In Vivo Sequential Study of Skeletal Muscle Capillary Permeability in Diabetic Rats: Effect of Anthocyanosides

Francine Cohen-Boulakia, Paul E. Valensi, Hatem Boulahdour, Roger Lestrade, Jean-François Dufour-Lamartinie, Claudine Hort-Legrand, and Abraham Behar

Alterations in the capillary filtration of macromolecules are well documented in diabetic patients and experimental diabetes. Various flavonoids including anthocyanosides and ginkgo biloba extracts have been shown to be effective against experimentally induced capillary hyperfiltration. The aim of the present study was to test the effects of anthocyanosides on capillary filtration in diabetic rats. For this purpose, we have validated the use of our previously described in vivo method for measurement of the capillary filtration of albumin (CFA) in rats. Male Wistar rats with streptozotocin (STZ)-induced diabetes were randomized in 3 groups to receive either ginkgo biloba (group A), *Vaccinium myrtillus* (group B), or no treatment (group C). The isotopic test of CFA consisted of intravenously injecting ^{99m}technetium-labeled albumin, inducing venous compression on a hindquarter, and measuring radioactivity externally on the limb before, during, and after removal of venous compression. After removal of the tourniquet, the radioactivity curve decreased. Interstitial albumin retention (AR) and the ratio of the amplitudes of the low- and high-frequency peaks (LF/HF ratio), an index of lymphatic function obtained by the fast Fourier transform of the last part of the radioactivity curve, were calculated. In STZ-treated animals, the isotopic test was performed at a mean age of 97 days (time 1) and after 6 weeks (time 2) and 12 weeks (time 3) of treatment, ie, 6 and 12 weeks after time 1. At time 1, AR was significantly higher in the 3 diabetic groups than in the control rats, without a significant difference between these groups. In group B, AR decreased significantly ($P = .015$) at times 2 and 3. In group C, AR increased significantly ($P < .0005$) from time 1 to time 3. In group A, AR increased slightly (NS) between time 1 and time 3. In groups A and C, the LF/HF ratio significantly increased with time ($P < .0005$) and the levels at time 3 were significantly higher versus control rats ($P < .0001$). In group B, the LF/HF ratio remained unchanged from time 1 to time 3 and similar to the values found in the control rats. In conclusion, these data show that (1) this new in vivo noninvasive method can be used to study CFA in skeletal muscle in diabetic rats, (2) it is reproducible and may be repeated over several months to evaluate spontaneous microcirculatory changes, and (3) anthocyanosides appear to be effective in preventing the increase in CFA and the failure of lymphatic uptake of interstitial albumin in diabetic animals.

VARIOUS MICROCIRCULATORY disorders have been described in diabetes. Several of them may occur before the onset of microangiopathic lesions and are supposedly crucial in the pathogenesis of microcirculatory complications in diabetes. Functional microangiopathy is characterized by increases in arteriolar blood flow and in capillary vasodilation, blood flow, and filtration and by rheological disorders.[1,2]

The changes in vascular permeability have been demonstrated by different methods. The capillary filtration of albumin (CFA) may be studied indirectly in vivo by using labeled albumin and measuring the systemic transcapillary albumin escape rate. This method has been used widely in humans[3,4] and sometimes in rats.[5] An increase in transcapillary albumin leakage has been extensively described in type 1 diabetes.[3,4] However, the measurement of the transcapillary escape rate investigates the whole transcapillary leakage of albumin and does not localize the phenomenon. In experimental diabetes, intravenous injection of labeled albumin has been used to assess albumin permeability in various tissues after death. This method has shown an increase in albumin permeability in eye, kidney, and nerve but has failed to demonstrate an increased leakage of albumin in skeletal muscle.[6] For several years, we have proposed human investigations of CFA in vivo in the forearm using an isotopic technique derived from the Landis method which measures interstitial albumin retention (AR) after venous compression. We have demonstrated with this method that the increase in CFA mostly in skeletal muscle is an early complication of diabetes and is mainly influenced by hypertension and neuropathy.[7,8]

The increase in macromolecular movement from the blood to the interstitium may result from different mechanisms,[1] including hemodynamic factors and metabolic and structural changes in the endothelial wall, in particular thickening of capillary basal membrane.[9]

Anthocyanosides of *Vaccinium myrtillus* are extracted from blueberries. They are currently used in ophthalmology for their capacity to improve vision and prevent diabetic retinopathy.[10-12] *Vaccinium myrtillus* is a flavonoid with a chemical structure linked to a phenylbenzopyrilium nucleus. Various flavonoids including anthocyanosides and ginkgo biloba extracts have been shown to be effective against experimentally induced capillary hyperfiltration.[13-15] With our in vivo method, we have also shown that a flavonoid fraction can improve and even normalize CFA in diabetic patients.[16] Regarding their mechanism of action, anthocyanosides are powerful inhibitors of aldose reductase activity,[17] a pathway involved in many diabetic complications. *Vaccinium myrtillus* inhibits cyclic adenosine monophosphate and cyclic guanosine monophosphate phosphodiesterase,[12] and is active as a scavenger of superoxide anions generated in xanthine oxidase system[12] and as an antioxidant on human low-density lipoproteins.[18] These effects together with

From the Laboratory of Biophysics, Paris 6 University, Paris; Laboratory of Nutrition and Metabolic Diseases, Jean Verdier Hospital, Paris-Nord University, Bondy; and Laboratory Leurquin—Mediolanum, Neuilly sur Marne, France.

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Address reprint requests to Professor Paul E. Valensi, MD, Hôpital Jean Verdier, Avenue du 14 Juillet, 93143 Bondy, France.

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the lack of significant adverse effects[10,12,14] led us to reconsider the putative clinical interest of this compound.

The aim of the present study was to test the effects of anthocyanosides on capillary filtration in diabetic rats. For this purpose, we have validated the use of our in vivo method for CFA measurement in rats to assess the therapeutic effect of anthocyanosides during several weeks of follow-up evaluation.

MATERIALS AND METHODS

Animals

Diabetes was induced in 45 5-day-old male Wistar rats by intraperitoneal injection of streptozotocin (STZ) at a dose of 70 mg/kg in citrate buffer (pH 4.5). The animals did not receive hypoglycemic agents during the trial. The study began when the rats reached a mean age of 3 months and ended when they were aged 6 months. Twenty male Wistar rats aged 59 ± 19 days and weighing 370 ± 100 g were used as controls. Ten of them were again tested at the age of 170 ± 25 days (body weight, 463 ± 62 g).

Experimental Protocol

After STZ administration, the animals were randomized in 3 groups of 15 rats to receive either ginkgo biloba (group A; IPSEN, Paris, France), *Vaccinium myrtillus* (group B; Leurquin Mediolanum, Neuilly-sur-Marne, France), or no treatment (group C) in drinking water. In groups A and B, medications were administered at a daily dosage of 40 mg/kg in 5 mL drinking water as previously tested.[12,19]

Three diabetic rats, one from each group, were tested on every day of the investigation. The isotopic test was repeated 3 times: at a mean age of 97 days (time 1) and after 6 weeks (time 2, age 137 days) and 12 weeks (time 3, age 180 days) of treatment, ie, 6 and 12 weeks after time 1. Similarly, 5 control rats were treated with ginkgo biloba and 5 others by *Vaccinium myrtillus.* These 10 rats were again tested after 6 and 12 weeks of treatment.

Blood glucose was periodically monitored with a strip (One Touch II; Johnson & Johnson, Milpitas, CA). Only STZ-treated rats with blood glucose greater than 10 mmol/L were considered diabetic and included in the study.

Isotopic Test of CFA

Procedure. Rats were anesthetized with inhalation of mixed 1% fluothane and oxygen. Human serum albumin was labeled with ^{99m}technetium and 37 MBq in 0.2 mL was injected intravenously into the tail. The animals were positioned prone. Radioactivity was measured on the 2 hindquarters with well-collimated INa crystal activated with thallium (51 mm × 51 mm). The detection field had a diameter of 18 mm and was located at 1 cm of the limb.[20] A multichannel analyzer (4,096 channels), with a time acquisition of 200 milliseconds per channel was used to obtain quantitative data. Acquisition was started at the steady state demonstrated by a constant radioactive curve achieved about 15 minutes after the albumin injection. The total number of impulses per channel varied from 2,000 to 8,000 ips. The mean background was 4 impulses per channel. Venous compression with a tourniquet (weight, 150 g) was applied on one limb 2.5 minutes after the beginning of the measurement and exerted for 6 minutes. After removing the tourniquet, the measurement of radioactivity continued for 5.5 minutes. Soon after, the same acquisition was performed on the other limb with the same procedure including venous compression.

Expression of the results. As previously shown in patients,[7,8,16] the radioactivity curve was characterized by 3 phases: (1) basal steady state (x), (2) an increase during venous compression leading to a maximal plateau (y), and (3) after removal of the tourniquet, a rapid decrease of radioactivity that returned to a mean value close to the basal level in normal animals. AR (percent) was calculated as an index of CFA with the formula, $AR = [(Z - X)/(Y - X)] \times 100$ (Fig 1). The reproducibility of the method was tested by comparing AR values for both hindquarters in 5 normal rats investigated twice in 2 days with a repeated injection of ^{99m}Tc-albumin.

The last part of the radioactivity curve recorded after removal of the tourniquet was also analyzed by the fast Fourier transform as previously described.[7,8,16,19] The time-function curve was transformed into a spectrum of peaks of discrete frequencies, a phase function and a frequency function B(f),

$$B(f) = \int_{-\infty}^{+\infty} A(t) \times \exp(-j \times 2\pi \times ft) \times dt,$$

where A(t) is the activity in ips and ft is the Fourier transform. Four peaks of different frequencies with amplitudes higher than the other harmonics were thus identified: high-frequency from 1,250 to 75 mHz and low-frequency from 75 to 4 mHz. The ratio of the amplitudes of the low- and high-frequency peaks (LF/HF ratio) was calculated and expressed as a percentage and may be considered an index of lymphatic function in rats (Fig 2). Indeed, in humans, we performed the same test with indium 111–labeled albumin and ^{99m}technetium-labeled erythrocytes and found that the LF/HF ratio was always normal with erythrocytes even when this ratio was increased with albumin,[21] which strongly suggests that the LF/HF ratio is an index for the lymphatic uptake of interstitial albumin.[8]

Reliability of the test. Since the labeling of human serum albumin with technetium is criticized for its long-term instability, we performed preliminary tests. In vitro 1 hour later (at laboratory temperature and at 37°C), we have always found a labeling yield of albumin between 95% and 98%. Moreover, any free fraction of technetium is immediately

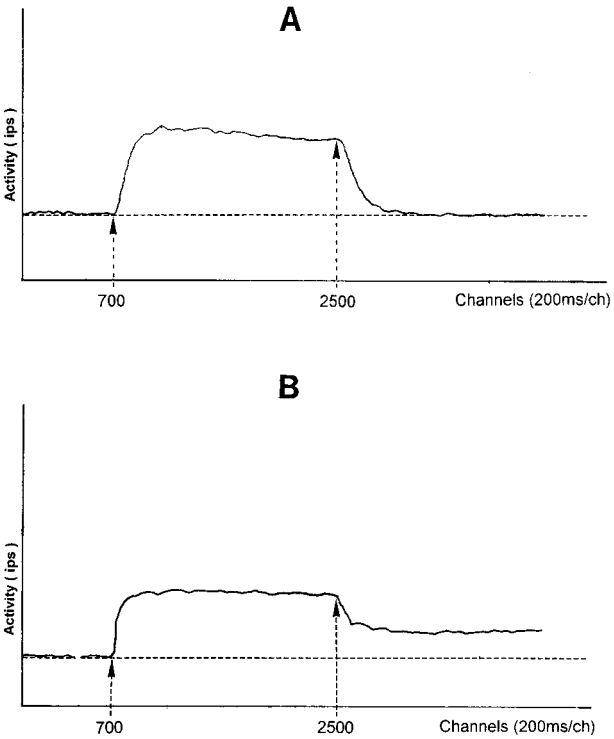

Fig 1. Radioactivity curve in a control rat (A) and a diabetic rat (B). The first arrow corresponds to the beginning of venous compression, and the second arrow to the removal of venous compression. AR was 0% and 25% in these 2 animals, respectively.

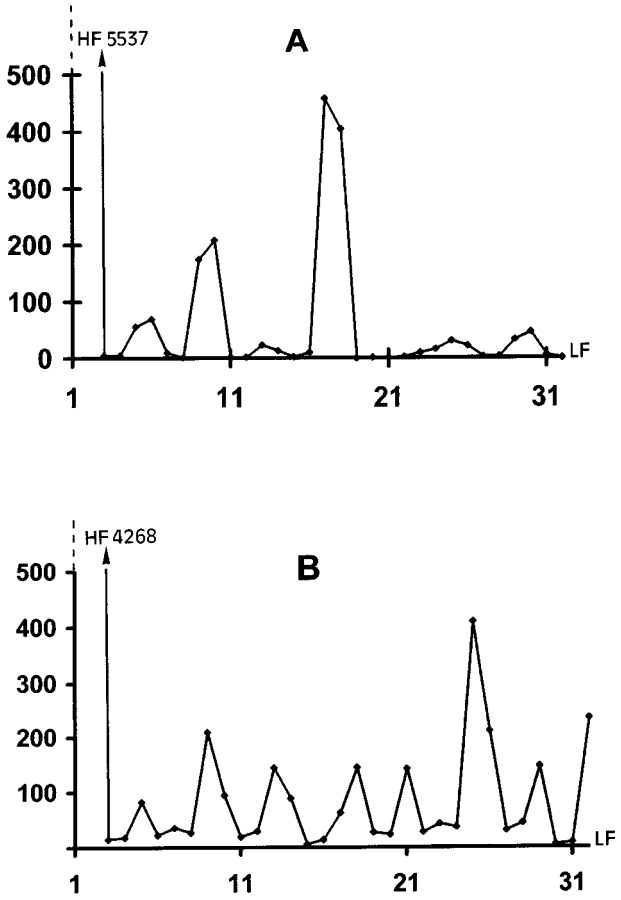

Fig 2. Fast Fourier transform of the last part of the radioactivity curve. In the control rat (A), the peak LF (harmonic 32)/HF ratio is $9 \cdot 10^{-6}$. In the diabetic rat (B), the LF/HF ratio is far higher, $5.46 \cdot 10^{-2}$.

eliminated by the kidneys and does not alter the measurements. The use of ^{131}I and ^{111}In (with DTPA) shows the same labeling yield.[22]

Statistical Analyses

The results are expressed as the mean ± SEM. Comparisons between groups were made using the Student *t* test. To study the changes in AR and the LF/HF ratio during the treatment of diabetic rats, we used the General Linear Model procedure to test the following: (1) differences between groups at each time. Levene's test was used to test the hypothesis that the variance of each parameter was similar in these groups; (2) overall changes with time. Mauchly's test was used to verify the assumption of sphericity of the variance-covariance matrix. If this assumption appeared to be violated, an adjustment of the degrees of freedom was used (Huynh-Feldt or Greenhouse-Geisser epsilon); and (3) the time × treatment interaction. The statistical tests were performed using SPSS software (SPSS, Chicago, IL).

RESULTS

Glycemia

In the control rats, the mean blood glucose was 5.0 ± 0.2 mmol/L. Two rats were eliminated from groups A and B and 3 from group C because they had glycemia less than 10 mmol/L. In addition, 3 rats died during anesthesia in groups A and C and 2 in group B. At the end of the study, there were 10 rats in group A, 11 in group B, and 10 in group C. For these 31 rats, blood glucose was 19.5 ± 1.2 mmol/L at time 1, 18.5 ± 0.6 mmol/L at time 2, and 16.1 ± 1.4 mmol/L at time 3.

CFA

Only the data for these 31 rats were considered. Age, body weight, and blood glucose at the time of CFA studies are shown in Table 1. These parameters did not differ significantly between the 3 diabetic groups at any time.

Control rats. There was no difference between the left and right hindquarters ($P > .05$) in the 20 normal rats, so we calculated the mean AR value for both sides in each rat. No significant difference for the mean AR for both hindquarters was observed between 2 isotopic tests performed at a 2-day interval in 5 normal rats. Moreover, AR was very similar at the first test (n = 20, 0.68% ± 0.32%) and in the 10 normal rats retested at a mean age of 170 days (n = 10, 0.75% ± 0.28%). Therefore, for this whole series of 30 untreated normal rats, AR was pooled and the mean AR was 0.72% ± 0.30%.

In the same way, no significant difference was found for the LF/HF ratio between the left and right hindquarters ($P > .05$) in the 30 untreated normal rats. The normal value for the LF/HF ratio was 0.15% ± 0.03%. There was no difference in the mean LF/HF ratio for both hindquarters between the 2 isotopic tests performed at a 2-day interval in 5 normal rats. The mean coefficient of variation for this parameter was 3%.

In the control rats treated with either ginkgo biloba or *Vaccinium myrtillus*, AR and the LF/HF ratio did not change significantly. After 6 and 12 weeks of treatment, the mean AR was 0.23% ± 0.20% and 0.38% ± 0.22% after ginkgo biloba and 0.05% ± 0.04% and 0.10% ± 0.05% after *Vaccinium myrtillus.*

Diabetic rats. At time 1, AR was significantly higher in the 3 groups of diabetic rats versus the untreated control rats ($P < .001$) and was similar in the 3 diabetic groups. In group A, AR increased from 9.6% ± 4.2% to 22.6% ± 2.9% between time 1 and time 3. In group B, AR decreased sharply from 14.0% ± 4.9% at time 1 to 3.2% ± 1.3% at time 3, and at time 3, AR did not differ versus the untreated normal rats. In group C, AR increased from 12.7% ± 4.1% at time 1 to 33.0% ± 3.3% at time 3. The overall changes in AR with time were significant ($P = .007$). There was a significant time × treatment interaction ($P < .0001$). At time 2, AR was significantly lower in

Table 1. Age and Blood Glucose at the Time of CFA Studies in the Three Groups of Diabetic Rats

Group	No. of Rats	Age (d)	Body Weight (g)	Blood Glucose (mmol/L)
Time 1				
A	10	96 ± 3	325 ± 16	19.6 ± 1.0
B	11	98 ± 3	320 ± 15	20.3 ± 1.3
C	10	97 ± 3	330 ± 18	18.0 ± 1.2
Time 2				
A	10	135 ± 4	349 ± 15	17.8 ± 1.4
B	11	138 ± 4	341 ± 17	18.5 ± 1.0
C	10	136 ± 4	379 ± 20	18.8 ± 1.7
Time 3				
A	10	178 ± 3	381 ± 15	15.7 ± 1.1
B	11	181 ± 3	346 ± 11	17.4 ± 1.2
C	10	180 ± 3	406 ± 18	14.6 ± 1.1

group B versus group C ($P < .0001$). At time 3, AR was lower in group B than in groups A and C ($P = .019$ and $<.0001$, respectively) (Fig 3).

Regarding the LF/HF ratio at time 1, it was not significantly different in the 3 groups of diabetic rats compared with the untreated normal rats and did not differ significantly between diabetic groups (Fig 4). In groups A and C, the LF/HF ratio increased from 0.35% ± 0.11% to 1.41% ± 0.30% and from 0.47% ± 0.16% to 2.33% ± 0.45%, respectively, between time 1 and time 3, and the levels at time 3 were significantly higher than the values in the untreated normal rats ($P < .0001$). In group B, the LF/HF ratio remained unchanged from time 1 to time 3 (0.40% ± 0.15% and 0.30% ± 0.11%, respectively) and similar to the values found in the untreated normal rats. The overall changes in the LF/HF ratio with time were significant ($P < .0001$). There was a significant time × treatment interaction ($P = .002$). At time 2, the LF/HF ratio was lower in groups A and B compared with group C ($P = .03$ and .05, respectively). At time 3, the pattern was similar ($P = .05$ and $<.0001$), with the lowest level in group C. There was no toxic effect related to the treatment in any group.

DISCUSSION

Our present data indicate that the measurement of CFA with a clinical in vivo isotopic test is applicable in rats. The present results also confirm that long-term treatment of STZ rats with *Vaccinium myrtillus* prevents the alteration of CFA induced by diabetes.

The alteration in the capillary filtration of macromolecules in diabetic patients is well documented.[3,4,7,8] In experimental studies, fluid filtration and vascular permeability are mainly measured ex vivo by extravasation of radiolabeled albumin or Evans blue in different organs after intravenous injection.[23] These invasive methods, which have been extensively validated, do not allow an investigation of albumin permeability in optimal physiological conditions. In the present study, we have applied a clinical in vivo isotopic test of CFA in diabetic rats.[7,8] This test is based on the Landis method which includes, after injection of labeled albumin and removal of venous compression, measurement of the retention (AR) and lymphatic uptake (LF/HF ratio) of interstitial albumin. This method has been validated with Wistar rats at the hindquarters, and accounts for capillary filtration in body tissues, probably for the most part in skeletal muscle. In these conditions, the between-day repeatability is less than 10%, indicating that this method is reproducible in rats.

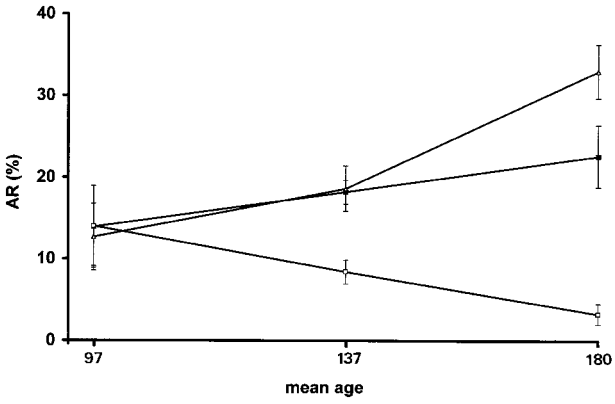

Fig 3. Changes in AR in the 3 groups of diabetic rats. Group A, ginkgo biloba (■); group B, *Vaccinium myrtillus* (□); group C, no treatment (△).

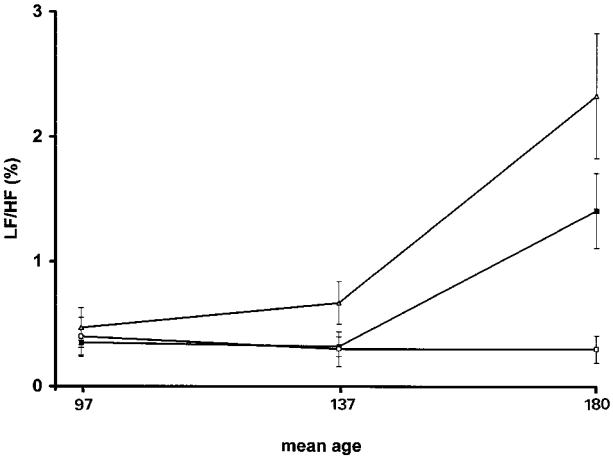

Fig 4. Changes in the index of lymphatic function (LF/HF ratio) in the 3 groups of diabetic rats. Group A, ginkgo biloba (■); group B, *Vaccinium myrtillus* (□); group C, no treatment (△).

In diabetic STZ rats, we have found an increase in AR and the LF/HF ratio with time, which indicates, in good accordance with many studies, that chronic diabetes induces a progressive increase in albumin permeability. We did not measure albumin permeability before STZ treatment, but the low AR obtained in nontreated Wistar rats suggests that this disorder occurs early after the induction of diabetes, before the first measurements at time 1.

The early onset of the increase in CFA is likely to indicate functional changes that may be related either to metabolic factors or to hemodynamic disorders. Increased blood flow has been previously described in experimental diabetes in mesenteric vessels[24] and glomerular capillaries.[25] In diabetic STZ rats, systolic hypertension has been reported 1 week after STZ injection,[26] but later, no difference has been noted between diabetic rats and their normoglycemic controls.[5] We cannot exclude that the initial hypertensive phase during the first week after STZ injection may have contributed to the increase in CFA found later in our experiments. The metabolic factors most likely reflect permanent structural alterations in long-lived extracellular matrix components that induce irreversible changes in blood vessel permeability in diabetic rats. The metabolic factors may involve an activation of the aldose reductase pathway as supported by the preventive effect of aldose reductase inhibitors on the increase in the vascular filtration of albumin in various tissues,[27,28] or an accumulation of advanced glycation end products[29] as suggested by the cleavers of advanced glycation end products which restore large artery properties in experimental diabetes.[30]

Among the pharmaceuticals likely to be of benefit in microcirculatory changes, flavonoids have been proposed for a long time. Flavonoids are widely distributed throughout the plant kingdom and are present in large amounts in foods.[31] They have been found to be effective against an induced disturbance

in capillary filtration in experimental conditions.[13-15] Moreover, several flavonoids appear to be powerful inhibitors of aldose reductase activity.[17,32-37] In particular, this has been demonstrated with anthocyanosides.[17] We have previously shown that a purified micronized flavonoid fraction can improve and even normalize CFA in diabetic patients.[16] Flavonoids can also prevent or delay the occurrence of cataract in rat lens perfused in a high-glucose solution[32] or in diabetic rabbits.[17] A beneficial effect on neuropathy in diabetic animals has also been reported.[33]

In the present study, our results indicate that long-term treatment with *Vaccinium myrtillus* but not with gingko biloba, reverses the alteration of CFA induced by diabetes. We have used the same daily dosage of 40 mg/kg successfully tested for the 2 agents in other situations, eg, the systemic capillary leak syndrome.[19,38] Nevertheless, in our model, a higher dosage of gingko biloba might have been more efficient. Many hypotheses have been proposed to explain the effect of *Vaccinium myrtillus* on capillary fluid filtration. We found that 6 weeks after STZ injection, AR and the LF/HF ratio were not different between *Vaccinium myrtillus*–treated diabetic rats and untreated animals, suggesting that *Vaccinium myrtillus* did not antagonize the acute effect of STZ on local hemodynamics. Nevertheless, the later decrease of CFA in the diabetic rats treated by *Vaccinium myrtillus* strongly suggests that this agent is more efficient for preserving the alteration in capillary filtration of macromolecules due to metabolic factors. Indeed, anthocyanosides have been shown to preserve vascular collagen,[39] and thus to limit irreversible changes in permeability due to permanent structural alterations in long-lived extracellular matrix components.[29] Lymphatic disorders have been shown to occur early in diabetic patients and even to precede the appearance of an increase in interstitial AR.[7] Lymphatic function is in fact enhanced in diabetes but is overwhelmed because of the increase in albumin capillary leakage.[40] The beneficial effect of *Vaccinium myrtillus* on the LF/HF ratio, which reflects lymphatic function in humans,[21] may therefore be considered a limiting effect against the interstitial accumulation of macromolecules and possibly glycated materials.

In conclusion, we have developed a new in vivo noninvasive method to study CFA in skeletal muscle in diabetic rats. This method is reproducible and may be used over several months to evaluate spontaneous microcirculatory changes. It is helpful when assessing the therapeutic effect of a medication. Anthocyanosides appear effective for preventing the increase in CFA and the failure of lymphatic uptake of interstitial albumin in diabetic animals. This result may be compared with the preventive effect of these agents on diabetic retinopathy.[41] Other controlled trials using anthocyanosides should be performed to evaluate their effects on retinopathy, nephropathy, and nerve function. As regards the mechanisms involved in these effects, further studies are required to evaluate the effect of this agent, given alone or with insulin, on structural changes in the endothelial wall related to metabolic factors or the effect on hemodynamic factors, particularly capillary blood flow.

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