IN INVOLVEMENT OF NITRIC OXIDE AND OTHER MOLECULES WITH REDOX POTENTIAL IN PRIMARY OPEN ANGLE GLAUCOMA

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ABSTRACT

**Purpose:** Nitric oxide (NO) and other molecules with redox potential are involved in cell signalling, including endothelial-dependent relaxation and the maintenance of vascular homeostasis. We investigated the availability of NO and the formation of reactive oxygen species (ROS) in the aqueous humour and its relationship to the pathogenic mechanisms of primary open-angle glaucoma (POAG).

**Methods:** We analysed biochemically aqueous humour samples from patients having anterior segment surgery that were divided into two separate groups: 1) patients having a Watson’s trabeculectomy because of worsening of the glaucoma evolution (GG; n=60), and 2) a comparative group of individuals having phacoemulsification for non-complicated cataracts (CG; n=60). Enzymatic-colo-

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INTRODUCTION

The aqueous humor is generated and evacuated in a constant and balanced manner in the eye’s anterior segment structure for its own nutrition as well as maintenance of the ocular tone (1). The intraocular pressure (IOP) regulation depends on the balance of complex mechanisms involved in producing and draining the aqueous humor, among which are the stability and survival of the cellular phenotypes involved and maintenance of homeostasis. Early diagnosis of ocular hypertension (OHT) is essential in preventing glaucoma. Glaucoma is still the main cause of irreversible blindness in the world, with a 2.4% incidence rate. It is estimated that 66.8 million people suffer from glaucoma, of which 6.7 million have bilateral blindness (2). In Spain there are around 300,000 people with diagnosed glaucoma and approximately 700,000 that suffer from it, without being diagnosed. Even though risk factors have been identified for glaucoma (3,4) and advancements have been made in the clinical-surgical treatment of it, the unstoppable advance of glaucoma and blindness due to optic atrophy has not been able to be avoided.

Nitric oxide (NO) is a short-lived hydrophobic gas, produced endogenously and that has a relatively simple chemical structure. NO is synthesized endogenously from L-Arginine, oxygen and NADPH, as shown in the reactions below, measured by nitric oxide synthase (NOS). Three isoforms have been identified: NOS1 (also called neural NOS, nNOS or NOS I), NOS2 (inducible NOS, iNOS or NOS II) and NOS3 (endothelial NOS, eNOS or NOS III). All the subtypes produce NO, L-Citrulline and NADP+ also specifying the hemo group and Ca$^{2+}$/calmodulin as co-factors (5,6).

Toward the end of the seventies, it was shown that the vessel dilator effects of nitro-glycerin and other nitrates were mediated by NO and that the latter could activate the soluble guanylyl cyclase in the smooth muscle cells of blood vessels. This allowed mechanisms for cyclic GMP synthesis by the specific enzyme to be known. Almost at the same time it was discovered that the relaxation of the blood vessels dependent on the action of acetylcholine needed an intact vascular endothelium and it was thought that there must be a specific substance that, when released by the endothelial cells, would act on...
the smooth muscle cells, or what we would consider to be a «relaxation factor derived by vascular endothelium» (7). In subsequent years, the conclusion was reached that this molecule was NO, through the evidence that endothelial cells produced enough NO to explain the relaxation seen. These findings confirmed the previous hypotheses, with the Nobel Prize for Physiology and Medicine given to Robert Furchgott, Louis Ignarro and Ferid Murad in 1998 for discovering the role of NO as a signalling molecule in the cardiovascular system and its role in maintaining health (8-10). NO is considered as a basic messenger for cell signalling systems in the brain and in the cardiovascular apparatus. Even though the synthesizing enzymes in the brain were identified (NOS1) and then in macrophages (NOS2) and in the endothelium (NOS3), these enzymes have also been found in other cells of the organism, taking part in different physiological and pathological processes, including eye diseases, in which its involvement in cataracts, uveitis and glaucoma has been described (11-13).

Oxidative stress is the result of one of the following processes: 1) increase in the formation and activity of oxidants, 2) decrease in the anti-oxidant defense mechanisms, or 3) failure of the oxidative damage repair mechanisms (14). The reactive oxygen species (ROS) are made up of free radicals, reactive and unstable anions that contain oxygen atoms, or molecules containing oxygen atoms that are capable of producing free radicals or that are chemically activated by them. Among the ROS the most relevant ones are the hydroxyl radical, superoxide anion, hydrogen peroxide and peroxynitrite. The highest ROS production happens during aerobic respiration, although they are also formed in the peroxysomes (fatty acid oxidation) and microsomes (cytochrome P450 system and xenobiotic metabolism), phagocytosis of pathogen agents or of lipopolysaccharides, metabolism of Arginine and some specific tissues (15). Under normal circumstances the ROS are eliminated mainly by the action of the superoxide dismutase enzymes (SOD), catalase and glutathione peroxidase (GSHPx). The formation and accumulation of ROS damages cells and tissues by way of macromolecule attack, such as polyunsaturated fatty acids of the membrane lipids, essential proteins and nucleic acids (16). Different diseases have been etiopathogenically linked with the ROS, and besides oxidative stress, they form part of the damaging mechanisms of Alzheimer’s, Parkinson’s, cancer and ageing. With regard to the visual system, ROS have also been implicated in the etiopathogenesis of different ophthalmologic processes, mainly cataracts, age-related macular degeneration and vitreo-retinopathies (17-19).

Applying all these concepts to our line of research on primary open-angle glaucoma (POAG) and trying to identify the molecules implicated in the progression of the glaucomatous disease, and studying their possible use as markers for the progress of glaucoma, we have designed this study with the aim of determining, by way of enzymatic-colorimetric methods, NO presence and that of the oxidative and antioxidant activities in the aqueous humor of patients with POAG who underwent anti-glaucoma surgery.

SUBJECTS, MATERIALS AND METHODS

We chose 120 consecutive patients of both sexes that came in for check-ups requested by the Ophthalmologists of the Glaucoma Department in the Ophthalmology clinics at the hospitals mentioned. After being adequately evaluated by using nomograms for calculating the risk of POAG progression, according to information still unpublished from Vinuesa-Silva et al (under review Arch Soc Esp Oftalmol), based on individual risk factors, the functional tests and morphological findings, those patients were considered to be at risk for vision loss due to their disease and therefore were on the road to anti-glaucoma surgery using Watson’s trabeculectomy technique (n=60). A comparative group of subjects with cataracts (CG; n=60) scheduled for phacoemulsification technique cataract surgery and intraocular lens implant were included in this study and analyzed with the CG. The inclusion and exclusion criteria of the subjects under study are found in table I.

During the first step of the intervention in both groups of patients, using standard paracentesis, a Rycroff cannula was inserted in the anterior chamber connected to an insulin syringe, absorbing an approximate volume of 0.1-0.02 µL of aqueous humor, avoiding collapse of the anterior chamber. These samples were directly deposited in sterile cryotubes that were labeled and put in a freezer at -20°C for one hour. Later they were sent off to storage in classification boxes in the deep freezer (-85°C) until processing.
The biochemical techniques that were used for all the aqueous humor samples are as follows:

1) Determination of the total nitric oxide concentration: By using a commercial preparation from R&D Systems. NO is a compound with a very short average lifespan, which is why conventional detection methods cannot be used. On the other hand, since most of the NO in the organism is found in the form of nitrite and nitrate we can use the concentration of these anions to perform a quantitative measurement of NO production. For this to be done, first of all the nitrate and nitrite must be converted and, later, convert this nitrite in a chromophore by using Griess’ reaction, and perform the spectrophotometric measurement at 550 nm (20, 21). The variability of the technique for determining the NO, just as it appears in the corresponding protocols, is from 10 to 92 µmols per liter.

2) Determination of the oxidant activity: By using a protocol that allows for malonyldialdehyde (MDA) to be determined as a product of lipid peroxidation, using the thiobarbituric acid (TBA) technique and TBA of reactive species (TBARS). The first step is to precipitate the proteins and separate the MDA from them. Then the MDA is made to react with the TBA (one hour boiling in the dark) and the result is the formation of a fluorescence-emitting compound (Figure 1). This compound is extracted using butanol and then the fluorescence of the supernatant put in a multiwell tray. The technique is already established and described extensively in previous works (19, 22, 23).

3) Determination of the total antioxidant status: Following an enzymatic-colorimetric technique and with a commercial preparation from Randox Laboratories, LLC. In this procedure the ferrimyoglobin and ABTS (2,2’-azino-die-[3-ethylbenzthiazoline sulphonate]) reaction is induced to obtain the ABTS cation radical, which gives off a greenish-blue color with an intensity that can be measured on a spectrophotometer at 600 nm. The presence of antioxidants inhibits the formation of this cation, so that the decrease in the intensity of this coloration produced is proportional to the antioxidant concentration in the sample (Figure 2). This technique has been described previously; it was used by our research team and validated in previous works (19, 22, 23).

As for the processing of the data, we must point out that the sample size was calculated by using Epiinfo StatCalc (Atlanta GA, USA). All of the information was recorded on an Excel worksheet (Microsoft Windows Professional, Microsoft Corporation, USA) designed for the purpose. The analysis of the results was done using SPSS 15.0 (SPSS Inc, Chicago, IL, USA). Student’s t test was used (with a 95% confidence interval) in order to compare the quantitative variables while the Chi squared test was used to analyze the qualitative variables.

RESULTS

Table II shows the general characteristics of the study subjects. Distribution by gender in both groups was the same, with a male-to-female ratio...
The age range was between 40 and 90 years old.

The biochemical tests performed on the aqueous humors of the subjects participating in the study has provided the following information:

1) Determination of the Total nitric oxide: The numbers obtained were the mean and the standard deviation (SD) of 2-3 measurements per patient. The results were significantly higher in the GG (75.885 µM; SD 29.203 µM) than in the CG (58.020 µM; 27.185 µM) with \( p = 0.0007 \). The median for both groups is shown in figure 3.

2) Determination of the oxidative activity (MDA/TBARS): The trial was repeated three times and the results are the mean and SD of the three measurements done per patient. Statistically significant and higher values were seen in the GG (0.442 µM; SD 0.0159 µM) than in the CG (0.089 µM; SD 0.030 µM) with \( p = 3.05551E-38 \). Figure 4 shows the median for both experimental groups.

3) Determination of the Total Antioxidant Status: The results obtained are the mean and the SD of two trials. The total antioxidant status was significantly lower in the GG (1.954 mM; SD 0.623 mM) than in the CG (4.010 mM; SD 1.341 mM) with \( p = 2.97074E-19 \). The median for both groups is shown in figure 5.

**DISCUSSION**

This study was designed to identify molecules that may intervene in the pathogenesis and progress of POAG. Due to the impossibility of recruiting a group of healthy people so as to surgically remove a sample of aqueous humor, this study used a group of patients with uncomplicated cataracts like the comparative group. Although the presence of oxidative stress in the cataracts has been previously proven (17) and taking into account that the numbers of this group are the result of the cataractogenesis process, we have started out with these numbers to compare the level of oxidative stress obtai-
ned from the glaucomatous patients during surgery according to the risk nomograms and the ophthalmologist’s criteria. The surgery was performed using the «ab external» trabeculectomy technique and the results were compared with those from healthy subjects.

The distribution analysis according to gender and age did not show significant differences between the two groups. Even though the age range was from 40 to 90, most of the patients in this study were over 60. This information is interesting in the context of our work because it has already been proven that both glaucoma and cataracts have a direct link with an increase in age and ageing (4). A research group from the Instituto de Oftalmobiología Aplicada (IOBA, Applied Ophthalmobiology Institute) performed work on a population in Segovia where they concluded that glaucoma prevalence in the group of people between 40 and 49 years old was 0.01% and in people between 60 and 69, it was 2.40% (25).

Our aims have been to determine the presence of NO, ROS and the status of the antioxidant mechanisms (TAS) in relation to glaucoma progression, using aqueous humor obtained at the beginning of the programmed surgery of the anterior segment. In order to make this section easier to read, it is structured in a number of sections:

1) The NO levels in the aqueous humor of both groups of subjects was analyzed. Statistically higher values were seen in the group of patients with glaucoma than in the group with cataracts. These results support those found by Chang et al. (13) that show the variability of NO in relation to glaucoma. Experiments have not been done to determine the enzyme implicated in the production of NO in the aqueous humor of the study subjects and therefore the cellular phenotype or phenotypes that over-express NO. Now, the global NO concentration is significantly increased in patients whose glaucoma has become worse, and it is the ophthalmologist’s criterion that determines the risk of progression and therefore the convenience or not of anti-glaucoma surgery. The amount of NO transformed into peroxynitrites has not been determined and therefore the presence of nitrosative stress cannot be guaranteed. Even in the light of this, taking into account all our results, we propose that the increase in NO formation in the aqueous humor in those patients with glaucoma, in the presence of oxidants, should induce the formation of peroxynitrites and it is very possible that nitrosative stress is induced as a result of this. This point is an essential goal to be handled when it comes to future experimentation within this line of research.

2) The enzymatic-colorimetric determinations of ROS and TAS show redox activity in the aqueous humor of both groups. The higher imbalance between the EROS formation and the TAS decrease in the GG is the most noteworthy. Several research groups have done studies that prove the existence of oxidative stress in aging (24) and well as with glaucoma, such as the work done by Ferreira et al. (26). Following along the same path our results show the same parameters as those obtained by this group of researchers, based on the presence of oxidative agents in the aqueous humor in pre-surgical stage glaucoma. This has been defined as the phase of the disease in which the progression of the morphological (papillary cup) and functional tests (visual field, optic fibre analysis) and/or unresponsiveness to hypotensor treatment (or not following through with it), independently from which medication is used, suggests anti-glaucoma surgery in order to avoid vision loss.

The glaucomatous optic neuropath is a neurodegenerative disease and therefore causes irreversible blindness in those patients affected by it (4). We know that, from a purely mechanical point of view, the main cause is a pressure increase inside the eye. However, this disease is characterized by the induction of apoptosis in retinal ganglion cells (4,15). What, then, are the cellular and molecular causes that surround the ganglion cell degeneration? Is this
just an isolated event in the retina? Is there a possibility of slowing down or reversing the degenerative process leading to blindness? Obviously there are still too many unanswered questions. In our study we confirm the findings of Ferreira et al. (26) and corroborate the implication of NO and oxidative stress as glaucoma etiopathogenesis mechanisms, emphasizing the importance of biochemical markers in the aqueous humor to evaluate the progression of the glaucomatous optic neuropathy.

From all this we infer that an understanding of the biochemical and molecular bases making up the etiopathogenesis of glaucoma in humans is essential for researching new lines of treatment that may improve on those currently used. Also, the identification of any process that may tell us about cell repair and replacement of those elements damaged during glaucomatous disease, will open up new horizons for vision expectations in patients with glaucoma, who would be the first to benefit from this research, including therapeutic strategies that allow for NO availability to be controlled as well as inhibition of ROS formation, or blockage of their activity, thus favoring the anti-oxidant defense status.

REFERENCES


