Red blood cell membrane integrity in primary open angle glaucoma: *ex vivo* and *in vitro* studies

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LABORATORY STUDY

Abstract

Purpose **There is increasing evidence that abnormal perfusion of the optic nerve head is an important factor involved in the pathophysiology of glaucoma. Transport and distribution of oxygen to the tissues takes place through the erythrocyte membrane. Red blood cell (RBC) acetylcholinesterase (AChE) is a marker of RBC membrane integrity. The aim of this study was to find out whether RBC membrane integrity is preserved in primary open angle glaucoma (POAG), and whether it is modified by the use of topical timolol maleate and pilocarpine.**

Method **RBC AChE activity was determined *ex vivo* by Kaplan's spectrophotometric method in 19 POAG patients undergoing topical treatment for glaucoma with timolol, pilocarpine or a combination of the two drugs, and compared with that in 20 controls. To assess the effect of antiglaucomatous therapy in our findings, we carried out an *in vitro* study in 26 non-glaucomatous patients in which we measured RBC AChE activity after incubation of blood with either timolol maleate, pilocarpine or a combination of the two drugs, using the same spectrophotometric method.**

Results **There was a significant increase in RBC AChE enzyme activity in POAG patients compared with the control group ($p < 0.002$). However, timolol and pilocarpine, individually or in combination, have the opposite effect, significantly decreasing RBC AChE activity ($p < 0.05$).**

Conclusion **This change in RBC AChE enzyme activity could suggest alterations in RBC membrane integrity in primary open angle glaucoma. Whether or not this finding has implications regarding the microcirculation at the optic nerve head needs to be investigated further.**

Key words Acetylcholinesterase, Glaucoma, Pilocarpine, Red blood cell, Timolol

There are various pathogenetic mechanisms of glaucoma that express themselves differently. Some patients may present with primary vascular dysfunction, while in others vascular insufficiency is secondary to the effects of intraocular pressure (IOP). It is therefore important to evaluate ocular haemodynamics in disease as well as the effect of glaucoma medications on blood flow to the eye.

There is increasing evidence that abnormal perfusion of the optic nerve head is an important factor involved in the pathophysiology of glaucoma.[1,2] Transport and distribution of oxygen to the tissues take place through the erythrocyte membrane. Red blood cell (RBC) acetylcholinesterase (AChE) is a lipid-glycoprotein complex molecule, located on the outer surface of the erythrocyte membrane, which is used as a marker of RBC membrane integrity.[3] Such integrity is of paramount importance in the maintenance of the normal blood rheology, and tissue oxygenation, especially at the microcirculatory level.

The purpose of this study was (i) to find out whether RBC membrane integrity is preserved in primary open angle glaucoma, in patients undergoing antiglaucomatous therapy, and (ii) to verify how topical antiglaucomatous medications such as timolol and pilocarpine could affect RBC membrane integrity.

Materials and methods

Ex vivo *study*

RBC AChE enzyme activity was determined *ex vivo* by Kaplan's spectrophotometric method[4] (Ellman's modified laboratory method) in blood from 19 primary open angle glaucoma (POAG) patients: 10 men and 9 women, age range 46–68

Table 1. Ex vivo *study: population profile*

	POAG ($n = 19$)	Controls ($n = 20$)
Men (n)	10 (52.6%)	10 (50%)
Women (n)	9 (47.4%)	10 (50%)
Age range (years)	46–68	45–66

POAG, primary open angle glaucoma.

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Table 2. *Red blood cell acetylcholinesterase (AChE) activity (U/min/mg Hb) in POAG:* ex vivo *study*

	POAG	95% CI	Control	95% CI	*p* value
AChE	265	184–419	205	131–304	< 0.002

years. It was compared with RBC AChE activity in 20 controls: 10 men and 10 women, age range 45–66 years (Table 1). There was no statistical difference in age and gender between the two groups. All 19 POAG patients were undergoing topical medical treatment to both eyes. Seven patients were on timolol 0.5% twice a day; 6 patients applied pilocarpine 4% four times a day; and the remaining 6 patients underwent treatment with both timolol 0.5% twice a day and pilocarpine 4% four times a day. Subjects were informed about the nature of the study and consent was obtained before the withdrawal of 5 ml of blood from the antecubital vein with a 21 gauge catheter. All blood samples were stored in heparin tubes until the time of testing.

In vitro *study*

To assess the effect of antiglaucomatous therapy in our findings, we studied 26 non-glaucomatous patients attending the eye clinic: 14 men and 12 women, age range 40–71 years. None suffered from high blood pressure, anaemia, diabetes, diseases of the central nervous system or any other disease known to affect AChE activity.[5] They were not undergoing any form of treatment, either topical or systemic, at the time of the study, and those who smoked were asked to refrain from smoking for 48 h before withdrawal of the blood sample.

We carried out an *in vitro* study in which patients' blood samples (8 ml) were divided into four aliquots of 2 ml each. One of the aliquots served as a control; subsequently a 10^{-5} M solution[6–8] (final concentration) of the active principle of timolol maleate, pilocarpine and a combination of the two drugs were incubated with the remaining three aliquots, at room temperature for 30 min. Previous studies[9] have reported induction of haemolysis as well as decreased RBC AChE activity in blood samples from healthy subjects after incubation with timolol maleate (0.09–3.9 mM). The final concentration of 10^{-5} M for timolol and pilocarpine was considered satisfactory for the spectrophotometric method used in the study as well as non-haemolytic.

RBC AChE enzyme activity was determined by Kaplan's spectrophotometric method. To eliminate any possible interference of timolol maleate and pilocarpine with the spectophotometric determinations, our blank contained the same drugs at precisely the same concentration as those in the blood samples.

MANOVA repeated measures and paired Student's *t*-test (two-tailed) were used for the statistical analysis of the *in vitro* study. The null hypothesis was rejected at the 0.05 level. The group Student's *t*-test was applied to the *ex vivo* study. The null hypothesis was rejected at the 0.05 level.

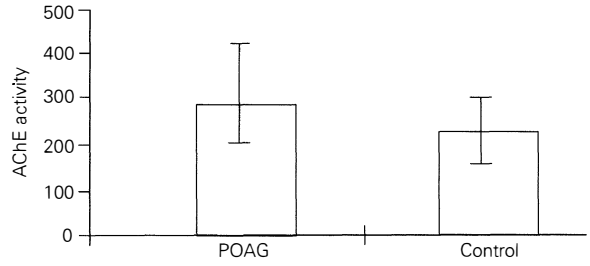

Fig. 1. *Red blood cell (RBC) acetylcholinesterase (AChE) activity (U/min/mg Hb; mean ± 95% CI) in primary open angle glaucoma patients and healthy controls:* ex vivo *study.*

Results

In the *ex vivo* study we verified a significant increase in RBC AChE activity (Table 2) in POAG patients (265 U/min/mg Hb) compared with the control group (205 U/min/mg Hb; $p < 0.002$) (Fig. 1).

In the *in vitro* study, pilocarpine 10^{-5} M significantly decreased RBC AChE enzyme activity (247 ± 36; $p = 0.008$), as did timolol maleate 10^{-5} M (264 ± 37.6; $p = 0.05$) (Table 3). The reducing effect was greater when the two drugs acted simultaneously (245 ± 45.1; $p = 0.002$) (Fig. 2).

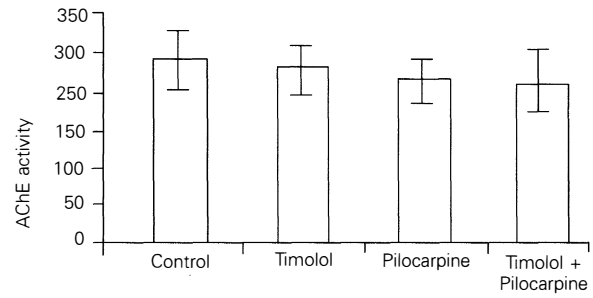

Fig. 2. *Influence of timolol and pilocarpine on RBC AChE activity (U/min/mg Hb; mean ± SD) in healthy individuals:* in vitro *study.*

Table 3. *Influence of timolol 10^{-5} M and pilocarpine 10^{-5} M on red blood cell acetylcholinesterase activity (AChE) activity (U/min/mg Hb):* in vitro *study*

	Control	Timolol		Pilocarpine		Tim + Pilo[a]	
	Mean ± SD	Mean ± SD	*p*	Mean ± SD	*p*	Mean ± SD	*p*
AChE	283 ± 41.8	264 ± 37.6	0.05	247 ± 36.0	0.008	245 ± 45.1	0.002

[a]A combination of timolol and pilocarpine.

Discussion

The results of this study verify that there is increased RBC AChE activity in POAG patients that is not the result of topical timolol or pilocarpine therapy. This increased enzyme activity could, in some cases, be translated into reduced RBC membrane fluidity,[10] making the passage of the RBC through the vessels more difficult. The exact implications of this result for the haemorheology at the optic nerve head remain to be further studied. However, our findings could be in agreement with those of other groups[11–13] who have described increased RBC aggregation and filtration index in POAG, these being indicators of increased blood viscosity.

Systemic hypertension has been implicated for many years as a risk factor for developing glaucoma.[14,15] Patients with essential hypertension were also found to have increased AChE enzyme activity when compared with age- and sex-matched healthy controls,[16,17] as well as lower blood filtration index[18] suggesting impaired RBC deformability.

Pilocarpine and, to a lesser extent, timolol seem to reduce AChE activity in the RBC *in vitro.* This could be the result of direct enzymatic inhibition of the AChE active center by the drug molecule, or it could be due to membrane phospholipid or cytoskeleton modulation as a result of the interaction of the drug molecule with the erythrocyte membrane itself. Considering the second hypothesis, it seems that the two drugs may reduce RBC plasma membrane deformability.

Timolol and pilocarpine have classically been used in the treatment of glaucoma because of their intraocular pressure reducing effect through their actions on the aqueous humour production–outflow cicle. We have now verified that they could have an effect on the microcirculation *in vitro*, by reducing RBC AChE activity, which counteracts the increased enzyme activity found in the peripheral blood of POAG patients.

Altered blood flow can be detected and measured with current technology. The effects of various medications on blood flow can also be evaluated. The problem is to ascertain whether altered blood flow is primary or secondary to the disease. Further investigations should aim to answer this question.

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