituents that may be beneficial for the prevention and treatment of diabetes. Although there are several drugs that have been used as therapeutic regimens for diabetic retinopathy, there is little evidence that food factors themselves can be directly beneficial for preventing diabetes-induced retinal vascular injury. Anthocyanins are the largest group of water-soluble pigments in the plant kingdom, and they are widely available in the human diet in cereals, beans, fruits, vegetables and red wine (Wu et al., 2006). Berry fruits, such as blueberries (*Vaccinium cyanococcus*), bilberries (*Vaccinium myrtillus*) and blackcurrants contain large amounts of anthocyanins, making them one of the major sources for dietary anthocyanin intake (Takikawa et al., 2010; Wu et al., 2006). Considerable attention has focused on the health benefits of bilberry, which include antioxidant, anticancer, anti-neurodegenerative, and anti-inflammatory activities (Seeram, 2008). *Vaccinium myrtillus* extract (VME) contains 15 different anthocyanins (Nakajima et al., 2004). Animal studies have demonstrated *V. myrtillus* anthocyanin is beneficial in improving vascular tone, blood flow, and vasoprotection (Colantuoni et al., 1991; Lietti et al., 1976). Recently, Matsunaga et al. reported that VME inhibits VEGF-induced proliferation of human umbilical vein endothelial cells and retinal vascular angiogenesis in oxygen-induced retinopathy mice by inhibiting the phosphorylations of extracellular signal-regulated kinase 1/2 (ERK 1/2) and protein kinase B (Akt) (Matsunaga et al., 2010).

The results of these studies lead to the question of whether dietary anthocyanin-rich VME can ameliorate diabetes-induced retinal vascular hyperpermeability. Moreover, the molecular

action of bilberry responsible for ameliorating diabetic retinopathy is not yet fully understood *in vivo*. Therefore, the present study was designed to examine if the administration of anthocyanin-rich VME inhibits the breakdown of BRB in streptozotocin (STZ)-induced diabetic rats.

Methods

Animals

All experiments were approved by the Korea Institute of Oriental Medicine Institutional Animal Care and Use Committee. Male 6-week-old Brown Norway (BN) rats were purchased from Charles River Laboratories (Wilmington, MA) and acclimated for 1 week prior to study. The animals were maintained in a 12-h light/12-h dark cycle, with normal pellet diet and water provided ad libitum. Diabetes was induced by a single injection of STZ (60 mg/kg, i.p.) in rats. Age-matched control rats received an equal volume of vehicle (0.01 M citrate buffer; pH 4.5). One week after the STZ injection, rats with a blood glucose level higher than 300 mg/dl were considered as diabetes-induced rats. The animals were divided into three groups as follows: (1) normal control rats (NOR; $n = 32$), (2) STZ-induced diabetic rats (DM; $n = 32$) and (3) STZ-induced diabetic rats treated with VME (DM + VME; 100 mg/kg, $n = 32$). The standardized VME containing 36% anthocyanins (glycosylated compounds) was purchased from Kukje Pharm (Sungnam, Korea). Additional information about the contents of total anthocyanins in VME was provided in the manufacturer's information sheet. VME was dissolved in deionized water at a concentration of 5 mg/ml. One group of diabetic rats received daily gastric gavage of VME at 100 mg/kg, and the other group was given the same amount of vehicle gavage for 42 d. The dose of VME was based on the daily dose of VME commonly prescribed in humans. The blood glucose level and body weight were monitored consecutively. Eight rats in each group were sacrificed at days 7, 14, 21 and 42 after the onset of diabetes.

Measurement of BRB permeability

The assay to measure BRB permeability was performed according to established protocols (Kim et al., 2014). Rats were injected 100 mg/kg of fluorescein isothiocyanate (FITC)–dextran (4.4 kDa; Sigma, St. Louis, MO) into the left ventricle under deep anesthesia. After the circulation for 10 min, the retinas were dissected, flat mounted onto a glass slide, and viewed by fluorescence microscopy (BX51, Olympus, Tokyo, Japan). For quantification of retinal vascular leakage, rats were perfused with PBS, and the retinas were carefully removed, weighed and homogenized to extract the FITC-dextran in 200 µl of distilled water. Plasma was also collected before perfusion. The fluorescence in each 100 µl sample was measured using a spectrofluorophotometer (Synergy™ HT, Bio-Tek, Winooski, VT).

Western blot analysis

Western blot was performed as previously described (Kim et al., 2014). Briefly, retinal proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (BioRad, Hercules, CA). For immunoblotting, the following primary antibodies were used: rabbit anti-occludin antibody (1:1000, Invitrogen Life Technologies, Carlsbad, CA), rabbit anti-zonula occludens-1 (ZO-1) antibody (1:2000, Invitrogen Life Technologies, Carlsbad, CA), rabbit claudin-5 antibody (1:1000, Invitrogen Life Technologies, Carlsbad, CA) and mouse anti-VEGF antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse β-actin antibody (1:5000, Sigma, MO). Either goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a secondary antibody. The immunoreactive bands were visualized with an enhanced chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ), and measured using FLA-2000 (Fujifilm, Tokyo, Japan).

In situ hybridization

In situ hybridization was performed using the RNAscope technique (RNAscope 2.0; Advanced Cell Diagnostics, Hayward, CA) as described (Wang et al., 2012). VEGF mRNA status was assessed using RNAscope probe for VEGF (RNAscope, Advanced Cell Diagnostics, Hayward, CA).

Statistical analysis

Data are expressed as the mean ± SE and analyzed by one-way analysis of variance followed by Tukey's multiple comparison test or by unpaired Student's *t*-test using GraphPad Prism 5.0 software (GraphPad, San Diego, CA). Differences with a value of $p < 0.01$ were considered statistically significant. Pearson's correlation coefficient was also calculated to analyze the statistical correlation between VEGF expression and the vascular permeability.

Results

Body weight and blood glucose level

As shown in Table 1, STZ caused a stable increase in blood glucose from 442.8 to 592.8 mg/dl after 42 d of diabetes, and this

Table 1. Metabolic and physical parameters.

	Duration of diabetes	NOR	DM	DM + VME
Body weight (g)	0 day	262.5 ± 7.5	227.0 ± 20.3	224.3 ± 22.7
	7 d	309.7 ± 13.7	238.0 ± 45.0*	234.2 ± 48.9
	14 d	333.9 ± 18.8	216.0 ± 72.9*	217.1 ± 76.4
	21 d	366.5 ± 22.2	223.2 ± 85.6*	229.2 ± 90.9
	42 d	413.8 ± 27.3	221.3 ± 112.6*	229.0 ± 123.0
Blood glucose (mg/dl)	0 d	140.5 ± 14.5	442.8 ± 121.8*	439.1 ± 125.1
	7 d	145.7 ± 19.5	490.6 ± 81.7*	435.1 ± 96.1
	14 d	146.8 ± 15.7	568.4 ± 52.0*	521.8 ± 46.2
	21 d	144.7 ± 17.6	555.5 ± 61.7*	555.5 ± 60.8
	42 d	145.5 ± 19.1	592.8 ± 57.6*	520.4 ± 77.3

NOR, normal control rats; DM, STZ-induced diabetic rats; DM + VME, diabetic rats treated with VME (100 mg/kg). All data are expressed as the mean ± SE.
*$p < 0.01$ versus NOR group.

increase was unaffected by VME. The body weight of diabetic rats was decreased when compared to the control subjects. However, the decrease in body weight due to hyperglycemia was not ameliorated by VME treatment.

Effects of VME on BRB breakdown in diabetic rats

To compare vascular permeability changes in STZ-diabetic rats, retinal vascular leakage was measured 7, 14, 21 and 42 d after the onset of diabetes (Figure 1). In normal rats, FITC dye was retained in retinal vessels without leakage. However, many areas of dye leakage from retinal vessels were observed in vehicle-treated diabetic rats. In diabetic rats, vascular leakage increased early with a 3.2-fold elevation over the control level ($p<0.01$) 7 d after the onset of diabetes. Vascular leakage reached the plateau at 42 d with a level 4.3-fold higher than that found in the controls ($p<0.01$). Although vascular leakage started to increase 7 d after the onset of diabetes (1.7-fold over the control value; $p<0.01$), vascular permeability of VME-treated diabetic rats remained significantly lower than that in diabetic rats at 7, 14, 21 and 42 d (1.7-, 1.9-, 1.5- and 1.3-fold less than that in diabetic rats, respectively; $p<0.01$). This result indicated that VME retards the onset of BRB breakage and protects BRB function in diabetic rats.

Effects of VME on retinal VEGF expression in diabetic rats

Retinal VEGF levels were measured by Western blot analysis in diabetic rats and compared to respective age-matched normoglycemic controls at different time points after the onset of diabetes. Following the induction of diabetes, the retinal VEGF levels in diabetic rats were higher than those of control rats during the time period of diabetes that lasted from 7 to 42 d (Figure 2). The retinal VEGF levels did not change with age in normal rats. Although the expression of VEGF gradually increased during 42 d in the VME-treated diabetic rats, VME lowered VEGF expression at all experimental times.

In situ hybridization was also used to identify the cells synthesizing VEGF mRNA in diabetic retinas. As early as 7 d after the onset of diabetes, there was a significant increase in VEGF mRNA levels compared to those of control rats, particularly in the inner nuclear layer (Figure 3). Throughout the study, the levels of retinal VEGF mRNA were significantly increased compared to normal rats. However, VME significantly reduced VEGF mRNA expression, and this effect of VME was lasted through the study period.

To evaluate the relationship between VEGF and the vascular permeability in the retinas, we analyzed the correlation between the expression levels of VEGF protein and the extravasation levels of FITC-dextran. As shown in Figure 4, the expression levels of VEGF protein in the retinas was positively correlated with the retinal vascular permeability ($r=0.9718$, $p<0.01$).

Effects of VME on tight junction protein loss

Endothelial cell-to-cell junctions serve as a barrier by regulating paracellular permeability (Antonetti et al., 1998). Thus, we performed western blots to evaluate the expression of tight junction proteins (ZO-1, occludin and claudin-5) at different time points after the onset of diabetes. The protein expression of ZO-1, occludin and claudin-5 was significantly lower in the vehicle-treated diabetic rats than age-matched controls ($p<0.01$). Treatment with VME restored the expression of endothelial cell-to-cell junction proteins in the diabetic rats (Figure 5). In particular, the expression levels of ZO-1 had a tendency to decrease with time in the vehicle-treated diabetic rats. VME inhibited this reduction of ZO-1 expression during the entire experimental period of 42 d ($p<0.01$). The expression levels of claudin-5 in VME-treated diabetic rats were gradually decreased but remained significantly higher than those in diabetic rats throughout the study ($p<0.01$). The expression levels of occludin in VME-treated diabetic rats remained higher than that in diabetic

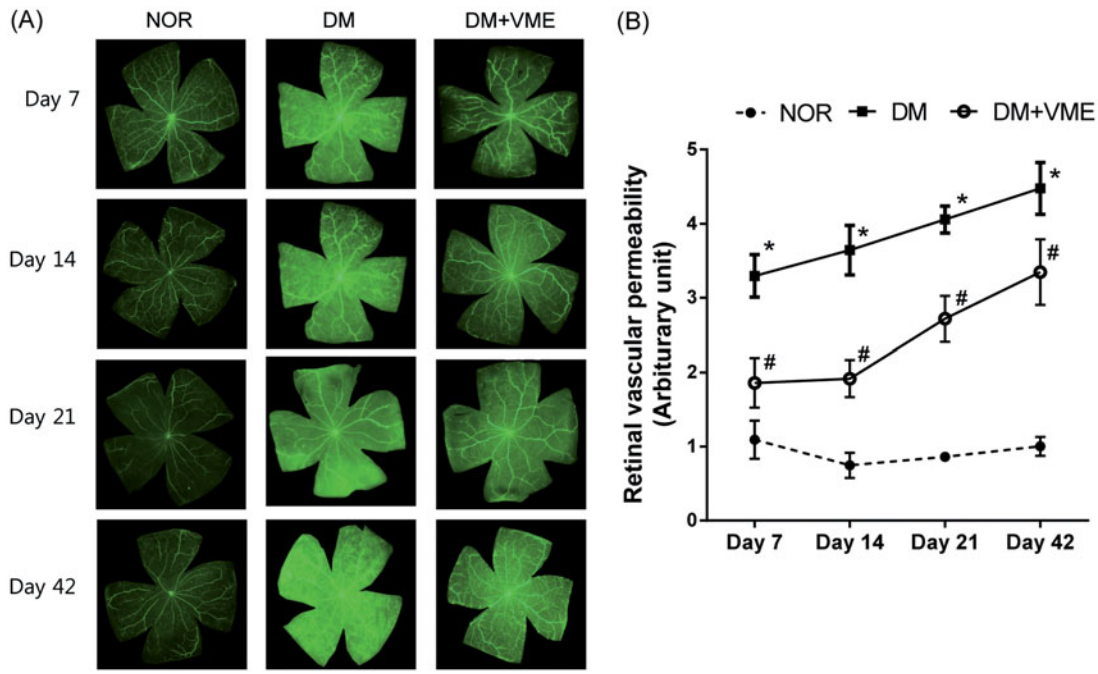

Figure 1. Blood–retinal barrier breakdown. (A) FITC-dextran angiography on retinal flat mounts. (B) Quantitative analysis of retinal vascular permeability. NOR, normal control rats; DM, vehicle-treated diabetic rats; DM + VME, diabetic rats treated with VME (100 mg/kg). Normal retinas showed no leakage of the tracer into the retina as is apparent by the clear delineation of the retinal capillaries. Diabetic retinas demonstrated a widespread breakdown of their BRB with tracer leakage into the neural retina and a loss of delineation of the retinal capillaries. However, treatment with VME significantly decreased retinal vascular permeability in diabetic rats. Values in the bar graphs represent the mean ± SE, $n=8$. *$p<0.01$ by one-way ANOVA with Tukey's test (NOR versus DM); #$p<0.01$ by one-way ANOVA with Tukey's test (DM versus DM + VME).

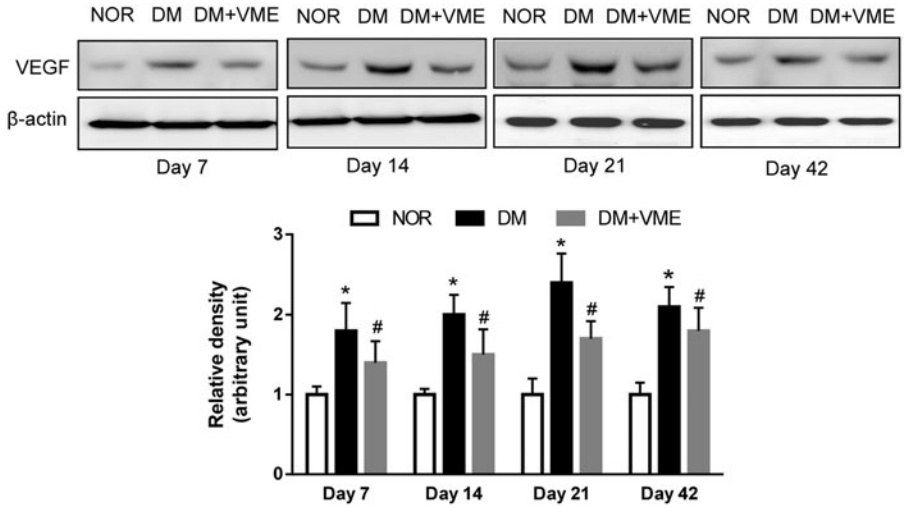

Figure 2. Effect of VME on the levels of retinal VEGF protein. The levels of VEGF protein were determined by Western blot analysis and quantified using densitometry. All data are expressed as the mean ± SE ($n = 8$). *$p < 0.01$ by Student's t-test (NOR versus DM); #$p < 0.01$ by Student's t-test (DM versus DM + VME).

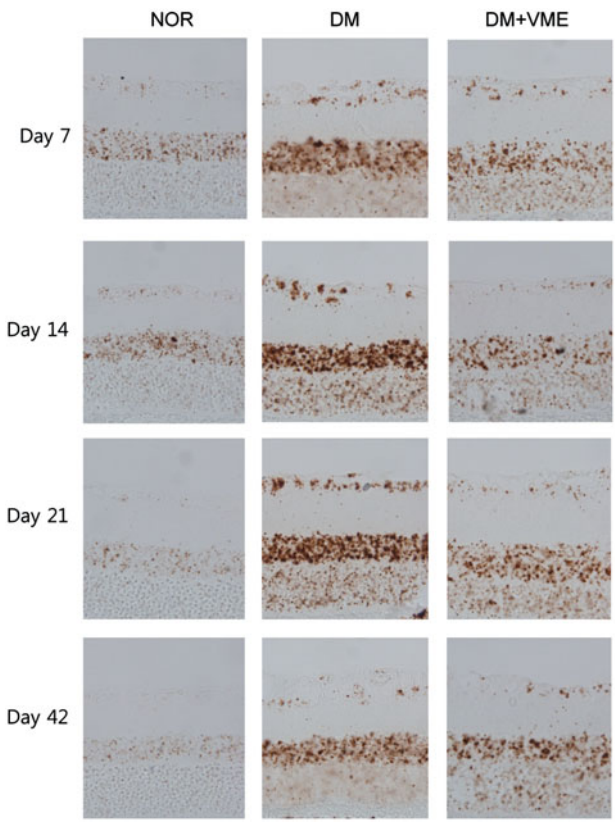

Figure 3. Effect of VME on the expression of VEGF mRNA in retinas. The expression of VEGF mRNA was determined by in situ hybridization. There was a significant increase in VEGF mRNA levels in vehicle-treated diabetic rats, particularly in the inner nuclear layer. VME reduced the expression of VEGF mRNA.

rats at day 7 ($p < 0.01$). However, the loss of occludin was also observed in VME-treated diabetic rats at days 14, 21 and 42.

Discussion

In recent years, many dietary supplements of various dosages and formulations have been sold through advertising their large number of beneficial effects. We evaluated the time-course in BRB breakage in diabetic rats treated with VME. Several rat strains have been used for diabetes-induced retinal vascular permeability studies (Ennis & Betz, 1986; Xu et al., 2001). STZ is

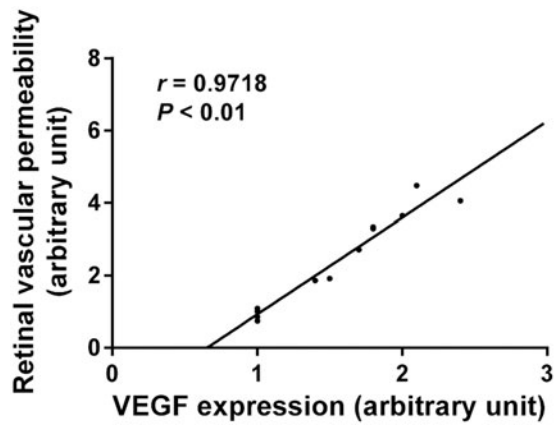

Figure 4. Correlations between the expression levels of VEGF protein and the extravasation levels of FITC-dextran in the retinas. Linear regression analysis revealed the positive correlation between VEGF levels and the vascular permeability in the retinas.

used to induce experimental diabetes in rodents. STZ-induced diabetic rats demonstrate characteristics of the diabetic retinopathy that occurs in humans, including blood vessel dilation, increased leukostasis, loss of endothelial cells and pericytes from capillary beds, microaneurysm formation and increased vascular permeability resulting from breakdown of the BRB barrier (Kusari et al., 2010). Zhang et al. found that retinal vascular leakage occurs earlier and lasted 3 d to 16 weeks in STZ-induced diabetic BN rats (Zhang et al., 2005). Rats that had received STZ but remained normoglycemic, showed no increase of retinal vascular permeability (Qaum et al., 2001). These data suggest that BRB breakage in STZ-induced diabetic rats is a consequence of hyperglycemia itself and not a toxic effect of STZ. Thus, we used the STZ-induced diabetic BN rat as a model for the studies of retinal vascular permeability.

The present study demonstrated that VME delayed or prevented the increase of diabetes-induced endothelial permeability along with its concomitant VEGF upregulation and loss of tight junction integrity. Diabetic macular edema is a common pathological feature in diabetic retinopathy and is responsible for vision loss. BRB breakdown, a characteristic sign of early diabetic retinopathy, and the subsequent increase in vascular permeability are thought to play major roles in the development of diabetic macular edema and progression of diabetic retinopathy.

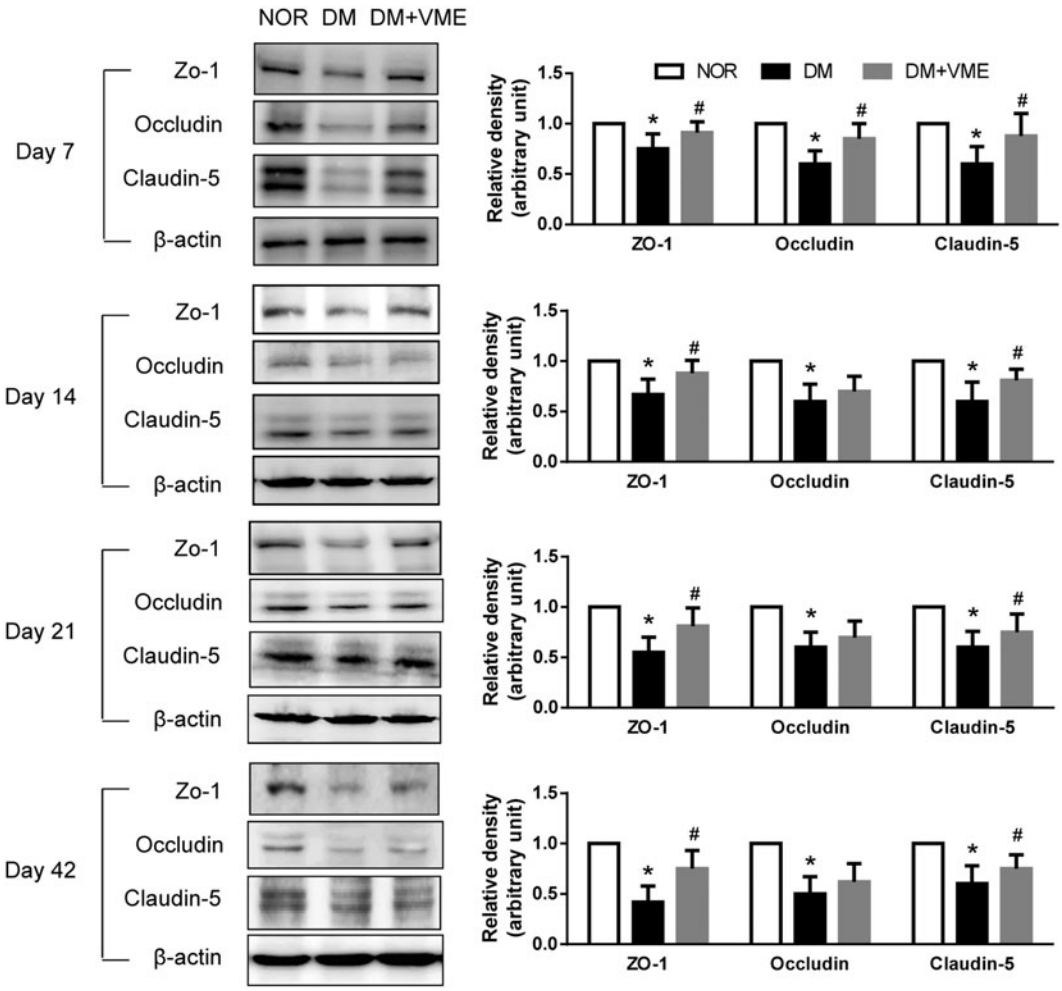

Figure 5. Effects of VME on diabetes-induced tight junction protein loss. Total protein was isolated, and a Western blot was performed. The diabetic retinas demonstrated less tight junction proteins (ZO-1, occludin and claudin-5) than the normal retinas. This loss of tight junction proteins was ameliorated by the treatment of VME. Values in the bar graphs represent the mean ± SE, $n = 8$. *$p < 0.01$ by Student's t-test (NOR versus DM); #$p < 0.01$ by Student's t-test (DM versus DM + VME).

VEGF is a potent angiogenesis and vascular permeability factor (Shweiki et al., 1992). In diabetes, elevated levels of retinal VEGF correspond with BRB breakdown in animals (Murata et al., 1995) and humans (Vinores et al., 1997). The induction of VEGF promotes BRB breakdown and the eventual development of end-stage diabetic complications. Numerous studies have suggested that VEGF plays a key role in vascular injuries and that the inhibition of VEGF significantly blocks diabetes-induced vascular changes (Crawford et al., 2009). We found that VME treatment blocked increases in VEGF protein and mRNA expression in diabetic rats.

In addition, we observed that the treatment of diabetic mice with VME significantly inhibited the reduction of tight junction proteins. Tight junction proteins are expressed in the endothelial cells of the blood–brain barrier and the BRB (Watson et al., 1991). Similar to our findings, several investigators have also reported an increase in vascular permeability in STZ-induced diabetic retinas coincident with a decrease in endothelial junction proteins (Leal et al., 2007; Navaratna et al., 2007). VEGF can cause hyperpermeability by inducing phosphorylation of tight junction proteins, such as occludin and ZO-1 (Antonetti et al., 1999; Esser et al., 1998). The treatment of VME delayed the reduction of endothelial cell-cell junction proteins in STZ-induced diabetic rats by blocking VEGF expression. In previous reports, a dietary bilberry reduced the expression of genes in the mitogen activated protein kinase (MAPK) pathway in the retinas from mice fed a high-fat diet (Mykkanen et al., 2012). The activation of p38 MAPK has been reported in the retinas of diabetic rats and is associated with BRB breakdown (El-Remessy et al., 2006). VEGF increased MAPK activation in retinal endothelial cells (Yamagata et al., 2006). These results indicate that VME may prevent VEGF-related vascular pathology in the diabetic retina.

There is considerable interest in anti-VEGF compounds and materials because of their therapeutic potential (Campa & Harding, 2011). Several natural and synthetic compounds have been proposed as inhibitors of VEGF. VME contains 15 types of anthocyanins (Matsunaga et al., 2009). Anthocyanins have various health benefits due primarily to their anti-oxidative and anti-inflammatory properties (Wang & Stoner, 2008; Zhu et al., 2013). The delphinidin and cyanidin contents of VME are higher than those of the other anthocyanidins. Delphinidin and cyanidin inhibit the release of VEGF stimulated by platelet derived growth factor in vascular smooth muscle cells (Oak et al., 2006). Recently, it has been reported that delphinidin acts as a potent VEGF receptor (VEGFR) inhibitor and inhibits VEGF-dependent tyrosine phosphorylation of VEGFR-2 in human umbilical vein endothelial cells (Lamy et al., 2006). Anthocyanidins differ in the positions of the hydroxyl and methoxy groups in their B-rings (Sekher Pannala et al., 2001). A previous structure–function analysis has suggested that the inhibitory effects of delphinidin are enhanced by the presence of three hydroxyl groups at the

B-ring compared to other anthocyanidins (Lamy et al., 2006). Furthermore, a free hydroxyl group at position 3 may be essential for potent inhibition because the presence of sugar residues at this position in delphinidin 3-*O*-beta-glucopyranoside eliminates the inhibitory properties of delphinidin. Thus, delphinidin clearly exhibited the highest inhibitory potency to inhibit VEGF-related pathways (Oak et al., 2006). The VEGF/VEGFR-2 signaling pathway is essential for vascular permeability (Ferrara & Davis-Smyth, 1997). Therefore, it is suggested that the inhibition of diabetes-induced BRB breakage by VME may be related to the inhibitory action of delphinidin on VEGF expression and VEGFR-2 activity.

In present study, the effective dose of VME in rats is 100 mg/kg. The total anthocyanin content of VME is 360 mg/g. Thus, the optimal dose of anthocyanins for BRB breakage is estimated to 36 mg/kg. Considering an average body weight of an adult of 60 kg (Manimaran et al., 2010), this dose for a 60 kg human is equal to 2.16 g/d. The total anthocyanins content of *V. myrtillus* is generally in the range of 300–700 mg/100 g of fresh fruit (Burdulis et al., 2009). The daily intake of *V. myrtillus* translates to approximately 300–700 g of fresh billberries. Depending on our study, it needs the excessive consumption of billberry to get the same relative amount of anthocyanins. However, the dietary intake of anthocyanins may be enhanced with the consumption of commercially available *V. myrtillus* extract.

Conclusions

In conclusion, the present findings indicated that VME inhibits diabetes-induced BRB breakage in diabetic rats by preventing VEGF expression. The inhibition of retinal VEGF expression and the preventive effect on loss of tight junction proteins by VME might delay the onset of diabetic retinopathy. It seems likely that treatment with VME is an effective therapy for prevention of diabetes-induced BRB breakdown.

Declaration of interest

This research was supported by a grant K13040 from the Korea Institute of Oriental Medicine (KIOM). The authors declare that they have no conflicts of interest to disclose.

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