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STEREOLOGICAL ANALYSIS OF CHANGES IN THE 3-DIMENSIONAL STRUCTURE OF THE RETINAL VASCULATURE DURING DIABETES

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ABSTRACT

This study generated quantitative estimates of changes in the 3-dimensional structure of the retinal vasculature during diabetes.
Male Wistar rats were rendered diabetic with streptozotocin and sacrificed in groups of 6 animals after 6 months and 1 year duration of diabetes together with similar numbers of control rats. The eyes were enucleated and fixed for transmission electron microscopy. Using trephine blades, circular tissue blocks were cut from the central retina and embedded in resin. Estimates of the 3-dimensional structure of the retinal capillaries were produced using the modern stereological method of 'vertical sections' specifically adapted to generate estimates of volume and surface area of structures in vertical sections of retina. After 1 year of diabetes the volume and surface area of retinal capillary basement membrane had increased compared to both the corresponding controls and the 6 month diabetics ($p \leq 0.05$). The volume of retinal capillaries also increased after 1 year of diabetes ($p \leq 0.05$). The volume of capillary endothelium and pericytes was greater in the 1 year diabetics than in the 1 year controls or 6 month diabetics ($p \leq 0.05$). The data produced by this study represents the first estimates of the 3-dimensional structure of the retinal vasculature and the changes which occur during the development of diabetes.

Key words: diabetes, retina, stereology, vertical sections.

INTRODUCTION

Morphometric analysis of changes occurring to the retinal vasculature during diabetes have previously dealt only with 2-dimensional structural changes such as increases in capillary basement membrane thickness (Fischer and Gartner 1983; Stitt et al. 1994; Sosula et al. 1972). The lack of information to date on changes in the 3-dimensional structure of the retina probably results from difficulties in the generation of isotropic uniform random (IUR)

sections in a multi-layered epithelium. Previous stereology techniques were model based and required the use of IUR sections in order to generate unbiased estimates of 3-dimensional structure. As sections from a layered tissue would possess a particular orientation their use would therefore have led to biased estimates.

The introduction of the new design based stereology methods now allows the unbiased, quantitative estimation of 3-dimensional structure including volume and surface area from vertical sections (Baddeley et al. 1986). The present study uses the method of 'vertical sections' which has been specifically adapted to the retina (Anderson et al. 1994) to generate the first stereological estimates of changes in the 3-dimensional structure of the retinal vasculature during diabetes.

MATERIALS AND METHODS

Experimental diabetes was induced in male Wistar rats by a single intraperitoneal injection of streptozotocin (45mg/Kg). Six animals were sacrificed after 6 months duration of diabetes and another six after 12 months together with similar numbers of age- and sex-matched control rats. Following enucleation of the eyes the anterior segment and vitreous were removed and the posterior cup fixed by overnight immersion in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer. Using a 2mm diameter trephine blade, 5-6 cylindrical cores were cut from the central retina around, but not including, the optic disc (Fig 1). This sampling method ensured that the samples of retina for stereological analysis were uniform random, that is, every part of the central retina had an equal chance of being sampled. The trephines of retina were then post-fixed in osmium tetroxide, dehydrated and embedded in Spurr resin in a flat electron microscopy (EM) mould.

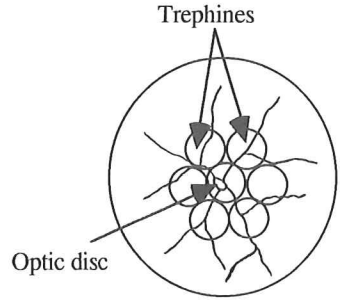

Fig 1. Diagram showing the position of the trephines taken from around the optic disc.

One semithin (1μm) and one ultrathin EM section were cut from each of 4 trephines taken from the right eye of each animal. The semithin sections were stained with toluidine blue and the EM sections with uranyl acetate and lead citrate.

The use of trephines as samples allowed sections of retina to be cut which fulfilled the criteria essential for the generation of vertical sections (Baddeley et al. 1986). Vertical sections must be taken at 90° to an arbitrary fixed plane in the specimen and they must be free to rotate about the vertical axis while still maintaining the 90° angle to the horizontal. The osmicated trephines of retina are flat, black cylinders, so when embedded in a flat EM mould and sectioned, the slice taken will be vertical and the orientation of the slice random (Fig 2).

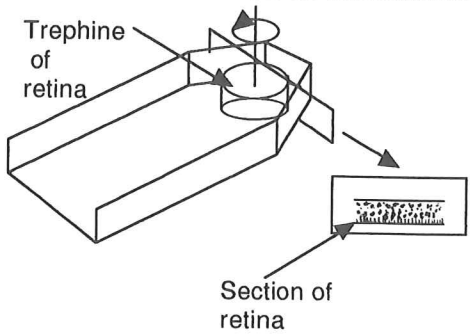

Fig. 2. Trephine of retina embedded in a flat EM mold.

Estimation of volume density (Vv)

A point-counting method was used to estimate volume density of the structure of interest, that is,

$$Vv = \frac{\text{Number of points over structure of interest}}{\text{Number of points over reference space}}$$

However, this method generates a ratio estimate of the volume of interest / reference volume. The use of such ratios as a method of expressing biological data can cause problems, for example the "reference trap" (Braendgaard and Gundersen 1986). Therefore, it is better to use total quantities which are more meaningful and sensitive indicators than ratios. Total quantity can be derived by multiplying the ratio by a reference volume (Vref), for example, in the case of Vv,

$$Vv \times Vref = V \text{ total}$$

Therefore, the first step in the present study was to estimate the volume of retina in each trephine to use as a reference volume. Each semithin section was viewed under a light microscope and the resulting images were transferred directly to an image analyser and adjusted for magnification. The 2-dimensional thickness of the retina was measured on the image analyser (Fenestra, Kinetic Imaging Ltd., Liverpool, England) and, as the diameter of the trephine was known (2mm), the volume of retina in each trephine could then be determined,

Volume of retina per trephine $= \pi r^2 \times h$ (where h = thickness of the retina) (1)

No further shrinkage of the 2mm trephines was measured in the polymerised resin blocks (measured with a micrometer loop).

The next step in the stereological analysis involved the estimation the Vv of capillaries in the retina. This was carried out on the image analyser using a digital stereology software package (Kinetic Imaging Ltd., Liverpool, England) which can generate and then superimpose a grid of points over an image. Again, using the semithin sections, Vv (capillaries/retina) was estimated by,

$$Vv\ (\text{capillaries/retina}) = \frac{\text{Points over capillaries}}{\text{Points over retina}} \quad (2)$$

An estimate was then made of the Vv of the structures of interest within the capillaries, that is, basement membrane (BM), endothelial cells and pericytes, by viewing the EM sections in a Hitachi H-7000 transmission electron microscope (Hitachi Scientific Instruments, Berkshire, England) fitted with a Gatan CCD TV camera. Images of every capillary in each section were transferred directly from the CCD camera to the image analyser and corrected for magnification. Again, using a computer-generated point grid,Vv was estimated. For example, for Vv (BM/capillaries),

$$Vv\ (\text{BM/capillaries}) = \frac{\text{Points over BM}}{\text{Points over capillaries}} \quad (3)$$

Ratio estimates of the Vv of the structures of interest in the capillaries were determined for each trephine.
The total volume of any structure of interest in a trephine was then determined by multiplying back to Vref, for example, for the total volume of BM in a trephine,

Volume of retina / trephine	X	Vv (capillaries/ retina)	X	Vv (BM/ capillaries)	=	Volume of BM/trephine)
(1)	X	(2)	X	(3)	=	(4)

The total volume of the structure of interest in the total volume of retina sampled from each eye was then determined by adding together the volumes obtained in (4) above for each of the four trephines from any one eye and the total amount of retina sampled from each eye determined by adding the Vrefs obtained in (1) from each of the four trephines.

Estimation of surface density (Sv).

The unbiased estimation of Sv of a structure in a vertical section is achieved by probing the structure with test lines whose length is weighted by sin θ, where θ is the angle between the line and the vertical (Baddeley et al. 1986). The cycloid represents such a line, therefore, the Sv of a structure of interest in a vertical section can be estimated using a cycloidal test system comprising cycloids and points (Baddeley et al. 1986). The mean number of intersections between the cycloids and the structure are counted as well as the number of points falling over the reference volume.

In the present study the Sv of capillary BM was estimated from the same EM images of retinal capillaries used to estimate Vv. The estimation of Sv was carried out on the image analyser using a digital stereology software package which can superimpose a test system comprising sin θ-weighted linear probes and points over each image ensuring that the short axis of the test line is orientated parallel to the vertical. The Sv of capillary BM for each trephine was estimated by counting the mean number of intersections between the test lines and both the inner and outer surfaces of the BM as well as the number of points falling over the BM. From these counts an estimate of Sv was generated using the relationship

$$Sv\ (BM/capillaries) = 2\ I / L \qquad (5)$$

(where S=surface area of interest, v=volume of object of interest, I= number of intersections, L=total length of test line falling on the object)

The ratio estimate of Sv for each trephine was converted to a total surface area of BM by multiplying by the total volume of BM in each trephine,

$$\text{Surface area of BM } (6) = (5)\ X\ (4) \qquad (6)$$

The surface area of the BM in the total volume of retina sampled from each eye was then determined by adding the results from (6) for each of the four trephines per eye.

Variation in the stereological estimates of volume and surface area between animals within the four groups was determined from the coefficient of variation: (CV)

$$CV = \frac{SD}{mean}$$

Statistical analysis was then carried out on the results using the Wilcoxan Rank Sum Test.

RESULTS

In order to compare the stereological estimates of the volume of retinal capillaries, basement membrane, capillary endothelial cells, pericytes and the surface area of basement membrane between the four groups of animals, the results were calculated as the volumes (μm^3) and surface areas (μm^{-1}) of the structures listed above which were present in $1.0x10^9$ mm^3 central retina. The values obtained are presented in Tables 1 below:

Table 1. The volumes of retinal capillaries, capillary endothelial cells, pericytes, basement membrane (BM) ($x10^6\mu m^3$) and the surface area of capillary BM ($x10^6\mu m^{-1}$) present in $1.0x10^9\mu m^3$ central retina from 1 year diabetic rats, 6 month diabetic rats, 1 year controls and 3 month control rats. CVs between animals are also shown.

	Volume of retinal capillaries	Volume of endothelial cells	Volume of pericytes	Volume of BM	Surface area of BM
1 year diabetic rat					
Rat1	4.50	1.30	0.34	1.17	4.60
Rat2	6.06	1.53	0.50	1.48	4.99
Rat3	2.39	0.87	0.14	0.65	2.16
Rat4	3.51	0.98	0.68	0.99	4.67
Rat5	4.59	1.59	0.22	1.55	4.53
Rat6	5.03	1.23	0.39	1.17	4.60
CV	26.4%	22.6%	46.8%	25.5%	22.3%
6 month diabetic rats					
Rat1	3.69	0.92	0.13	0.56	2.65
Rat2	2.92	0.78	0.08	0.53	2.05
Rat3	3.34	0.72	0.27	0.65	3.11
Rat4	3.09	1.12	0.06	0.47	2.15
Rat5	2.61	0.85	0.17	0.36	2.07
Rat6	3.04	1.02	0.12	0.54	2.51
CV	10.8%	15.3%	48.0%	17.7%	15.7%
1 year control rats					
Rat1	2.95	0.84	0.28	0.32	2.37
Rat2	2.96	0.88	0.27	0.36	2.77
Rat3	3.03	0.77	0.19	0.39	2.61
Rat4	2.41	0.76	0.14	0.28	2.15
Rat5	3.80	1.05	0.19	0.45	3.12
Rat6	3.54	1.04	0.33	0.36	2.55
CV	13.1%	27.1%	14.7%	11.6%	14.4%
3 month control rats					
Rat1	2.96	3.23	0.24	0.89	6.05
Rat2	3.04	3.86	0.73	1.36	9.69
Rat3	2.35	2.26	0.68	0.71	5.29
Rat4	3.40	2.63	0.68	0.77	8.63
Rat5	2.29	4.23	0.69	0.62	5.96
Rat6	3.36	2.31	0.67	0.91	7.89
CV	15.2%	24.3%	27.5%	27.0%	21.9%

Wilcoxan Rank Sum Tests carried out on the above estimates showed that after 1 year duration of diabetes there was a significant increase in the total volume and surface area of retinal capillary basement membrane in the central retina compared to both the corresponding 1 year old control animals and the 6 month diabetic rats ($p \leq 0.05$). A significant increase also occurred in the total volume of capillary endothelium and pericytes after 1 year of diabetes compared to the 6 month diabetics or the 1 year controls ($p \leq 0.05$). However, the younger controls had greater volumes of endothelium and pericytes compared to the other 3 groups of animals. Also, the total volume of retinal capillaries was greater in the 1 year diabetic rats compared to the 6 month diabetics or the controls ($p \leq 0.05$).

DISCUSSION

The present study illustrates the application of stereology to the retina producing the first estimates of the 3-dimensional structure of the retinal vasculature and the changes which occur during diabetes.

It is probable that stereology has not previously been used as a tool to study the retina due to difficulties in calculating a reference volume and creating accurate vertical sections. We have introduced the concept of using cores or trephines of retina as samples which allow the volume of retina sampled to be easily determined and also allow sections to be cut which fulfill the criteria essential for the generation of vertical sections (Baddeley et al. 1986).

The stereological estimates produced here show that the volume and surface area of retinal capillary BM increase during diabetes which correlates well with the previously reported increases in the 2-dimensional thickness of the BM (Fischer and Gartner 1983; Anderson et al. 1994; Sosula et al. 1972). Results also show an increase in the volume of retinal capillaries, and changes in the volumes of capillary endothelium and pericytes as a result of diabetes.

Information on 3-dimensional structure is particularly useful for biological systems as it is more relevant to cell function than 2-dimensional measurements. Thus, information on the 3-dimensional changes occurring to the retinal vascular system during diabetes may be a better reflection of any functional changes occurring in the diseased state.

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