European Review for Medical and Pharmacological Sciences 2012; 16: 1453-1457

Oxidative stress in the closed-eyelid test: management of glaucoma

N. PESCOSOLIDO, R. MALAGOLA**, G. SCARSELLA*, F. LENARDUZZI**, L. DAPOTO**, M. NEBBIOSO**

Department of Cardiovascular, Respiratory, Nephrology and Geriatric Sciences; *Department of Biology and Biotechnology Charles Darwin; **Department of Sense Organs, Sapienza University of Rome, Rome, Italy

Abstract. – BACKGROUND AND OBJECTIVES: To evaluate the role of antioxidant drugs in the tonometric increase that follows the closed eyelid test (CET), a predicitive test for glaucoma, after administration of antioxidant substances was observed.

MATERIALS AND METHODS: 30 subjects of 54.57 ± 5.62 years, 13 males and 17 females, were examined by measuring the ocular pressure after 1 hour from the CET, both in normal conditions and after the administration of antioxidants such as: vitamin A (50,000 IU/die), vitamin E (600 mg/die), and vitamin C (1000 mg/die). The increases in temperature of the iridocorneal angle and of the iris were also measured in the same conditions with an infrared Thermo-Precision tonometer (Sola Electro-Optics, China) both before and after CET.

RESULTS: The results showed increased pressure after CET and decreased pressure after the administration of each antioxidant substance, although vitamin A was found to be more effective and with statistically significant values compared to vitamins E and C.

CONCLUSIONS: Considering the responses obtained after administration of antioxidant drugs, the ocular hypertension induced after CET could be a response to mixed stress, oxidative and thermic, with degenerative effects on the trabecular meshwork (TM). Besides, in light of these considerations the research results underline that the open angle glaucoma (OAG) should be considered a multifactorial degenerative disease.

Key Words:
Antioxidants, Closed eyelid test, Oxidative stress, Trabecular meshwork, Vitamins A-C-E.

Introduction

The open angle glaucoma (OAG) is a multifactorial disease that causes progressive loss of either optic disc or retinal nerve fibers and then deficit of visual function[1]. Therefore also an early glaucoma can cause changes in the normal psychophysics and electrophysiological testing such as: automated visual fields, contrast sensitivity, visual evoked potentials, and pattern electroretinography[2]. Hence, research in the past years has been aimed at defining the most effective means of screening patients at risk and, consequently, sorting out those patients requiring treatment. Studies on possible glaucoma predictors started already a century ago, when the darkroom test was proposed as a provocation test for angle closure glaucoma[3].

Further investigations demonstrated that eyelid closure, or the use of a subaqueous mask with open eyelids, can be responsible of an increase in ocular pressure due to an increase in local temperature[4]. The results presented in this study could explain the role of oxidative stress in the tonometric increase after closed-eyelid test (CET). The importance that oxidative stress can have in the etiopathogenesis of OAG is confirmed also by the fact that the serum antibodies directed against the gluthathione S-transferase are more frequent and have higher values in patients affected by OAG compared to controls[5].

Moreover the international literature reports studies which show that trabeculocytes, alike the ciliary epithelium, have both glutathione peroxidase and glutathione reductase[6,7].

Based on this knowledge, the following experiment has been undertaken with the aim to confirm *in vivo* the influence of oxidative stress in the anterior chamber, considering also temperature as a possible concurrent cause. To evaluate the potential role of oxidative stress we proposed to effectuate the test after administration of the three known antioxidant substances: vitamin A (retinol), vitamin E (α-tocopherol) and vitamin C (ascorbic acid).

Corresponding Author: Marcella Nebbioso, MD; e-mail: marcella.nebbioso@uniroma1.it

Materials and Methods

We examined 60 eyes of 30 subjects, 17 females and 13 males, with a mean age of 54.57 ± 5.62 years (range 47 to 64 years), without any evident acute or chronic disease. Eligibility was determined through a detailed medical and ocular history, and a comprehensive eye examination. Eye examination included best-corrected visual acuity (BCVA) for far and near vision, slit lamp biomicroscopy, measurements of intraocular pressure (IOP) with Goldmann applanation tonometry at four different times, corneal pachimetry, gonioscopy, dilated fundus examination, and optic disc evaluation. Measurements of the standard automated perimetry were performed on the 30 to 2 Swedish interactive threshold algorithm standard program of the Humphrey Field Analyzer (Carl Zeiss Meditec, Inc., Dublin, CA, USA).

Inclusion criteria were as follows:

- IOP in the range of 17 to 22 mmHg, without any treatment;
- Refraction values between -4 and +4 spheric diopters;
- BCVA for far distance equal or over 8/10;
- Open angle at gonioscopy;
- Normal corneal pachimetry (550 to 620 μm);
- Horizontal C/D ratio of 0.2 to 0.6 at slit lamp examination;
- Normal SAP (30 to 2 Swedish interactive threshold algorithm standard program).

In this group, the mean IOP value was 19.50 ± 2.15 mm Hg. BCVA ranged between 8/10 and 10/10 (logMAR 0.14 to -0.3).

In compliance with the Helsinki Declaration, informed consent was obtained from all subjects before enrolment. The chosen subjects underwent during the first day the CET, evaluating the increase of ocular pressure after eyelid closure of one hour in a quiet atmosphere.

Subsequently the test was repeated, at the same hour, after administration of one pill a day of 50,000 U.I. of retinol (Arovit®, Roche S.p.A., Milan, Italy) for 7 consequent days.

After 30 days from the suspension of the first therapy, vitamin E was administered with a dosage of 2 capsules/die of 300 mg (Ephynal 300®, Roche S.p.A., Milan, Italy), for one week, after which the test was repeated, always at the same hour.

After 30 days from the suspension of the second therapy, vitamin C was administered with a dosage of 2 capsules/die of 500 mg (C-Tard®, Home Products S.p.A, Milan, Italy) for one week, after which the test was repeated, always at the same hour.

The ocular pressure has been measured always by the same operator using a Goldmann applanation tonometer after instillation of a drop of oxybuprocaine hydrochloride solution at 0,4% (Novesina 0.4%®, Novartis Farma S.p.A., Varese, Italy) and the use of a fluorescein-stained sterile filter paper strip (Haag-Streit AG, Köniz, Switzerland). Two measurements were taken in a semi dark environment and the arithmetic average has been considered.

The evaluation of the temperature of the anterior chamber (sclerocorneal angle and iridal surface) was performed with an infrared thermoprecision tonometer (Sola Electro-Optics, Shanghai, China) both before and after CET.

The results obtained were statistically elaborated by using the Student's *t*-test for paired samples.

Results

The evaluation of the temperature of the anterior chamber both before and after CET are reported in Table I. The values represent the average and the standard deviation of the mean. The

Table I. The closed eyelid test (CET) has been performed in eyes with primary open angle glaucoma. Temperature increase after CET in the sclerocorneal angle and on the iridal surface. The values represent the average and the standard error of the mean (SEM), while between brackets the standard deviation (SD) is indicated as a variability index of the sample.

Temperature degrees centigrade	Sclerocorneal angle	Iridal surface
Before the CET	33.2 ± 0.4 (1.1)	35.3 ± 0.3 (0.9)
After the CET 60 min	36.5 ± 0.3 ** (1.0)	37.1 ± 0.2 * (0.8)

The significance limits between before and after the test have been examined with the paired Student's *t*-test to minimize the individual deviations, and codified in this way: $^{**}p < 0.01\%$; $^{*}p < 0.05\%$; otherwise $p > 0.05\%$.

Table II. Tonometric increase in mmHg (Δ) and in percentage (%) after "closed-eyelid test" (CET) in basal conditions and after seven consequent days of treatment with vitamin A (50,000 U.I./die), vitamin E (600 mg/die) and vitamin C (1000 mg/die).

Increased pressure	Δ basal	Δ after vitamin A	Δ after vitamin E	Δ after vitamin C
In mmHg	11.08 ± 3.65	4.16 ± 3.12	8.75 ± 2.89	8.33 ± 2.70
In percentage	58.63 ± 24.13	23.24 ± 17.58	46.66 ± 17.03	41.63 ± 14.55

significance limits between before and after the test have been examined with the paired Student's *t*-test and have resulted statistically significant after 60 min ($p < 0.01$ and $p < 0.05$).

The increased pressure obtained in mmHg and in percentage after CET with the statistical significances, Student's *t*-test, of the 30 examined patients are shown in Table II.

In Table III one can see how after the treatment with antioxidant substances, vitamin A, E, and C, there is a significant pressure decrease after CET when compared to the other values obtained after CET in basal conditions. The antioxidant substance that majorly decreased the pressure was, at least in the tested dosages, vitamin A, with statistically significant values compared to vitamin E and C. No difference between tonometric increases has been encountered between vitamin C and E. A very interesting finding is the remarkable effect observed after administration of vitamin A in which the tonometric increase is little less than 25%.

Discussion

Our research has been undertaken with the aim to confirm *in vivo* the influence of oxidative stress in the anterior chamber, considering also the temperature as a possible concurrent cause. To evaluate the potential role of oxidative stress we proposed to effectuate the test after administration of the three known antioxidant substances: vitamin A (retinol), vitamin E (α-tocopherol), and vitamin C (ascorbic acid).

The importance that oxidative stress can have in the etiopathogenesis of OAG is showed by the fact that the serum antibodies directed against the glutathione S-transferase are more frequent and have higher values in patients affected by OAG compared to controls[5]. Subsequently, Saccà and Izzotti[8] (2004) evaluated the damage to the deoxyribonucleic acid (DNA) in the trabecular meshwork (TM) of patients affected by glaucoma. The damage has been verified by measuring the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a precise indicator of the oxidative damage to DNA, in tissue samples removed during filtration surgery. Furthermore, the Authors examined on the same samples the presence of two genes that codify for glutathione S-transferase, GSTM1 and GSTT1 and that, according to literature, seem to undergo a homozygous deletion in a high percentage of the population[9]. Therefore the results demonstrated an actual existence of oxidative damage to DNA in the TM of patients affected by OAG. Besides, the deletion of the GSTM1 gene seems to predispose to an aggravation of the oxidative damage of trabecular DNA and to a more accentuated prevalence of OAG[9]. This leads to the hypothesis of stress dependent oxidative build up of DNA damage in the trabecular area.

When the relation between oxidant factors and the antioxidant defense system in a biological structure gets modified in favor of the former, we

Table III. Tonometric increase in mmHg (Δ) after "closed-eyelid test" (CET) in basal conditions and after seven consequent days of treatment with vitamin A (50000 U.I./die), vitamin E (600 mg/die) and vitamin C (1000 mg/die).

Δ basal in mmHg	**Δ after vit. A**	***p***
11.08 ± 3.65	4.16 ± 3.12	.001 **
Δ basal	**Δ after vit. E**	
11.08 ± 3.65	8.75 ± 2.89	.000 **
Δ basal	**Δ after vit. C**	
11.08 ± 3.65	8.33 ± 2.70	.001 **
Δ after vit. A	**Δ after vit. E**	
4.16 ± 3.12	8.75 ± 2.89	.008 **
Δ after vit. A	**Δ after vit. C**	
4.16 ± 3.12	8.33 ± 2.70	.012 **
Δ after vit. C	**Δ after vit. E**	
8.33 ± 2.70	8.75 ± 2.89	.339

The statistical significances (*p*) of tonometric increases in basal conditions, after treatment, and between groups that underwent different treatments have been elaborated with the paired Student's *t*-test.

assist to an oxidative stress. The alteration in the redox potential can start a series of reactions that results in cellular damage[10]. The group of neutralizer antioxidants block the chain reactions started by the free radicals at an intermediate level making them inactive. They include some non enzymatic systems that can be divided in: liposoluble antioxidants, such as α-tocopherol (vit. E), retinol (vit. A) and carotenoids and hydrosoluble antioxidants such as ascorbic acid (vit. C)[11-14].

With regard to *vitamin E or tocopherol*, this is an essential nutrient that is not synthetized by the organism so has to be introduced with food, and is found particularly in vegetable oils and green vegetables. It consists of the mixture of four physiologically active tocopherols, α, β, γ, and δ, of which the α-tocopherol is the most biologically active form, and is commonly called vitamin E[13]. Thanks to its structure, this compound lies in the biological membranes with its aromatic ring located on the polar surface and the side chain closely associated to the polyunsaturated fatty acids of the phospholipids. It acts at a molecular level as a neutralizer of the free radicals that get formed in these structures representing at this level the main cellular defense system against the peroxidation of membrane lipids[12,14]. This vitamin interrupts the chain of radical reactions by transforming itself in a radical that is more stable and less aggressive.

Vitamin A or retinol is a superior alcohol which is present in foods of vegetal origin in form of carotenoid precursors, α, β, and γ-carotene, of which the most active form is β-carotene.

The animal organism is capable of transforming these substances in vitamin A, therefore, they can be considered as A provitamins. Retinol, together with phospholipids and proteins, has an important role in maintaining the morphological and functional integrity of the cellular membranes rendering stable the lipoproteic composition of the membranes and regulating their permeability[14]. β-carotene and α-tocopherol can be considered defensive agents against singlet oxygen, the activated form of oxygen, with a double action: they can, in fact, react directly with the singlet oxygen or deactivate it (quenching) without reacting.

The ascorbic acid or vitamin C is present in fresh vegetables and is a strong reducing agent as for it can easily and reversibly transform itself in dehydroascorbic acid. This capacity is what gives it its redox system properties, with a protective action related mainly to its role as an antioxidant substance towards other vitamins such as vitamins A and E[14].

The reported findings indicate that the increased pressure induced by CET is, at least partly, a clinical expression of a "sensitivity" status of the ocular hydrodynamic pathway towards oxidative stress in which vitamin A would act most effectively, perhaps stabilizing better the membranes to thermic increase. The increased pressure induced after CET could represent a general index of the resistance to autoxidation of ocular anterior segment tissue, therefore highlighting important aspects of metabolism in the anterior chamber especially as an answer to external stress as can be that induced by eyelid closure. Evaluating the basic event of stress (thermic increase), considering that also only the application of a subaqueous mask with open eyelids for one hour gives according results, the obtained data seems to confirm the results of Nguyen et al[6], Yang et al[5] and Saccà et al[15] on the susceptibility of trabeculocytes to oxidative stress that after years could lead to a degenerative cellular condition typical of primary OAG.

In conclusion, the clinical implications of our researches suggest that additional in vitro studies are needed, and *in vivo* animal assays will need to be performed to ascertain the true clinical implications of our findings for OAG patients.

References

1) American Academy of Ophthalmology. ONENetwork: The Opthalmic News and Education Network. Primary Open-Angle Glaucoma Suspect Preferred Practice Pattern (PPP); 2010. Available at: http://one.aao.org/CE/PracticeGuidelines/PPP.aspx. Accessed March 24, 2011.
2) Nebbioso M, Gregorio FD, Prencipe L, Pecorella I. Psychophysical and electrophysiologi-cal testing in ocular hypertension. Optom Vis Sci 2011; 88: E928-939.
3) Grönholm V. Untersuchungen über den einfluss der papillenweite, der accomodtion und der convergenz auf die tension glaucomätoser und normal augen. Arch Augenheilk 1910; 66: 346.
4) Bucci MG, Pescosolido N. The closed-eyled test in the management of glaucoma. Glaucoma 1983; 2: 84-89.
5) Yang J, Tezel G, Patil RV, Romano C, Wax MB. Serum auto-antibody against glutathione S-transferase in patiens with glaucoma. Invest Ophthamol Vis Sci 2001; 42: 1273-1276.

6) Nguyen KP, Karageuzian LN, Anderson PJ, Epstein DL. Glutathione reductase of calf trabecular meshwork. Invest Ophthalmol Vis Sci 1985; 26: 887-890.

7) Coca-Prados M, Escribano J, Ortego J. Differential gene expression in the human ciliary epithelium. Prog Retin Eye Res 1999; 18: 403-429.

8) Saccà SC, Pascotto A, Camicione P, Capris P, Izzotti A. Oxidative DNA damage in the human trabecular meshwork: clinical correlation in patients with primary open-angle glaucoma. Arch Ophthalmol 2005; 123: 458-463.

9) Seidegard J, Vorachek WR, Pero RW, Pearson WR. Hereditary differences in the expression of the human glutathione transferase active on trans-stilbene oxide are due to a gene delection. Prot Natl Acad Sci USA 1988; 85: 7293-7297.

10) Pescosolido N, Librando A, Puzzono M, Nebbioso M. Palmitoylethanolamide effects on intraocular pressure after Nd:YAG laser iridotomy: an experimental clinical study. J Ocul Pharmacol Ther 2011; 27: 629-635.

11) Meister A. Glutathione metabolism and its selective modification. J Biol Chem 1988; 263: 17205-17208.

12) Packer L, Landvik S. Vitamin E: introduction to biochemistry and health benefits. Ann N Y Acad Sci 1989; 570: 1-6.

13) Burton GW, Ingold KH. Vitamin E: application of the principles of physical organic chemistry to the exploration of its structure and function. Acc Chem Res 1986; 19: 194-201.

14) Fidanza A. Le vitamine. Biochimica, Fisiologia, Nutrizione, Terapia. Roma, Agnesotti Ed 1990.

15) Saccà SC, Izzotti A, Rossi P, Traverso C. Glaucomatous outflow pathway and oxidative stress. Exp Eye Res 2007; 84: 389-399.

16) Pescosolido N, Belcaro G, Rusciano D, Steigerwalt RD, Nebbioso M. Retrospective study of glaucoma and closed-eyelid test: long-term outcomes in an Italian native population. Panminerva Med 2012 (in press).

17) Pescosolido N, Cavallotti C, Rusciano D, Nebbioso M. Trabecular meshwork in normal and pathological eyes. Ultrastruct Pathol 2012; 36: 102-107.