Original article

Morphological and morphometric changes in rat optic nerve microvessels in a glaucoma experimental model

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Aim: To study the morphological and morphometric changes produced in the capillaries of the optic nerve (ON) head and initial portion after the experimental increase in intraocular pressure (IOP).

Material and methods: Wistar rats underwent cauterization of three episcleral veins, which produced an immediate increase in the IOP, and was maintained for 3 months. Sagittal sections of the eyeball were studied with immunohistochemical techniques, using a primary antibody to GLUT-1. The GLUT-1 positive capillaries were counted, and measurements were made of the area, perimeter and mean diameter.

Results: Microscopic examination of sections of the ON of control rats revealed a lower density and larger caliber of capillaries in the prelaminar region as compared with the other regions of the OP (p < .05). Comparison between the control and the experimental groups showed a reduction in capillary density (except in the prelaminar region) and a smaller size in all the areas of the OP studied, but less evident in the initial portion (p < .05).

Conclusions: The increase in IOP was associated with significant qualitative and quantitative changes in the capillaries of the laminar and poslaminar regions of the OP head. These changes appear to return toward parameters compatible with normality in the initial portion of the OP, an area where the vascular collapse was less evident. These findings might explain the significant reduction in ocular blood flow seen in patients with primary open-angle glaucoma.

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Cambios morfológicos y morfométricos en los capilares del nervio óptico de rata en un modelo experimental de glaucoma

RESUMEN

Objetivo: Estudiar los cambios morfológicos y morfométricos producidos en los capilares de la cabeza del nervio óptico (NO) y de su porción inicial, después de la elevación experimental de la presión intraocular (PIO).

Material y métodos: Se utilizaron ratas Wistar que fueron sometidas a cauterización de 3 venas epiesclerales, con el resultado inmediato de elevación de la PIO, manteniéndose esta durante 3 meses. Se realizaron secciones sagitales del globo ocular y se aplicaron técnicas inmunohistoquímicas, mediante un anticuerpo para GLUT-1. Se procedió al recuento de los capilares GLUT-1 positivo y se midieron área, perímetro y diámetro medio.

Resultados: El examen microscópico de las secciones del NO de las ratas controles demostró una menor densidad de capilares y un mayor calibre de los mismos en la región prelaminal, respecto a las demás regiones del NO (p < 0,05). Cuando se compararon los grupos control y experimental se observó una disminución en la densidad de capilares (excepto en la región prelaminal) y un menor tamaño de los mismos en todas las zonas del NO analizadas, menos evidente en la porción inicial (p < 0,05).

Conclusiones: El aumento de la PIO se relaciona con cambios cualitativos y cuantitativos de los capilares de las regiones laminar y poslaminal de la cabeza del NO, y parecen recuperarse hacia parámetros compatibles con la normalidad en la porción inicial del NO, donde el colapso vascular es menos evidente. Estos hallazgos podrían explicar la reducción significativa del flujo sanguíneo ocular observada en pacientes con glaucoma primario de ángulo abierto.

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Introduction

The topographic region corresponding to the optic nerve head (ONH) is an anatomical crossroads with very peculiar characteristics, making it vulnerable to numerous neuropathies, notably glaucoma. A number of risk factors are considered in said disease, mainly increased intraocular pressure (IOP). In addition, several clinical-epidemiological and experimental studies have demonstrated that vascular insufficiency arises at the ONH in the course of glaucomatous disease which leads to diminished blood supply for its structural components.1,2

ONH vascularization is complex and is mainly derived from the posterior ciliary arteries and the central retinal artery.3,4 Alterations in the posterior ciliary circulation in the ONH have been identified as one of the courses of glaucoma5 as well as common ischemic diseases.6

ONH capillaries are characterized by: (1) the presence of occlusive unions between endothelial cells which prevent the exchange of substances between blood and the interstitial space; (2) shortage of pinocytosis vesicles which provide limited trans-endothelial transport of a large range of substrates; and (3) the existence of specific membrane transport proteins involved in regulating the passage of molecules through capillary walls. However, even though the capillaries of the optic nerve (ON) prelaminary region (PLR) exhibit a morphology similar to the capillaries of other regions of this nerve structure, they feature intense transendothelial permeability regulated by the presence of abundant pinocytosis vesicles.7

A range of transport molecules have been utilized for studying the characteristics of the blood–brain barrier, including the glucose transporter family (GLUT)8 which enables glucose, the main energy substrate of the brain, to enter the cell. Specifically, isofrom GLUT-1 has been utilized as a marker for the hemato-ocular barrier9 due to its presence in the membrane of endothelial cells and on the astrocyte “feet” surrounding the capillary.

Previous studies carried out by our group utilizing an experimental ocular hypertension rat model similar to that developed by Shareef et al.,9 has evidenced that chronic high IOP induces an obstruction in the anterograde axon transport10 and an increase in the expression of nitric oxide synthase 1 and 2,11 compatible with the death of retina ganglion cells, one of the characteristics of glaucomatous optic neuropathy.

Due to the importance of microvascularization for the ONH and the role it plays in glaucomatous disease, in this study we have analyzed whether experimental chronic IOP increase gives rise to significant changes in the ON head. In this regard, we have used the GLUT-1 isofrom of the glucose carrier to immunohistochemically mark the capillaries of the various ON regions: PLR, laminar region (LR) and post-laminar region (PR) of the ONH, as well as its initial portion, evaluating the size and density of said capillaries.
Subjects, material and methods

Animals

The subjects of the experiment were Ten Wistar male rats weighing between 250 and 300 g, which were divided in 2 groups: control (n = 5) and experimental (n = 5). The experiments were carried out according to the European Union guidelines (86/609/EU) for the use of lab animals, substituted by the new Royal Decree 1201/2005, and approved by the Scientific Committee of Málaga University.

Experimental protocol

The animals of the experimental group were anesthetized with 8% chloral hydrate (0.1 ml/30 g weight) and subsequently submitted to cauterization of two episcleral veins of the upper rectus and one of the temporal rectus of the right eye, which produced immediate IOP increase. The animals were maintained for three months with raised IOP. The IOP values of both groups of animals were recorded with a Tono Pen XL tonometer (Mentor, Norwell, MA, USA).

Immunohistochemical processing

After three months with raised IOP, the animals were sacrificed with intraperitoneal 8% chloral hydrate, perfused with a cleansing solution of saline phosphate tampon 0.1 M with a pH of 7.4 and subsequently with 4% paraformaldehyde fixing solution in 0.1 M phosphate tampon with pH 7.4. After the perfusion, the right ocular globe of all animals were extracted and post-fixed in the same fixing solution at 4 °C during 4 h, transferred to 70% ethanol and included in paraffin in an automatic tissue processor (Myr, Tarragona, Spain). Sagittal sections with the thickness of 6 μm were pre-incubated in citrate tampon (pH 6.0) in a pressure container and treated with 0.06% H2O2 during 15 min. Subsequently, they were incubated overnight at 4 °C with the anti-GLUT-1 antibody (Rabbit Polyclonal Antibody AB 1340, Chemicon, IL, USA) at a dilution of 1:1,000 and with the biotinylated secondary goat anti-rabbit antibody (Dako A/S, Glostrup, Denmark) at a dilution of 1:600 during one hour at room temperature. Subsequently they were treated with peroxidase biotin-avidin complex (Vectastain-ABC kit, Vector Lab. Inc., Burlingame, CA, USA) during one hour and developed with DAB (3,3’-diaminobenzidine, Sigma-Aldrich, Madrid, Spain), utilizing peroxidase substrate during 5 min. After completing the immunohistochemical technique and in order to evidence the cell nuclei in the thickness of the retina, the sections were counterstained with Harris hematoxylin (Merck, VWR International Eurolab SL, Barcelona, Spain), dehydrated, set on Entellan (Merck, VWR International Eurolab SL, Barcelona, Spain) and examined under a microscope Nikon H550L microscope, Tokyo, Japan (Fig. 1a). Likewise, a section of each animal processed with the same protocol was included as negative control, omitting the primary antibody.

Morphometric analysis

The study comprised the 3 ONH regions: PLR, LR and PR, as well as the initial portion. For both groups at least 3 sections of each animal were chosen, drawing all the positive GLUT-1 capillaries taking as reference an area of 0.049152 mm². Three microscopic fields were used in each section for PLR, PR, and the initial ON portion and 4 in the LR. The study only included vessels having a cross-section with diameters comprised between 3 and 10 μm (Fig. 1a) in order to ensure that the assessed structures were true capillaries, excluding extraneous elements such as venules or small lymphatic vessels.12 In each region the number of capillaries/mm² of tissue were counted, and the mean capillary area, perimeter and diameter were measured with the Visilog Image Analysis application (Noesis, Paris, France) connected to a video camera (Polaroid Corp, Waltham, MA, USA) in turn connected to a light microscope (Elipse E400, Nikon, Tokyo, Japan) (Fig. 1b).

Statistical analysis

The statistical analysis of data was carried out by means of the SPSS 13.0 application (IBM, NY, USA), utilizing the Turkey nonparametric test and a multiple comparison test with significant differences. The significance level was considered to be p < 0.05.

Fig. 1 – (a) Optic nerve post-laminar region, showing immunohistochemically marked capillaries with antibodies anti-GLUT-1 (400×). (b) Anterior image taken with the Visilog Image Analysis application showing in color GLUT-1 positive capillaries. The true color of this figure can only be appreciated in the electronic version of this article.
Capillary prelaminar. (48.67 ± 2.92 µm²), and the smallest area of capillaries in the initial portion of the ON (30.15 ± 2.41 µm²) (p < 0.05) (Fig. 3a).

**Results**

IOP records of the control group, obtained at two-week intervals for a period of 3 months, exhibited a mean value of 14.85 ± 0.65 mmHg, while the experimental group exhibited a significant IOP increase which reached a mean value of 33.5 ± 1.06 mmHg (Fig. 2).

**Capillary size**

Control group: the morphometric study demonstrated that the largest area capillaries are located in the PLR (48.67 ± 2.92 µm²), and the smallest area of capillaries in the initial portion of the ON (30.15 ± 2.41 µm²) (p < 0.05) (Fig. 3a).

Capillary perimeter (Fig. 3b) and mean capillary diameter (Fig. 3c) exhibited the same tendency.

In the experimental group the capillary area diminished progressively at the ONH from the PLR up to the PR (p < 0.05). However, in the initial portion of the ON the mean size of capillaries is bigger than those in the PR (p < 0.05) (Fig. 3a–c).

A comparative study between the control and experimental group animals revealed that chronic increased IOP produces a significant reduction of said parameters in all the studied regions (p < 0.05). Accordingly, the area is approximately twice as small in the PLR and the LR, and 2.5 times in the PR. It must be emphasized that the PR capillaries exhibited the most significant size reduction (≈61%).

**Capillary density (number of capillaries/mm²)**

Control group: it was observed that the ONH PLR is the area exhibiting the lowest density of GLUT-1 positive capillaries (33.7 ± 2.5/mm²) and that it increases progressively in the remaining ON regions. In the LR, the number of capillaries/mm² was 86 ± 5 and in the PR an initial portion of the ON was 121 ± 9 and 121 ± 5, respectively (Fig. 3d).

Experimental group: in this group a progressive increase of the density of GLUT-1 positive capillaries was observed, from the PLR up to the initial portion of the ON (Fig. 3).

The comparative study between the control and experimental groups revealed that the chronic increased IOP did not modify the capillary density in the PLR. In the other regions of the study a significant reduction of this parameter was observed (p < 0.05) in the experimental group animals, with a loss of GLUT-1 positive capillaries of approximately 39% in the...
LR, 41.5% in the PR and 25% in the initial portion of the ON (p < 0.05).

Fig. 4a and b illustrate GLUT-1 marking of the various regions comprising the ON in the control and experimental groups, respectively.

Discussion

This study aims at verifying the effect that chronic increased IOP produces in the microvasculature of the ON. The chosen model was the episcleral vein cauterization model described by Shareef [4] that produces immediate IOP increases which persists 3 months after surgery. In addition, Wistar rats were chosen as experimentation animal due to low cost, ease of maintenance and possibility of obtaining high IOP for an extended period of time. [13]

Our results demonstrate that increased IOP during 3 months produces a significant reduction in the area, perimeter and mean diameter of GLUT-1 positive capillaries in the experimental group when compared to the control group. These results confirm the findings of de Feher et al. [14] about ONH capillaries in humans, which observed progressive occlusion of light with IOP increases. On the other hand, the size reduction described in the present study could be related to the ONH blood flow self-regulation mechanism in response to IOP increases, [15] so that when the ONH is compressed due to higher pressure, blood flow diminishes and causes vasoconstriction in the region. [5] Fuchsjäger-Mayrl et al. [16] observed that patients with open-angle primary glaucoma and ocular hypertension produced a reduction of ocular blood flow when compared to healthy subjects and indicated that ONH vascular anomalies could be an early event in the development of glaucoma.

In the capillaries of the initial ON portion we have found that the animals with increased IOP exhibited a significant increase of all analyzed parameters referring to PR. We believe that this size increase could be due to the lower vascular resistance in this area which is further away from the region submitted to the maximum compression.

The results of the present study also illustrate that the density of GLUT-1 positive capillaries in the PLR is lower to that of the remaining ON regions and is not modified after IOP has increased. These data match those of other studies [17, 18] carried out in groups of control and high IOP monkeys. However, the other ON regions exhibit a significant capillary density reduction vis-à-vis the control group.

Considering that the effects of increased IOP express mainly in the lamina cribosa, [19] a region which is particularly sensitive to IOP variations, it is possible that the LR capillaries compress easily when the lamina cribosa curves inwards, producing according to our results a significant 39% of capillaries density compared to the control group. We have also observed this behavior in the PR, with an estimated 41.5% reduction of this parameter. Third party research carried out in human patients with primary open angle glaucoma [20] demonstrate in this region that capillary density reduction runs in parallel to the loss of ON axons.

Said vascular aggression would cause hypoxia on the cellular component which makes up the ON. Compromised cells would comprise ganglion cell axons, astrocytes and microglia. [21] Varela and Hernández [22] described an increased expression of acid fibrillary gylal protein (selective marker for astrocyte activity) in the PLR and LR in different experimental glaucoma models. It is probable that said glial cells play a role in maintaining the blood-retina barrier. [23] This activation could be an attempt by the glial cell to maintain the retinal function and morphology after IOP had increased.

Microglia is a neuron damage sensor which plays an important role in the defense of the central nervous system. [24] Increased microglia activity has been demonstrated in glaucomatous animals with the likely purpose of modulating the early changes that take place in the ON. [25] Wang [26] stated that these cells play a neuroprotective role vis-à-vis the ganglion cell axons against possible alterations in the blood-barrier in glaucoma. Neufeld [27] indicated that these cells migrate from the ON to the parapapillary region and are probably responsible for the peripapillary choroidal atrophy observed in glaucomatous patients. Lam [25] has observed accumulations of these cells in the parapapillary vessels but not in the chorioretinal peripapillary area. It can be seen that the protective versus cytotoxic role of this cell is controversial and its function in the physiopathology of glaucoma is not clearly established.

Finally, the results of the present study demonstrate that in the group with increased IOP the capillary density in the initial
portion of the ON increases significantly compared to that of the FR. This could be due to the greater distance of this region from the vascular collapse zone brought about by increased IOP.

In summary, the effects of IOP on microvascularization of the optic nerve express mainly in the LR and PLR of the ONH, with significant reduction in the size as well as the density of these capillaries.

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Conflict of interests

No conflict of interests has been declared by the authors.

REFERENCES