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Evaluation of oxidative stress levels in the conjunctival epithelium of patients with or without dry eye, and dry eye patients treated with preservative-free hyaluronic acid 0.15 % and vitamin B12 eye drops

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Abstract
Purpose Increased levels of oxidative stress have been seen in animal models of dry eye and in the conjunctival epithelial cells of patients with Sjögren's syndrome. The aims of this study were to compare the levels of oxidative stress in patients with dry eye and patients without dry eye and to evaluate the effects of treatment with preservative-free eye drops containing hyaluronic acid 0.15 % and vitamin B12 on oxidative stress and dry eye symptoms.
Methods Three cohorts of patients who were to undergo planned cataract surgery were enrolled: patients with dry eye randomized to either no treatment (n=29) or treatment (n=32) with hyaluronic acid/vitamin B12 eye drops, and patients without dry eye (n=42). Patients were assessed by Schirmer's type I test, fluorescein clearance test (FCT), Break Up Time (BUT), and Ocular Surface Disease Index (OSDI). Lipid peroxidation, a marker of oxidative stress, was assessed by LP-CHOLOX test.
Results Compared with patients without dry eye, patients with dry eye had significantly increased levels of oxidative stress, higher OSDI and FCT scores, and significantly lower Schirmer's test and BUT scores. Treatment with eye drops containing hyaluronic acid 0.15 % and vitamin B12 was associated with significantly reduced levels of oxidative stress and OSDI and FCT scores and significantly increased Schirmer's test and BUT scores.
Conclusions These findings indicate that oxidative stress is associated with dry eye and that hyaluronic acid/vitamin B12 eye drops may attenuate oxidative stress and inflammation, improving dry eye symptoms. Further study in controlled clinical trials is warranted.

Key words Dry eye · Fluorescein clearance test · Preservative-free hyaluronic acid eye drops · Reactive oxygen species · Lipoperoxides

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Introduction

Dry eye syndrome, also known as keratoconjunctivitis sicca and xerophthalmia, is a common eye disorder resulting from tear deficiency or excessive evaporation of tears [1–3]. Dry eye can be caused by aging, diseases such as Sjögren's syndrome, Stevens-Johnson syndrome, ocular cicatricial pemphigoid, rheumatoid arthritis, diabetes mellitus, or systemic lupus erythematosus (SLE), and the use of medications including antihistamines, antihypertensive agents, oral contraceptives [2, 3]. Environmental factors (pollution, smoke, low humidity, wind) and activities which cause reduced blink rate (including the use of visual display units) also contribute to the development of dry eye [1–3].

It has recently been reported that there is a correlation between dry eye and the increased presence of products from lipid peroxidation and myeloperoxidase enzymatic activity and xanthine oxidoreductase and xanthine oxidase activity in the tear film [4–6]. The consequent production of reactive oxygen species (ROS) is the cause of oxidative stress that can be involved in the propagation of cellular injury that leads to eye pathology in various conditions, including not only dry eye disease, but also conjunctivochalasis, age-related macular degeneration, and UV light-induced and tobacco smoke-induced ocular surface epithelial damage [5]. Oxidative stress is biologically relevant in vivo, and is intimately linked with an integrated series of cellular events [7–13]. Interaction between these various components is not necessarily a cascade, but might be a cycle of events, of which oxidative stress is a major component [14–16]. Therapeutically, it is possible that inhibition of oxidative stress might act to break the cycle of cell death [17].

Evaluation of the differences in oxidative stress on the ocular surface in dry eye patients could lead to a new approach to dry eye therapy that is based not only on tear film substitution, but also on the reduction of inflammation through the inhibition of oxidative stress on the ocular surface [6, 18].

The aim of this study was to provide an assessment of oxidative stress levels in the conjunctiva of dry eye patients, compared with the conjunctiva of patients without dry eye. Furthermore, this study also aimed to evaluate the effects of preservative-free eye drops containing hyaluronic acid 0.15 % and vitamin B12 on oxidative stress levels in the conjunctiva of patients with dry eye.

Materials and methods

The prospective, randomized, controlled study enrolled consecutive healthy and dry eye patients who were going to undergo planned cataract surgery at the Department of Ophthalmology at the University of Genoa in Genova, Italy. Patient enrolment and sample collection were performed at San Martino Hospital in Genova, Italy, from September 2011 to May 2013.

The study was conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki, and its design was approved by the institutional ethics committee of San Martino Hospital in 2011. Informed consent was obtained from all patients prior to their participation in the study.

Patients were seen from 7 to 9 weeks before surgery. Patients were examined and underwent pre-operative tests at the enrolment visit. Those with dry eye were assigned to treatment or no treatment by simple randomization using a list of random numbers with a probability of 50 % for each group. Three groups of patients were included in the study: patients with dry eye who were not receiving any therapy (group 1), patients with dry eye without any therapy before enrollment (group 2a) who started treatment with preservative-free eye drops containing hyaluronic acid 0.15 % and vitamin B12 (Artelac® Rebalance, Bausch & Lomb, Italy) four times a day 1 month before surgery and were then examined for all parameters except oxidative stress 1 day before surgery (group 2b), and a control group of patients without dry eye (group 3). Oxidative stress testing was performed on conjunctiva biopsy specimens obtained immediately before cataract surgery.

To avoid an ethical problem, patients in group 1 (untreated dry eye) underwent conjunctiva biopsy and then cataract surgery within 1 week after enrolment so they were not left without therapy for a longer time. Furthermore, all patients in this group started therapy with artificial tears after surgery.

The primary endpoint of the study was the evaluation of oxidative stress in the conjunctiva in patients with dry eye who were untreated, patients with dry eye treated with eye drops containing hyaluronic acid 0.15 % and vitamin B12, and the control group of patients without dry eye.

Inclusion and exclusion criteria

Inclusion criteria for dry eye patients (groups 1 and 2) were a tear film fluorescein clearance test (FCT) score of >3 [19, 20], Schirmer's test type 1 result of <10 at 5 min, tear break up time (BUT) of ≤10 sec, an Ocular Surface Disease Index (OSDI) score indicating mild-to-severe dry eye [21], and no previous therapies for dry eye.

Inclusion criteria for the control group (group 3) were a tear film FCT score of ≤3, Schirmer's test type 1 result of ≥10 at 5 min, BUT of >10 sec, an OSDI score indicating the absence of dry eye, no use of topical ocular therapies or systemic therapy with drugs that could potentially interfere with the ocular surface unit (such as β-blockers), and the absence of systemic pathologies commonly associated with dry eye syndrome (including, but not limited to, rheumatoid arthritis, scleroderma, SLE, Stevens-Johnson syndrome, pemphigoid).

Assessments

Patients were assessed by Schirmer's I test, tear film FCT, BUT, and OSDI, and levels of oxidative stress were determined by LP-CHOLOX tests on conjunctiva samples.

Laboratory tests were performed at Research Laboratory DIMES (certified ISO9001:2000, 2008) General Pathology Section, at the University of Genoa. The research unit obtained biopsy samples of conjunctiva from patients immediately before undergoing cataract surgery from inferior bulbar and conjunctiva biopsy (two conjunctiva samples: the first one of size 2×2 mm from the superior bulbar conjunctiva and the second one of size 2×2 mm from the inferior bulbar conjunctiva).

The conjunctiva samples were used to evaluate basal levels of oxidative stress. Lipid peroxidation, an important indicator of oxidative stress [22], was evaluated using the LP-CHOLOX test (Diacron International, Grosseto, Italy). This test evaluates a class of hydroperoxides derived from lipid peroxidation, both saturated and unsaturated. In human tissues, such as the plasma, these hydroperoxides are mostly represented by oxidized cholesterol. This method is based on the ability of the peroxides to facilitate the oxidation of Fe^{2+} to Fe^{3+}. The Fe^{3+} binds to thiocyanate developing a color complex that can be measured photometrically (505 nm). The increase of absorbance is directly proportional to the concentration of lipoperoxides present in the sample [21, 23, 24] and the values are related to specific standard solution (400 μEq/L) [25].

The LP-CHOLOX test was carried out on samples of the human conjunctival epithelium from patients with dry eye syndrome and from healthy subjects, according to the procedure described in commercial kit datasheet (Diacron International, Grosseto, Italy). The tissue samples were transferred overnight at 37 °C into a microprotube containing the indicator mixture dissolved in alcohol (Reagent R1). Later, 90 μl of the reduced iron (Reagent R2) was added to the microprotube, mixed by shaking, and after 4 min at 37 °C the absorbance was read at the instrument.

Statistics

Based on initial results, a sample size of approximately 30 patients per group was considered sufficient to determine between-group differences. Distribution data were studied and resulted in a normal distribution except for fluorescein clearance test (FCT). On this basis the parametric two-tailed *t* test and Mann Whitney test were used to evaluate the differences between the patients without dry eye and the untreated dry eye group, and between the untreated and treated dry eye groups. Data are reported as mean and standard deviation (SD) unless otherwise stated.

Results

A total of 60 patients with dry eye (29 untreated and 31 treated) and 42 patients without dry eye were enrolled. The majority of patients (68.9 %) were female. Patient characteristics are presented in Table 1.

Schirmer's test results indicated that tear volumes were significantly lower for treated and untreated patients with dry eye than patients without dry eye ($P<0.001$ for both differences). While there was no significant difference between the treated and untreated patients with dry eye at enrollment (group 1 vs. group 2a), there was a significant difference after 1 month of therapy (Table 2).

Treated and untreated patients with dry eye had significantly higher FCT test scores than patients without dry eye ($P<0.001$ for both), but treated patients had a significantly lower score than untreated patients ($P<0.05$) (Table 2).

BUT scores were significantly lower for treated and untreated patients with dry eye than those without dry eye ($P<0.001$ for both). There was no significant difference between the treated and untreated dry eye groups at enrolment, but there was a significant difference after 1 month of therapy ($P<0.01$) (Table 2).

Mean OSDI scores were significantly higher for treated and untreated patients with dry eye than patients without dry eye ($P<0.001$); however, treated patients with dry eye had a significantly lower OSDI score than untreated patients ($P<0.001$) (Table 2).

Compared with patients without dry eye, untreated patients with dry eye had a significantly higher mean oxidative stress test score ($P<0.001$), but patients with dry eye treated with preservative-free hyaluronic acid 0.15 % and vitamin B12 eye drops did not. Furthermore, patients with treated dry eye had a significantly lower oxidative stress test score than patients with untreated dry eye ($P<0.001$) (Table 3).

Discussion

Recent studies have demonstrated increased levels of oxidative stress in the tears, saliva, and conjunctiva of Sjögren's syndrome patients [4–6]. A close relationship between lipid peroxidation-related membrane damage, protein oxidation, ROS production, and inflammatory processes in dry eye has been observed in patients with Sjögren's syndrome and animal models of dry eye [1, 4–6, 26–30]. These findings suggest that oxidative stress is likely to be a causative factor in the pathogenesis of dry eye disease.

Table 1 Patient characteristics

Mean ± SD	Untreated dry eye (*n*=29)	Treated[a] dry eye (*n*=32)	Without dry eye (*n*=42)
Age, years (mean ± SD)	74±6	72±3	75±7
Female, n (%)	20 (69.0%)	21 (65.6%)	30 (71.4%)

SD standard deviation

[a] treated with preservative-free eye drops containing hyaluronic acid 0.15 % and vitamin B12 (Artelac® Rebalance, Bausch & Lomb, Italy) for 1 month

Table 2 Comparison of dry eye severity at enrollment (groups 1, 2a, and 3) and after 1 month of therapy (group 2b) with preservative-free hyaluronic acid 0.15 % and vitamin B12 eye drops (Artelac® Rebalance, Bausch & Lomb, Italy)

	Patient group (mean ± SD)			
	Untreated dry eye at enrollment (Group 1, *n*=29)	Treated dry eye at enrollment (Group 2a, *n*=31)	Treated dry eye after 1 month of therapy (Group 2b, *n*=31) *	No dry eye at enrollment (Group 3, *n*=42)
Schirmer's I test, mm	6.0±2.4	6.5±2.2	11.5±3.8	14.5±2.4
Tear film FCT, score	4.7±0.7	4.5±0.9	3.5±0.8	0.8±0.9
BUT, s	6.0±2.0	6.1±1.8	9.6±2.2	13.2±1.9
OSDI, score	42.2±14.7	38±7.4	15.6±7.0	5.3±4.7
Comparison between groups, *p*-values				
	Group 1 vs. 2a	Group 1 vs. 2b	Group 1 vs. 3	
Schirmer's I test	NS	<0.001	<0.001	
Tear film FCT	NS	<0.001	<0.001	
BUT	NS	<0.001	<0.001	
OSDI	NS	<0.001	<0.001	
		Group 2a vs. 2b	Group 2a vs. 3	
Schirmer's I test		<0.01	<0.001	
Tear film FCT		<0.05	<0.001	
BUT		<0.01	<0.001	
OSDI		<0.001	<0.001	
			Group 2b vs. 3	
Schirmer's I test			<0.05	
Tear film FCT			<0.001	
BUT			<0.05	
OSDI			<0.01	

BUT tear break up time; *FCT* fluorescein clearance test; *NS* not significant; *OSDI* Ocular Surface Disease Index; *SD* standard deviation

P-value is Mann Whitney for FCT, *t* test for other assessments

Our results showed an increased level of oxidative stress, as evaluated by a new method (LP-CHOLOX test), in patients with dry eye conjunctiva compared with healthy subjects. The increased level of oxidative stress in patients with dry eye was accompanied by significant increases in FCT and OSDI scores and a significant reduction in tear volume, as measured by Schirmer's test and a decreased BUT. These findings concur with the previous studies in patients with Sjögren's syndrome that showed increased oxidative stress.

Our study also assessed the effects of the use of eye drops containing hyaluronic acid and vitamin B12 on dry eye symptoms and oxidative stress in patients with dry eye. Patients treated with these eye drops had significantly reduced oxidative stress, OSDI score, and FCT score, and increased Schirmer test and BUT scores compared with untreated patients with dry eye. These results indicate that eye drops containing hyaluronic acid 0.15 % and vitamin B12 improve oxidative stress as well as symptoms of dry eye. Our findings

Table 3 Oxidative stress test comparison among groups. Oxidative stress levels are expressed as microEq of hydroperoxides produced per mg of conjunctiva sample. The sample weights ranged from 7 to 35 mg and their relative absorbance values were 0.08±0.02, 0.06±0.02, and 0.002±0.01 per mg of tissue, respectively, for patients with untreated and treated dry eye, and patients without dry eye

	Patient group (mean ± SD)					
	Untreated dry eye (Group 1, *n*=29)	Treated dry eye after 1 month of therapy (Group 2b, *n*=31) *	No dry eye (Group 3, *n*=42)	*P*-value Group 1 vs. 3	*P*-value Group 2b vs. 3	*P*-value Group 1 vs. 2b
Oxidative stress (LP-CHOLOX test)	20.4±5.4	10.3±4.1	9.4±3.3	<0.001	NS	<0.001

The P-value was calculated by *t* test

are supported by recent studies that have also shown that hyaluronic acid and vitamin B12 have antioxidant activity in ophthalmology and other diseases involving ROS [31–38]. The results should not have been influenced by cataract surgery as this was performed after conjunctiva biopsy in all patients.

The main limitations of this study are the number of patients and the follow-up duration. The long-term effects of hyaluronic acid 0.15 % and vitamin B12 eye drops were not studied, and it is not possible to conclude from our study that they provide long-term clinical benefit. Moreover, we were not able to determine whether the treatment provided ongoing benefit with respect to reduced oxidative stress and improved symptoms of dry eye after discontinuing the medication. However, we have demonstrated that, at least during therapy, treatment with hyaluronic acid 0.15 % and vitamin B12 eye drops is associated with a significant reduction in oxidative stress in the conjunctiva of patients with dry eye.

Conclusion

Increased oxidative stress is present in patients with dry eye compared with those without dry eye. Treatment with eye drops containing hyaluronic acid 0.15 % and vitamin B12 reduces oxidative stress markers in addition to improving the symptoms of dry eye. Further study of the effects of this treatment in controlled clinical trials is warranted.

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Conflicts of interest The authors have no other potential conflicts of interest to declare.

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