

## Breakdown of the Inner and Outer Blood Retinal Barrier in Streptozotocin-Induced Diabetes

ANÁLIA DO CARMO\*, PEDRO RAMOS, ALDINA REIS, RUI PROENÇA  
AND J. G. CUNHA-VAZ

Centre of Ophthalmology, Institute for Biomedical Research of Light and Image, University of Coimbra,  
Coimbra, Portugal

(Received Cleveland 3 March 1998 and accepted in revised form 18 June 1998)

Using vitreous fluorophotometry and quantitative fluorescence microscopy the authors studied the permeability of the blood-retinal barrier (BRB) to fluorescein in control and in 8 days streptozotocin-induced diabetic rats.

Vitreous fluorophotometry showed that fluorescein permeates BRB in control and in diabetic rats. However, in diabetic rats the permeability to fluorescein was significantly increased as compared to control rats. The vitreous penetration ratio (VPR) values for total and free fluorescein at 60 min, were higher for diabetic rats ( $231.2 \pm 12.9 \text{ min}^{-1}$  for total fluorescein and  $1299.24 \pm 58.0 \text{ min}^{-1}$  for free fluorescein) than for control rats ( $95.5 \pm 3.5 \text{ min}^{-1}$  for total fluorescein and  $646.6 \pm 55.0 \text{ min}^{-1}$  for free fluorescein) ( $P < 0.05$ ).

Quantitative confocal fluorescence microscopy confirmed these findings and identified the site of leakage across the BRB by comparing the relative importance of the fluorescein leakage across the outer and inner BRB.

In control rats the fluorescence levels remained relatively low in the photoreceptor layer, next to the outer BRB but in the inner nuclear layer, next to the inner BRB reached values that were almost ten times higher. These results suggest that in retinas of control rats fluorescein penetrates predominantly through the inner BRB.

In diabetic rats the fluorescence levels in the photoreceptor and in the inner nuclear layer were significantly increased as compared to the fluorescence levels in controls rats. Nevertheless, in the inner nuclear layer the fluorescence levels were also generally higher than the fluorescence levels at the photoreceptor layer. The rates of fluorescence levels between the inner nuclear layer and the photoreceptor layer were apparently 3:1, 60 min after the single intravenous injection of fluorescein. Also, the fluorescein penetration in the inner nuclear layer of the diabetic rats is higher than that observed in the inner nuclear layer of the control rats ( $P < 0.001$ ).

These findings suggest that the permeability to fluorescein of both components of the BRB is increased 8 days after the induction of diabetes by streptozotocin and that the permeability of the retinal vasculature is preferentially affected.

© 1998 Academic Press

*Key words:* blood-retinal barrier; diabetes; quantitative confocal microscopy; fluorophotometry.

### 1. Introduction

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 (RPE) (Cunha Vaz et al., 1975; Palestine and Brubaker, 1980; Foulds, 1990). 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. For these reasons the BRB has an important role in the homeostasis of the retina (Cunha Vaz and Maurice, 1967; Tsuboi et al., 1984; Törnquist, Alm and Bill, 1990).

In a variety of pathological conditions, namely in diabetes, the BRB breaks down. However, the con-

tribution of each BRB component to that breakdown is not known (Vinores et al., 1990; Murata, Ishibashi and Inomata, 1993).

Previous studies, in streptozotocin induced diabetic rats, using horseradish peroxidase as the tracer suggested that this breakdown occurred preferentially at the level of the retinal pigment epithelium (RPE) (Tso et al., 1980), whereas other studies detected leakage only from the retinal vasculature (Ishibashi et al., 1980) or failed to detect any leakage (Wallow, 1983). Lanthanum perfusion has also been used to demonstrate leakage at the level of the RPE but not at the level of the retinal vasculature (Caldwell et al., 1985; Caldwell and Slapnik, 1992).

Conflicting results have also been reported using fluorescein as the marker. Kirber et al. (1980) suggested that 4 weeks after the induction of diabetes by streptozotocin there was an increased permeability to fluorescein at the RPE but not at the level of the retinal blood vessels (Kirber et al., 1980), nevertheless an increase in permeability to fluorescein was not

\* Correspondence and reprint requests: IBILI—Dept of Ophthalmology, Azinhaga de Sta Comba, Celas, 3000 Coimbra, Portugal.

detected either in RPE or in retinal blood vessels by Grimes (1985). In these studies however, the interpretation of the results was biased by technical problems related with the fluorescein concentrations used and by the limits of light microscopy resolution.

Fluorescein is used in daily clinical practice as a biological marker of BRB permeability because of its low toxicity and high fluorescence. Since the conclusions from the available studies were highly controversial and realizing that fluorescein is used clinically as a marker BRB permeability to fluorescein was studied by vitreous fluorophotometry and examined the contribution of each BRB component to permeability by quantitative confocal fluorescence microscopy, 8 days after the induction of diabetes.

## 2. Materials and Methods

### Drugs

Fluorescein was purchased from Faure. Streptozotocin was obtained from Sigma Chemical Co, St Louis. Ketamine was obtained from Parke-Davis. All other chemicals were of analytical grade.

### Animal Models

All the animals were watered and fed *ad libitum*, and were maintained on a 12 hr light/12 hr dark cycle.

To induce diabetes male Wistar rats (250–300 g body weight) were anaesthetized with ether and injected peritoneally with a freshly prepared solution of streptozotocin (STZ) (60 mg/ml/kg in 0.1 M citrate buffer, pH = 4.5). At the same time control rats were injected with an equal volume of 0.1 M citrate buffer. All the animals were assessed regularly for weight, blood glucose and polyuria. Blood for these analyses was collected via tail clipping under ether anesthesia. The blood glucose levels were checked by a colorimetric method based on a standard method. The criteria for diabetes was persistent hyperglycemia (> 350 mg 100 ml<sup>-1</sup>), polyuria and impaired growth.

### Vitreous Fluorophotometry

Ocular fluorophotometry was performed with a fluorophotometer (Fluorotron Master) fitted with a small animal adapter (Coherent) according to Ishiko et al. (1996) in ten control rats and in ten diabetic rats.

Vitreous fluorophotometry was performed before injection of citrate buffer or streptozotocin and 8 days following. All the animals were anaesthetized with ketamine hydrochloride (90 mg kg<sup>-1</sup>), the pupils were dilated with 1% tropicamide, cornea and conjunctiva were anesthetized with a topical anaesthetic (oxibuprocaine 4 mg ml<sup>-1</sup>) to help the examination procedure. To be sure that oxibuprocaine did not have any vasoactive effects the eyes were scanned before and after the application of the anaesthetic although, no differences in vitreous fluorescence were detected.

To prevent corneal dehydration, each cornea was humidified with a NaCl 0.9% solution. Recordings of the vitreous fluorescence were made for each eye by scanning from behind the retina to the crystalline lens. Background fluorescence of the vitreous was determined for each rat prior to fluorescein injection. The fluorescence intensity due to fluorescein was measured 30 min and 60 min after the intravenous injection via rat tail vein of 2% fluorescein-Na (14 mg kg<sup>-1</sup> body weight). Determination of the vitreous fluorescence was made using two different methods. In one of the methods, vitreous fluorescence was determined right after the retina peak 30 and 60 min after the fluorescein injection. In the other method, vitreous fluorescence was determined in the vitreous plateau, right before the crystalline lens.

Total and free plasma fluorescein levels, were determined from arterial blood collected in heparinized tubes 10, 15 and 50 min after the injection of fluorescein, according to Vine et al. (1984).

Determination of the vitreous penetration ratio (VPR) for fluorescein required a combination of the fluorescein concentration in plasma and in the vitreous body. This combination was performed by curve fitting using a simplified model of the eye as described by Lund-Andersen et al. (1985).

### Quantitative Confocal Fluorescence Microscopy

Since vitreous fluorophotometry does not allow to distinguish between the permeability of RPE and the permeability of the retinal blood vessels we combined this method with quantitative confocal fluorescence microscopy to quantify the permeability of each component of the BRB.

After an experimental interval of 8 days, 50 rats from the control group and 50 rats from the diabetic group were anaesthetized and injected i.v. with 2% fluorescein-Na via the tail vein (14 mg kg<sup>-1</sup> body weight). Ten control rats and ten diabetic rats were killed at each time interval (5, 30, 60 and 120 min) after fluorescein injection and the eyes were enucleated. To determine background fluorescence ten control rats and ten diabetic rats were enucleated without being injected with sodium fluorescein.

All the eyes were then embedded in O.C.T. compound and were quick-frozen in methanol immersed in liquid nitrogen for 24 hr. Sections were cut, placed on glass slides and evaluated. Each cryostat section of 10  $\mu$ m was placed on a glass slide, which was always maintained in ice and immediately analysed under transmission and epifluorescence light without being mounted or covered with a cover-slip.

The chosen method was based on a preliminary study, where rats were injected with 2% fluorescein-Na via the tail vein (14 mg kg<sup>-1</sup> body weight) and the enucleated eyes were analysed according three different methods in order to choose the best method to quantify the fluorescence in retina. Ten control and

ten diabetic rats were used in each method. In the first method, the eyes were removed after the fluorescein injection, immediately embedded in OCT and frozen in methanol immersed in liquid nitrogen. The frozen eyes were then freeze-dried at  $-35^{\circ}\text{C}$  for 12 hr to fix fluorescein, and then embedded in paraffin according to the method described by Patricia Grimes (1985). In the second method, the eyes were removed after the fluorescein injection, immediately embedded in OCT and frozen in methanol immersed in liquid nitrogen and sectioned in a cryostat. Each cryostat section was placed on a glass slide covered with poly-L-lysine, immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 at  $4^{\circ}\text{C}$  for 10 min, and analysed without being mounted or covered with a cover-slip. The third method, was the one that we used throughout our study. The examination of the sections revealed that the structure was better preserved using the third method and also that there were no statistical differences in fluorescein distribution between the three methods.

The equipment used for quantitative fluorescence microscopy consisted of a confocal microscope (BioRad MRC 600) previously described (Wells et al., 1990) equipped with a microscope (Nikon Optiphot-2), an epifluorescence system connected to a photomultiplier in which aperture was maintained constant throughout, and to a digitizer. The illumination system consisted of a Kr-Ar laser, a  $20\times$  and  $40\times$  fluor objectives and a BHS filter block which included an excitation filter 488 nm, a  $45^{\circ}$  dichroic reflector which reflects  $< 510$  nm and a barrier filter at 510 nm.

Eye sections were evaluated under transmission light to check for any structural abnormalities in the retina. Epifluorescence was used to quantify background fluorescence and fluorescence from the injected fluorescein. Transmission and fluorescence images were collected, digitized and down-loaded to an optical disk (Mitsubishi). Image analysis was performed on a PC computer (Research Machines), using software from BioRad. The gray scale was calibrated according to the instructions of the BioRad assistance. The black level and the gain was adjusted to allow the intensity range from the specimen to span from 0 to 255 in the digitized image. The pixel area as well as the objective, pinhole aperture, black level, and gain were maintained constant in all the experiments. Fluorescence intensity was measured in the photoreceptor layer and in the inner nuclear layer of the digitized eye sections from the control and diabetic rats.

From each eye 20 sections were made. In each section, fluorescence was measured at ten different locations of the inner nuclear layer and at ten different locations of the photoreceptor layer, all of them with the same dimension, randomly-selected and excluding retinal vessels, in order to obtain a mean signal for the fluorescence intensity of each retinal layer in each retina section. The measurements obtained for each

retinal layer were then adjusted by subtracting the tissue background fluorescence intensity determined under similar conditions, to yield the fluorescence intensity attributable to fluorescein. The mean measurements obtained from the photoreceptor layer and from the inner nuclear layer of diabetic rats were then compared to the values obtained from the same retinal layers of control rats.

### Statistical Analysis

All data are presented as mean  $\pm$  S.E.M. Statistical analysis was performed using Statistica W/5.0 from Statsoft. Student's *t*-test for independent samples was used to compare differences between the control and the diabetic groups and student's *t*-test for dependent samples was used to compare differences between the same group (control or diabetic). Receiver operating characteristic (ROC) curve was performed using MedCalc.

## 3. Results

### Diabetic State

Eight days after streptozotocin administration, the average plasma glucose concentration of the diabetic rats was  $414 \pm 42.2$  mg  $100\text{ ml}^{-1}$ . The average plasma glucose concentration measured in control rats was  $96 \pm 12$  mg  $100\text{ ml}^{-1}$  (Table I). All streptozotocin injected rats exhibited hyperglycemia, polyuria and decreased weight (Table I).

### Vitreous Fluorophotometry

The natural vitreous background fluorescence, determined before the fluorescein injection, in control and in diabetic rats was  $3.16 \pm 0.34$  ngEq  $\text{ml}^{-1}$ .

Vitreous fluorescence values were determined immediately after the retina peak, and directly before the crystalline lens. The ROC analysis for the two methods revealed that the area under the ROC curve (AUC) was  $0.62 \pm 0.104$  for the values determined immediately after the retina peak and  $1.00 \pm 0.0$  for the values determined directly before the crystalline lens. For this reason the results presented here are those that were determined directly before the crystalline lens, as these

TABLE I  
*Glycemia, polyuria and body weight in control and STZ-induced diabetic rats*

	Glycemia (mg $100\text{ ml}^{-1}$ )	Polyuria (ml $24\text{ hr}^{-1}$ )	Weight (g)
Control rats	$96 \pm 12$	$10 \pm 2.0$	$250 \pm 5.0$
Diabetic rats	$414 \pm 42.2^*$	$138 \pm 12^*$	$237 \pm 6.5$

Each value was the result from 10 control and 10 diabetic rats. Data are mean  $\pm$  S.E.M. values of separate experiments. Significantly different values are indicated: \*  $P < 0.05$  vs. control rats (Student's *t*-test for independent samples).

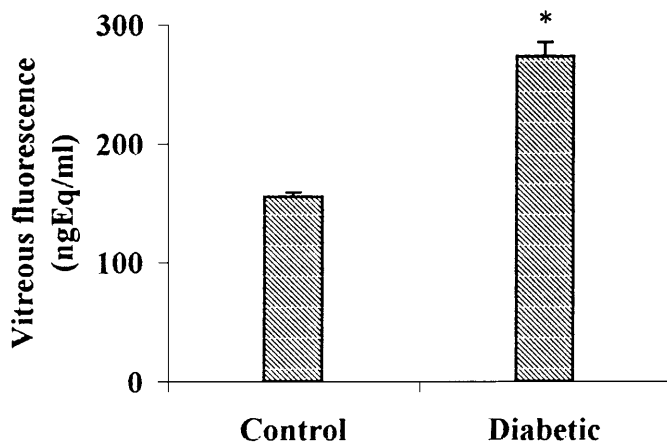


FIG. 1. Vitreous fluorescence measured in the vitreous plateau before the crystalline lens from ten control and ten diabetic rats, 60 min after the fluorescein injection using vitreous fluorophotometry. Data are mean  $\pm$  S.E.M. values of separate experiments. Significantly different values are indicated: \* $P < 0.01$  vs. vitreous fluorescence from control rat (Student's  $t$ -test for independent samples).

have less contribution from fundus fluorescence scattering.

Using this method of analysis it was not possible to determine the vitreous fluorescence 30 min after the fluorescein injection since the scan profile does not yield a defined vitreous plateau at this time interval. In the 60 min scan there was a clear vitreous plateau and the vitreous fluorescence was relatively constant from animal to animal, within each animal group.

According to this method of analysis, vitreous fluorescence value 60 min after the injection of fluorescein in control rats was  $155.18 \pm 3.54$  ngEq ml<sup>-1</sup> whilst in diabetic rats it was  $272.84 \pm 11.88$  ngEq ml<sup>-1</sup> ( $P < 0.05$ ) (Fig. 1).

These results showed that fluorescein penetrates the BRB of control and diabetic rats, and also that the fluorescence in the vitreous of the diabetic rats is significantly higher than the fluorescence in the vitreous of the control rats, (Fig. 1).

The quantification of plasma fluorescein revealed that in control and in diabetic rats after the fluorescein injection, the blood level of fluorescein rises until

TABLE III

Vitreous Penetration Ratio (VPR) values from total and free fluorescein concentrations at 60 min after the i.v. injection of  $14 \text{ mg kg}^{-1}$  of fluorescein in control and in diabetic rats

	VPR (min <sup>-1</sup> )	
	Total	Free
Control rats	$95.5 \pm 3.5$	$646.6 \pm 55.0^*$
Diabetic rats	$231.22 \pm 12.9$	$1299.24 \pm 58.0^*$

Data are mean  $\pm$  S.E.M. values of separate experiments from ten control and ten diabetic rats, 60 min after the fluorescein injection using vitreous fluorophotometry. Data are mean  $\pm$  S.E.M. values of separate experiments. Significantly different values are indicated: \* $P < 0.01$  vs. retina from control rats (Student's  $t$ -test for independent samples).

5 min after the injection and then falls rapidly (Table II). Nevertheless, from 5–60 min after the fluorescein injection, plasma fluorescein concentration decreases faster in diabetic rats than in control rats—70.0% and 81.5% respectively (Table II).

In diabetic rats, the percentage of free fluorescein was slightly higher than in control rats, at all time intervals. In control rats at 5, 30, 60 and at 120 min after the injection of fluorescein, the free fluorescein was respectively 13.5, 15, 17 and 15% of the total plasma fluorescein. In diabetic rats, at the same time intervals the free fluorescein was respectively 15, 18.2, 22.1 and 20.2% of the total plasma fluorescein (Table II).

To correct for individual variations in plasma fluorescein concentrations the vitreous fluorescence values, we determined the VPR values using the ratio between the vitreous fluorescence determined by the plateau method, in the 60 min scan and the integral of plasma fluorescein. The VPR values for total and free fluorescein at 60 min, were higher for diabetic rats ( $231.2 \pm 12.9 \text{ min}^{-1}$  for total fluorescein and  $1299.24 \pm 58.0 \text{ min}^{-1}$  for free fluorescein) than for control rats ( $95.5 \pm 3.5 \text{ min}^{-1}$  for total fluorescein and  $646.6 \pm 55.0 \text{ min}^{-1}$  for free fluorescein) (Table III).

TABLE II

Total and free fluorescein blood concentrations (ng ml<sup>-1</sup>) at different time intervals after the i.v. injection of  $14 \text{ mg kg}^{-1}$  of fluorescein in control and diabetic rats

Time after fluorescein injection (min)	Control		Diabetic	
	Total	Free (%)	Total	Free (%)
5	$53.41 \pm 1.15$	13.5	$51.26 \pm 2.87$	15
30	$27.77 \pm 1.01$	15	$17.94 \pm 2.21^*$	18.2
60	$16.02 \pm 0.72$	17	$9.48 \pm 1.75^*$	22.1
120	$4.91 \pm 0.85$	15	$4.87 \pm 1.66$	20.2

Total and free fluorescein concentrations were determined using fluorometry. The mean from each experiment was the result from ten control and ten diabetic rats. Data are mean  $\pm$  S.E.M. values of separate experiments. Significantly different values are indicated: \* $P < 0.01$  vs. plasma fluorescein in control rats (Student's  $t$ -test for independent samples).

TABLE IV

*Fluorescence intensity in the photoreceptor layer of the retinas from control and diabetic rats, at different time intervals, after fluorescein injection (14 mg kg<sup>-1</sup> i.v.)*

Time (min)	Fluorescence intensity in the photoreceptor layer (pixels)	
	Retinas from control rats	Retinas from diabetic rats
5	0.62 ± 0.21	39.89 ± 3.31*
30	0.70 ± 0.06	81.20 ± 5.60*
60	0.73 ± 0.04	30.38 ± 4.30*
120	0.76 ± 0.04	0.82 ± 0.03

Data are mean ± S.E.M. values of separate experiments. The mean from each experiment was the result of measurements in the photoreceptor layer of the retina from ten control and ten diabetic rats according to the section material and methods. \* $P < 0.001$  vs. retinas from control rats (Student's *t*-test for independent samples).

TABLE V

*Fluorescence intensity in the inner nuclear layer of the retinas from control and diabetic rats, at different time intervals, after fluorescein injection (14 mg kg<sup>-1</sup> i.v.)*

Time (min)	Fluorescence intensity in the inner nuclear layer (pixels)	
	Retinas from control rats	Retinas from diabetic rats
5	0.36 ± 0.04	32.81 ± 1.22*
30	5.01 ± 0.81	113.45 ± 9.15*
60	7.47 ± 0.87	91.14 ± 6.47*
120	0.98 ± 0.04	3.21 ± 0.36*

Data are mean ± S.E.M. values of separate experiments. The mean from each experiment was the result of measurements in the inner nuclear layer of retina from ten control and ten diabetic rats according to the section material and methods. \* $P < 0.001$  vs. retinas from control rat (Student's *t*-test for independent samples).

These results showed that in diabetic rats 8 days after the induction of diabetes by streptozotocin, the BRB permeability to fluorescein was clearly increased in comparison to control rats.

#### *Quantitative Fluorescence Microscopy*

All eye sections from control and diabetic rats examined in this study had fairly well preserved structure when observed in the transmission mode. No apparent focal sites of fluorescein leakage were detected, either from the RPE or through the retinal blood vessels in any of the control or diabetic eyes, at any time interval. This observation is confirmed by the relatively constant measurements of fluorescence intensity in each retinal layer.

Tables IV and V compare dye fluorescence intensities in the photoreceptor layer and in the inner nuclear layer from retinas of control and diabetic rats, at increasing time intervals after the injection of fluo-

rescein. At all time intervals, fluorescence intensity in retinas from diabetic rats at the photoreceptor layer and at the inner nuclear layer was higher than in retinas from control rats.

Five min after the fluorescein injection the fluorescence intensity in the photoreceptor layer of the retina from control rats was  $0.62 \pm 0.21$  pixels and in the retinas from diabetic rats was already  $39.89 \pm 3.31$  pixels (Table IV). During this same time interval in the inner nuclear layer the fluorescence intensity was  $0.36 \pm 0.04$  pixels in retinas from control rats and  $32.81 \pm 1.22$  pixels in retinas from diabetic rats (Table V).

Thirty min after the injection of fluorescein, the fluorescence intensity in the photoreceptor layer of the retinas from control rats was  $0.70 \pm 0.06$  pixels and in retinas of diabetic rats was  $81.20 \pm 5.60$  pixels (Table IV). During this same time interval, in the inner nuclear layer the fluorescence intensity was  $5.01 \pm 0.81$  pixels in retinas from control rats and  $113.45 \pm 9.15$  pixels in retinas from diabetic rats (Table V). At this time, the retinas from diabetic rats reached maximal fluorescence intensities at both the photoreceptor and the inner nuclear layers.

Sixty min after the injection of fluorescein, the fluorescence intensity in the photoreceptor layer of the retina from control rats was  $0.73 \pm 0.04$  pixels and in retinas from diabetic rats was  $30.38 \pm 4.3$  pixels (Table IV). At this same time interval at the inner nuclear layer the fluorescence intensity was  $7.47 \pm 0.87$  pixels in retinas from control rats and  $91.14 \pm 6.47$  pixels in retinas from diabetic rats (Table V). Fluorescence intensity in the retinas from control rats reached its maximum value at the inner nuclear layer. At this time interval, in the diabetic group, the fluorescence intensity of the photoreceptor layer decreased more (changed from  $81.20 \pm 5.6$  pixels at 30 min to  $30.38 \pm 4.30$  pixels at 60 min, Table IV), than did that of the inner nuclear layer (changed from  $113.45 \pm 9.15$  pixels at 30 min to  $91.14 \pm 6.47$  pixels at 60 min, Table V). In retinas from diabetic rats, at this time, fluorescence intensity in the inner nuclear layer was three times greater than in the photoreceptor layer.

Two hrs after the infection of fluorescein, the fluorescence intensity at the photoreceptor layer, was similar in the control and in the diabetic groups. In contrast, at the inner nuclear layer of the retinas from the diabetic group, fluorescence intensity ( $3.21 \pm 0.36$  pixels) remained three times greater than in the inner nuclear layer of the retina from the control group ( $0.98 \pm 0.04$  pixels) (Table IV and V).

These results show that in retinas from control rats, fluorescence intensity at the photoreceptor layer, did not change significantly with time after the injection of fluorescein (Table IV). In the inner nuclear layer of these rats, we observed a progressive increase in fluorescence intensity until 60 min after the injection of fluorescein (from  $0.36 \pm 0.04$  pixels at 5 min to  $7.47 \pm 0.87$  pixels at 60 min) (Table V).

In the retinas from diabetic animals, the fluorescence intensity at the photoreceptor layer and at the inner nuclear layer increased until 30 min after the fluorescein injection, decreasing thereafter (Table IV, and V). At the inner nuclear layer the fluorescence intensity at 30, 60 and 120 min after the fluorescein injection was significantly higher than the fluorescence intensity at the photoreceptor layer (Table IV and V).

#### 4. Discussion

In this study the BRB permeability to fluorescein eight days after the induction of diabetes by streptozotocin was measured using vitreous fluorophotometry and quantitative confocal fluorescence microscopy.

The quantification of the vitreous and plasma fluorescein in the experimental animal model revealed that an intravenous injection of fluorescein  $14 \text{ mg kg}^{-1}$  of body weight is appropriate since at all time intervals vitreous and plasma fluorescein concentrations remained below  $113 \mu\text{g ml}^{-1}$ , which is the concentration associated with saturation of the organic anion transport of the BRB (Cunha-Vaz and Maurice, 1967).

VPR values were determined for total and free plasma fluorescein. The VPR values for diabetic rats were significantly higher than VPR values for control rats. These results show that 8 days after the induction of diabetes the permeability of BRB to fluorescein is already altered which confirm previous observations (Waltman et al., 1978, Jones, Cunha-Vaz and Rusin, 1982, Krupin, Waltman and Becker, 1982).

Since vitreous fluorophotometry gives information about the permeability of the blood ocular barrier, but does not provide any information about the relative contribution to the permeability of the retinal pigment epithelium or of the retinal blood vessels a confocal microscope was used to perform quantitative fluorescence microscopy. For information about the outer BRB leakage, the fluorescence was measured in the photoreceptor layer and for the inner BRB leakage, in the inner nuclear layer.

Quantitative fluorescence microscopy clearly demonstrated that fluorescein permeates the BRB in both the control and the diabetic rat retina, which is in agreement with previous studies (Kirber et al., 1980; Enea et al., 1989; Proença et al., 1990; Viores et al., 1990; Viores et al., 1993). In the retinas of diabetic rats the level of fluorescein at 5, 30 and 60 minutes was significantly higher than in the retinas of control rats, which confirms the results obtained with vitreous fluorophotometry and shows a marked alteration of the BRB in STZ-induced diabetes.

There were, however, interesting differences within each animal group, between fluorescence levels at different time intervals after the fluorescein administration and between fluorescence levels in the immediate vicinity of the inner and outer components

of the BRB. In the control rats, the fluorescence levels remained relatively low in the photoreceptor layer at all time intervals, indicating very little fluorescein penetration into the retina of the control rats through the outer BRB. The fluorescence levels in the inner nuclear layer reached values that were almost ten times higher, 30 and 60 minutes after the fluorescein administration suggesting that fluorescein penetrates the normal retina predominantly through the inner BRB. The higher value registered 5 min after injection in the outer BRB may be attributed to the larger surface between blood and retina of the outer BRB.

In the diabetic rats, the fluorescence levels were also generally higher in the inner nuclear layer when compared to the photoreceptor layer. Five min after fluorescein administration the fluorescence levels were similar in both sites of the retina, but when compared to control values, the increase of fluorescein penetration is already much more marked in the vicinity of the inner BRB.

This increased vulnerability of the inner BRB in experimental streptozotocin-induced diabetes became even clearer 30 and 60 min after the fluorescein administration. The rates of fluorescence levels between the inner nuclear layer and the photoreceptor layer were apparently 3:1, 60 min after the single intravenous injection of fluorescein.

What appears to be clear from the results presented here is that although the permeability to fluorescein of both components of the BRB are altered in STZ-induced diabetes, the permeability of the retina vasculature is preferentially affected in this experimental situation.

Previous studies reported conflicting results using fluorescein as a marker of the BRB permeability in diabetes (Kirber et al., 1980; Grimes 1985). Differences among the experimental protocol and among the technical procedures used in those previous studies may explain the different results. In the studies that used vitreous fluorophotometry, plasma values were not taken into account and in the studies that used quantitative fluorescence microscopy the conventional fluorescence microscope used had much less resolution than the confocal microscope used in this study. Yet, our results are in agreement with studies using extravascular albumin as a marker of the BRB permeability, indicating that in streptozotocin diabetic rats the BRB failure is mainly localized at the level of the inner nuclear layer (Viores et al., 1990, 1993).

The mechanism of this preferential BRB breakdown in the retinal vessels of STZ-induced diabetic rats remains to be elucidated. It may be due either to an alteration of the junctional complexes as proposed by Gardner et al. (1998) or enhanced endothelial pinocytotic transport as described by Hofman et al. (1998) in vascular endothelial growth factor induced retinopathy in monkeys, or both.

In summary, the present study suggests that fluorescein is a good marker of the BRB permeability

and that quantitative confocal fluorescence microscopy is a useful technique for identification of the site of BRB breakdown. This study shows that the BRB breakdown that occurs 8 days after induction of diabetes by STZ occurs predominantly at the level of the retinal blood vessels, with associated failure of the RPE, albeit to an apparently lesser degree. Further studies are needed to clarify the nature of the BRB breakdown in diabetes.

### Acknowledgements

The authors want to thank Dr Barbara Oliveiros for the statistic analysis and to Mrs Graciete Abreu and Miss Carla Duarte for technical assistance.

This work was supported by a grant from the 'Junta Nacional de Investigação Científica'.

### References

- Caldwell, R. B. and Slapnick, S. M. (1992). Freeze-fracture and lanthanum studies of the retinal microvasculature in diabetic rats. *Invest. Ophthalmol. Vis. Sci.* **33**, 1610–19.
- Caldwell, R. B., Slapnick, S. M. and McLaughlin, B. J. (1985). Lanthanum and freeze-fracture studies of retinal pigment epithelial cell tight junctions in the streptozotocin diabetic rat. *Curr. Eye Res.* **4**, 215–19.
- Cunha-Vaz, J. G., Shakib, M. and Ashton, N. (1966). Studies on the permeability of the blood-retinal barrier. *Br. J. Ophthalmol.* **50**, 441–6.
- Cunha-Vaz, J. G. and Maurice, D. (1967). The active transport of fluorescein by the retinal vessels and the retina. *J. Physiol.* **191**, 467–8.
- Cunha-Vaz, J. G., Faria de Abreu, J., Campos, A. and Figo, G. (1975). Early breakdown of the blood-retinal barrier in diabetes. *Br. J. Ophthalmol.* **59**, 649–56.
- Enea, N. A., Hollis, T. M., Kern, J. A. and Gardner, T. M. (1989). Histamine H1 receptors mediate increased blood-retinal barrier permeability in experimental diabetes. *Arch. Ophthalmol.* **107**, 270–4.
- Foulds, W. S. (1990). The choroidal circulation and retinal metabolism—An overview. *Eye* **4**, 243–8.
- Gardner, T. W., Antonetti, D. A., Khin, A. J., Barber, E., Lieth, E., Ratz, M. and Hollinger, L. A. (1998). Reduced retinal vascular occludin accompanies increased permeability in diabetic rats. *Invest. Ophthalmol. Vis. Sci.* **39**, S673.
- Grimes, P. A. (1985). Fluorescein distribution in retinas of normal and diabetic rats. *Exp. Eye Res.* **41**, 227–38.
- Hofman, P., Blaauwgeers, H. G. T., Tolentino, M. J., Adamis, A. P., Werf, F., van der, Cardozo, J. N., Vrensen, G. F. and Schlingeman, R. O. (1998). Ultrastructural cellular basis of microvascular hypermeability in VEGF induced retinopathy in monkeys. *Invest. Ophthalmol. Vis. Sci.* **39**, S1116.
- Ishiko, S., Yoshida, A., Kitaya, N., Abiko, T., Mori, F., Kado, M., Takahashi, M. and Saito, K. (1996). Fluorophotometry with a small animal adapter. *Jpn. J. Ophthalmol.* **40**, 310–16.
- Jones, C., Cunha-Vaz, J. G. and Rusin, M. (1982). Vitreous fluorophotometry in the alloxan and streptozotocin treated rat. *Arch. Ophthalmol.* **100**, 1141–5.
- Kirber, W. M., Nichols, C. W., Grimes, P. A., Winegrad, A. I. and Laties, A. M. (1980). A permeability defect of the retinal pigment epithelium. *Arch. Ophthalmol.* **98**, 725–8.
- Lund-Andersen, H., Krogsaa, B., la Cour, M. and Larsen, J. (1985). Quantitative vitreous fluorophotometry applying a mathematical model of the eye. *Invest. Ophthalmol. Vis. Sci.* **26**, 698–710.