Evaluation of Lipid Oxidative Stress Status in Sjögren Syndrome Patients

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PURPOSE. We evaluated the levels of lipid oxidative stress markers and inflammatory cells from tears and conjunctiva of patients with Sjögren syndrome (SS) and normal subjects.

METHODS. We examined 31 eyes of 16 patients (16 females) with SS and 15 eyes of 10 healthy controls (2 males and 8 females) in this prospective study. All subjects underwent a Schirmer test, measurement of tear film break-up time, vital stainings, confocal microscopy of the conjunctiva, tear collection for hexanoyl-lysine (HEL), ELISA, and conjunctival brush cytology. Brush cytology samples underwent immunohistochemistry (IHC) staining with HEL and 4-hydroxy-2-nonenal (4HNE). Hematoxylin-eosin and IHC staining with HEL and 4HNE also were performed on conjunctival samples of SS patients and controls.

RESULTS. The tear stability and vital staining scores were significantly worse in eyes of SS patients compared to the controls. Conjunctival inflammatory cell density was significantly higher in SS subjects compared to controls. The numbers of conjunctival cells stained positively for HEL and 4HNE were significantly higher in SS patients compared to controls. Tear HEL concentrations correlated significantly with staining scores and inflammatory cell density in confocal microscopy. Conjunctival specimens also revealed higher numbers of cells stained positively for inflammatory markers, as well as HEL and 4HNE in the IHC stainings.

CONCLUSIONS. Increase of the oxidative stress status in the conjunctiva of SS patients appears to have a role in the pathogenesis of dry eye disease. A close relationship may exist between reactive oxygen species (ROS) production, lipid peroxidation related membrane damage, and inflammatory processes in dry eye. (Invest Ophthalmol Vis Sci. 2013;54:201–210) DOI:10.1167/iovs.12-10325

Sjögren’s syndrome (SS) is a multifactorial autoimmune exocrinopathy, mainly affecting the salivary and lacrimal glands, that is associated with immunologic abnormalities, and a severe form of dry eye and/or dry mouth. The clinical spectrum is diverse, varying from an organ-specific autoimmune disease to a systemic disorder affecting several organs.1 SS occurs worldwide and in all age groups. The peak incidence has been reported to be in the fourth and fifth decades of life, with a female-to-male ratio of 9:1.2 The incidence of primary SS reported in the literature varies from less than 1:1000 to more than 1:100.3

Although the mechanisms involved in the pathogenesis of the inflammatory processes in SS are not completely clear, the manifestations of SS generally are the result of T-cell infiltration, which causes acinar and ductal cell death, and hyposecretion of the tears or saliva.4 It is widely believed that in genetically predisposed individuals, various environmental factors, including viral infections, lead to epithelial cell activation and prolonged inflammatory responses resulting in features of systemic autoimmunity. However, there still are many aspects about the pathogenesis of dry eye disease in SS that remain unexplained.

Several studies described elevation of oxidative stress status in patients with autoimmune disease, such as rheumatoid arthritis, through analysis of reactive oxygen species (ROS) in the synovial fluid, serum, and urine.5–8 However, to our knowledge there still are no studies related to the status of oxidative stress in the ocular surface of patients with SS.

The purpose of our study was to investigate the relation between ocular surface lipid oxidative stress status, inflammatory cell infiltrates and clinical ocular surface findings in patients with SS dry eyes and compare the results to those of normal subjects.

METHODS

Subjects

We recruited 31 eyes of 16 SS patients (16 females) aged between 34 and 78 years (mean 62.8 ± 12.9 years) as well as 15 eyes of 10 normal
subjects (8 females and 2 males) aged from 38 to 88 years (mean 65.5 ± 18.3 years) sequentially from the Dry Eye Subspecialty Clinic of the Department of Ophthalmology, Keio University School of Medicine in this prospective study. The SS patients were diagnosed according to the revised Japanese Sjögren’s syndrome diagnostic criteria as follows: (1) Histopathology positive for a focus score ≥1 (pericapillar lymphoid cell infiltration ≥50) in a 4-mm² minor salivary gland and/or lacrimal gland biopsy. (2) Oral examination positive for abnormal findings in sialography ≥ Stage 1 (diffuse punctate shadows of less than 1 mm) and/or decreased salivary secretion (flow rate ≤10 mL/10 min according to the chewing gum test or ≤2 g/2 min according to the Saxon test), and decreased salivary function according to the salivary gland scintigraphy. (3) Ocular examination positive for Schirmer’s test ≤5 mm/5 min and Rose Bengal test ≥3 points according to the van Bijsterveld score; and/or Schirmer’s test ≤5 mm/5 min and positive fluorescein staining test ≥1 points. (4) Serologic examination positive for anti-Ro/SS-A antibody and/or anti-La/ SS-B antibody. SS was diagnosed when the patients met at least two of the above four criteria.9 Informed consent was obtained from all subjects. Examination procedures were board reviewed and the study was conducted in accordance with the tenets of the Declaration of Helsinki. None of the patients and controls had a history of Stevens-Johnson syndrome; chemical, thermal, and radiation injury; tobacco; or any other systemic/ocular disorder; or underwent any ocular surgery or had contact lens use that would create an ocular surface problem. All patients were using nonpreserved artificial tears for 8 weeks at the time of initial examination, and underwent tear function tests. All patients were using nonpreserved artificial tears for 8 weeks at the time of initial examination, and underwent tear function tests.

Confocal Scanning Laser Microscopy (CSLM)

In vivo CSLM was performed on all subjects with a new generation confocal microscope, the Rostock Corneal Software Version 1.2 of the Heidelberg Retina Tomograph II (RCM/HRT II; Heidelberg Engineering GmbH, Dossenheim, Germany). After the administration of topical anesthesia with 0.4% oxybuprocaine, the subject’s chin was placed in the chin rest. The objective of the microscope was an immersion lens covered by a polymethylmethacrylate cap (Tomo Cap; Heidelberg Engineering GmbH). Comfort gel (Bausch & Lomb, GmbH, Berlin, Germany) was used as a coupling agent between the applanating lens cap and conjunctiva. By adjusting the controller, the center of the TomoCap was applanated onto the center of the temporal interpalpebral bulbar conjunctiva, and in vivo digital images of the conjunctiva were directly visualized on the computer screen. When the first superficial cells were seen, the digital micrometer gauge was set at zero. By pressing on the foot pedal, sequence images were recorded by a charge-coupled device (CCD) color camera (five frames/s) while moving the focal plane forward gradually into the conjunctival stroma. The laser source used in the Heidelberg Retina Tomograph II/Rostock Corneal Module is a diode laser with a wavelength of 670 nm. Two-dimensional images consisted of 384 × 384 picture element, covering an area of 400 × 400 μm. Transversal field of view was captured using the “400 FOV” field lens. At least five sequences (100 images per sequence) of conjunctival CSLM images were taken for each eye. The morphologic characteristics of the conjunctiva were observed in patients with SS and compared to those of normal control subjects. Three images with high resolution and contrast were selected for inflammatory cell counting from the basal epithelial layer. We chose the basal epithelial cell layer only to analyze the inflammatory cell densities, since inflammatory cells appeared like round hyperreflective bodies with the basal epithelial area providing an excellent contrast for inflammatory cell calculations. Inflammatory cells were marked manually inside each 400 × 400 μm frame by placing a blue dot for each cell and then were calculated automatically with the Cell Count software (Heidelberg Engineering GmbH), and the results were expressed as cell density ± SD (cells/mm²).

Conjunctival Brush Cytology

The brush cytology specimens were obtained after administration of topical anesthesia with 0.4% oxybuprocaine. Two adjacent nonoverlapping areas of interpalpebral nasal and temporal conjunctiva were used for sampling. Conjunctiva was scraped seven times with the Cytobrush® (Mediscan AB, Malmö, Sweden), with the examiner holding the brush 2 cm away from the brush end, applying a gentle pressure to the conjunctiva. After sampling, the brushes were placed immediately in 1 mL of Hank’s buffered solution, and the containers were shaken to detach the cells from the brush. The suspended cells were collected using the Millipore filter technique using filters with 8 μm pore size. One slide was allocated for the assessment of conjunctival inflammatory cells number by Diff Quik staining.

Surgical Technique for Conjunctival Resection and Histopathologic Assessment of Specimens

 Conjunctival resections (5 × 5 μm sections) were collected during resection performed for diagnostic lacrimal gland biopsy in Sjögren syndrome patients. Conjunctival resection samples of age- and sex-matched healthy control subjects were obtained during cataract surgery performed at Ishida Eye Clinic. All surgical procedures were performed by the same surgeon (MK). All procedures were performed only from those subjects who provided informed consent. The procedures in our study were ethic board reviewed (Ethic board approval number 16-5-1). Tissue samples were fixed overnight in formalin solution and processed for paraffin embedding. Sections 5 μm in size were cut from paraffin wax blocks and mounted on precoated glass slides. Inflammatory cells were detected by hematoxylin and
eosin staining. Lipid oxidative stress status was visualized by immunohistochemistry (IHC) with anti-hexanoyllysine (anti-HEL) and anti-4-hydroxy-2-nonenal (anti-HNE) primary antibodies.

**Immunohistochemical Staining**

Oxidative stress induced lipid peroxidation was assessed by IHC detection of hexanoyl-lysine (HEL, early phase oxidative stress marker) and 4-hydroxy-2-nonenal (4HNE, late phase oxidative stress marker) protein adducts. The avidin–biotin–peroxidase complex (ABC) method was used for immunostaining. Brush cytology samples were fixed in paraformaldehyde 4% before the staining procedure. Tissue samples were fixed overnight in formalin solution and processed for paraffin embedding. Sections 5 μm thick were cut from paraffin wax blocks, mounted on precoated glass slides, deparaffinized, and rehydrated. Antigen retrieval was achieved by microwaving in 10 mmol/L sodium citrate buffer for 5 minutes, then cooling for 20 minutes. To block nonspecific background staining, sections were treated with normal horse serum (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. The sections then were incubated for 30 minutes with biotin–peroxidase activity was blocked using 3.0% H₂O₂ in methanol for 3 minutes. The sections were incubated for 30 minutes with horseradish peroxidase–anti-mouse IgG serum (Vector Laboratories), followed by avidin–biotin–alkaline phosphatase complex (Vector Laboratories) for 30 minutes. The sections were washed in PBS buffer, developed in prepared 3,3′-diaminobenzidine (DAB) chromogen solution, counterstained lightly with hematoxylin, dehydrated, and mounted.

**Quantification of Oxidative Damage**

All brush cytology specimens from each patient and control subjects were evaluated using light microscopy at 40× magnification for the presence of positive immunohistochemical staining for oxidative stress markers (HEL and 4HNE). The number of positively stained HEL- and 4HNE-stained cells was counted within a total of 100 conjunctival epithelial cells, in a masked fashion. The counting technique was repeated 5 times in nonoverlapping random areas for each specimen and cell density results were expressed in percentage of stained cells.

**ELISA for Tear HEL**

**Measurement of Tear HEL Levels.** A commercially available HEL ELISA (JaICA) was used to determine the tear HEL concentration, as reported previously.13 We also investigated the correlation between tear HEL levels and numbers of cells stained positively with HEL in conjunctival resection specimens.

**Statistical Analysis**

Data were processed using Instat, GraphPad software version of InStat 3.0 (GraphPad, San Diego, CA). The Mann-Whitney test was used to compare the percentages between the SS subjects and normal controls. The correlation between tear HEL levels and number of cells stained positively with lipid oxidative stress markers in conjunctival brush cytology specimens, as well as the correlation between corneal damage scores and numbers of cells stained positively with lipid oxidative stress markers and inflammatory cells were studied by using the Pearson correlation analysis. A probability level of less than 5% was considered statistically significant.

**RESULTS**

**Patient Characteristics**

There were no age-related differences between the patients and control subjects. All patients complained of dry eye symptomatology, including redness, foreign body sensation, tiredness, discomfort, and irritation.

**Tear Functions Tests and Ocular Surface Findings**

The mean BUT and Schirmer test scores were decreased significantly, while the mean fluorescein and Rose Bengal corneal staining scores were increased significantly in SS patients compared to normal subjects (Table 1).

**In Vivo Confocal Microscopy Conjunctival Inflammatory Cell Densities**

The mean conjunctival inflammatory cell densities in eyes of SS patients and healthy control subjects were 471.7 ± 218.4 cells/mm² and 41.8 ± 29.9 cells/mm², respectively. The differences were statistically significant as shown in Figure 1C ($P < 0.001$). In all patients with SS, in vivo confocal microscopy consistently displayed inflammatory infiltrates within the epithelium mainly consisting of polymorphs, dendritic cells and/or lymphocytes. Figures 1A-3 and 1B-3 display representative in vivo confocal scans from a patient with SS, and an age- and sex-matched healthy control subject.

**Immunohistochemistry Stainings for HEL and 4HNE, and Quantification of Lipid Oxidative Stress Damage**

All conjunctival specimens from SS patients revealed marked staining for HEL and 4HNE in the epithelial cells from brush cytology specimens compared to control subjects (Figs. 2, 3). The average percentages of positively-stained epithelial cells for 4HNE and HEL antibodies in the SS subjects were 86.91 ± 7.25% and 81.81 ± 11.44%, respectively. In the control group, the average percentages of positively-stained conjunctival epithelial cells for 4HNE and HEL were 26.37 ± 22.10% and 30.80 ± 20.28%, respectively. The differences were statistically significant ($P < 0.0001$).

**ELISA for Measurement of Tear HEL Levels**

The tear HEL concentration was significantly higher in the eyes of patients with SS (1412.3 ± 301.1 pg/mL) compared to the eyes of healthy control subjects (811.9 ± 396.6 pg/mL, Fig. 4, $P = 0.0005$).

**Table 1.** Comparison of Tear Functions and Ocular Surface Vital Stainings between SS Patients and Healthy Control Subjects

<table>
<thead>
<tr>
<th>Tear Functions and Ocular Surface Vital Staining Scores</th>
<th>SS Patients</th>
<th>Control Subjects</th>
</tr>
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<tbody>
<tr>
<td>Tear BUT, s</td>
<td>2.8 ± 0.7*</td>
<td>8.5 ± 2.8</td>
</tr>
<tr>
<td>Schirmer test 1, mm</td>
<td>3.6 ± 2.0*</td>
<td>13.3 ± 8.6</td>
</tr>
<tr>
<td>Fluorescein staining (0–9 points)</td>
<td>3.1 ± 1.9*</td>
<td>0.5 ± 0.7</td>
</tr>
<tr>
<td>Rose Bengal (0–9 points)</td>
<td>4.0 ± 2.1*</td>
<td>0.1 ± 0.3</td>
</tr>
</tbody>
</table>

* $P < 0.0001$ Mann-Whitney Test.
Immunohistochemistry Staining for Conjunctival Samples
Conjunctival tissues from SS patients presented marked positive staining for oxidative stress damage markers (HEL and 4HNE), and increased number of lymphocytes and other inflammatory cells compared to controls (Fig. 5).

Correlations of Tear Functions and Ocular Surface Vital Stainings between Inflammatory Cell Density, HEL Positively-Stained Cells, and Tear HEL Concentrations
The tear functions and ocular surface vital staining scores were inversely correlated with inflammatory cell density, HEL positively-stained cells, and tear HEL concentrations (Table 2).

Correlation of Tear HEL Concentration with Conjunctival HEL Staining
The tear HEL concentrations showed a significant linear positive correlation with percentages of positively-stained cells for HEL in conjunctival brush cytology specimens \( (r = 0.67, P < 0.0001) \) (Fig. 6A).

Correlation of Conjunctival HEL Staining and Tear HEL Concentration with Inflammatory Cell Density
The inflammatory cell density showed a significant linear positive correlation with percentages of positively-stained cells for HEL \( (r = 0.71, P < 0.0001) \) and with the tear HEL concentration \( (r = 0.65, P < 0.0001) \) as shown in Figures 6B and 6C.

Correlation of Inflammatory Cell Density and Corneal Epithelial Damage with Conjunctival 4HNE Staining
The percentages of positively-stained cells for 4HNE showed a significant linear positive correlation with corneal fluorescein staining scores \( (r = 0.65, P = 0.0005) \) and with inflammatory cell density \( (r = 0.81, P < 0.0001) \) as shown in Figures 7A and 7B, respectively.

DISCUSSION
Cells generate energy by reducing oxygen to water during which ROS are generated. The generation of free radicals has been reported to lead to tissue injury through induction of inflammation in several disease states, including sepsis, ischemic heart and brain disease, atherosclerosis, asthma, atopic dermatitis, and atopic keratoconjunctivitis.14–22

In relation to the autoimmune diseases, increased oxidative stress status, especially elevation of ROS, has been described in patients with systemic lupus erythematosus, rheumatoid arthritis, and Sjögren’s syndrome.23–27

Levels of oxidized proteins are elevated in autoimmune diseases, probably arising from increased inflammatory activity. Previous studies revealed activation of T-cells in the synovial...
joint, correlating strongly with levels of oxidative stress and
depletion of intracellular glutathione in rheumatoid arthritis
patients. The cellular activation of the synovial fluid T
lymphocytes has been shown to be suppressed after adminis-
tration of N-acetyl-L-cysteine, which also was shown to restore
intracellular glutathione levels. In addition, serum, synovial
fluid, saliva, and salivary gland concentrations of 8-oxo-7-
hydrodeoxyguanosine (8-oxodG) and 8 OHDG (markers for
oxidative DNA damage), 4HNE and HEL (markers of lipid
peroxidation), thioredoxin (TRX), and methyl glyoxal have
been reported to be higher in autoimmune disease patients
compared to controls. Kurimoto et al. confirmed the
presence of lipid peroxidation, detected by presence of 4HNE,
8-OHDG, and TRX stainings in the ducts of salivary glands of SS
patients. The lymphocytic infiltration in the lacrimal glands of
SS patients is similar to that observed in the salivary glands.
Histopathology of exocrine glands in SS patients shows
infiltration with CD4 lymphocytes, which transcribe IL-2 and
IFN-gamma. In addition, the conjunctiva of patients with SS
and non-SS appear to harbor inflammatory cells positive for
CD4 and mainly for CD4+. The conjunctival samples also
exhibit increased immunoreactivity for HLA-DR and HLA-DQ,
expressed by lymphocytes and epithelial cells. On the other hand, antibodies to SS-A/Ro antigens are found commonly in sera of patients with SS. Saegusa et al. showed that UVB irradiation-induced oxidative stress led to cell surface expression of SS-A/Ro on keratinocytes, which was blocked by N-acetylcysteine, an anti-oxidant substance. All these previous studies suggest that oxidative stress and inflammation coexist, and may be important in the pathogenesis of SS.

ROS generated from environmental insults and pathologic
conditions render the human eye particularly vulnerable to
oxidative damage, which has been reported to have an
important role in several ocular diseases, including age-related
macular degeneration, cataract, uveitis, retinopathy of prematurity, corneal inflammation, atopic keratoconjunctivitis, and conjunctivochalasis. Whether oxidative stress–related ocular surface damage is a primary cause or merely a downstream consequence of the inflammatory process still is an open question. Nakamura et al. demonstrated in a blink suppressed dry eye mouse model that superficial punctate keratopathy (SPK) was associated with an increase of oxidative stress markers, antioxidant-related genes, and ROS. These results suggest a strong relationship between the accumulation of oxidative stress and corneal epithelial alterations in blink-suppressed dry eye.

The tear fluid has been shown to contain a sufficient
amount of free radical scavengers, such as glutathione
peroxidase, superoxide dismutase, catalase, lactoferrin, and
calcium. An imbalance between radical-scavenging sys-
tems and free radical generation in the tears of dry eye patients
may result in ocular surface epithelial injury as reported
previously in a biochemical study by Augustin et al. Lipid
peroxide levels (measured by thiobarbituric acid method) and
myeloperoxidase activity were determined as parameters of
oxidative tissue damage and inflammatory activity in that study.
Although the oxidative stress status in salivary gland of SS
patients has been reported to be elevated, to our knowledge
there are no studies related to the status of oxidative stress in
the conjunctiva of SS patients and relevant features of the
dry eye disease in SS.
In our study, we investigated the lipid oxidative stress-related changes in the tear film and conjunctiva of patients with SS and control subjects. Our efforts to investigate the lipid oxidative stress changes interestingly revealed higher percentages of cells stained positively for HEL (early phase lipid peroxidation marker) and 4HNE (late phase lipid peroxidation marker) in bulbar conjunctival samples, suggesting an increased lipid peroxidation status in SS. The lipid peroxidation marker HEL also was significantly increased in tears of patients with SS compared to controls.

While the oxidative stress could be the consequence of ROS production by inflammatory cells, it might be that oxidative stress also could be an inciting factor in the generation of ocular surface inflammation. Oxidative free radicals directly oxidize various macromolecules, including lipids. More than 200 aldehyde species arise from the oxidative degradation of cellular membrane lipids, also known as lipid peroxidation (LPO), including 4HNE and malondialdehyde (MDA). It also has been described in the literature that lipid peroxides and their breakdown products, such as HEL and 4HNE, can directly or indirectly affect many functions integral to cellular and organ homeostasis. HEL, for example, is a major and toxic aldehyde generated by free radical attack on 0-6 polyunsaturated fatty acids (arachidonic, linoleic, and linolenic acids), and is considered to be a toxic messenger of oxygen free radicals. 4HNE undergoes many reactions with proteins, peptides, phospholipids, and nucleic acids, and has cytotoxic, mutagenic, and genotoxic signal effects. 4HNE has been identified as the most cytotoxic breakdown product generated from lipid peroxidation.

The cellular consequences of 4HNE synergistic reactivities with biomolecules containing amino and thiol groups include growth inhibition, decreases in glutathione levels, decreases in sulphydryl- and thiol-containing proteins, inhibition of enzyme activities, inhibition of calcium sequestration by microsomes, inhibition of protein synthesis and degradation, and alterations in signal transduction and gene expression profiles. Excessive production of 4HNE during periods of oxidative stress may

**Figure 3.** Representative immunohistochemistry stainings for the late lipid oxidation marker in brush cytology specimens from an SS patient and a control subject. Note the extensive lipid oxidative stress damage in the 4HNE immunohistochemistry staining from brush cytology samples of an SS (A-1) patient compared to the healthy control subject (B-1). Note the significantly higher percentage of cells stained by 4HNE in patients with SS (C). (A-2, B-2) The negative control from the immunohistochemistry.

**Figure 4.** Comparison of average tear HEL levels between SS patients, and age- and sex-matched healthy control subjects.

*p < 0.001 Mann-Whitney Test*
lead to the saturation, inhibition or degradation of the normal metabolic pathways responsible for maintaining its intracellular homeostasis, and thereby result in its accumulation and consequent reactivity with other cellular constituents.\textsuperscript{51,52} The cornea possesses an armamentarium of nonenzymatic and enzymatic defense systems to combat oxidative stress, including high levels of albumin, ascorbate, ferritin, superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferases, aldose reductase, alcohol dehydrogenases, and aldehyde dehydrogenases (ALDH), which enable this tissue to maintain its structural integrity. The ALDH enzymes catalyze the NAD(P)$^+$-dependent irreversible oxidation of a wide spectrum of endogenous and exogenous aldehydes. ALDH proteins are found in all subcellular regions, including cytosol, mitochondria, endoplasmic reticulum, and nucleus, with several found in more than one compartment.

Among the ALDH isozymes, ALDH1A1 encodes a homotrimer distributed ubiquitously in the adult epithelium of various organs, including testis, brain, eye lens, liver, kidney, lung, and retina. ALDH1A1 is one of three highly conserved cytosolic isozymes, in addition to ALDH1A2 and ALDH1A3, that catalyze the oxidation of the retinol metabolite, retinaldehyde, to retinoic acid (RA). RA synthesis is critical for normal growth, differentiation, development, and maintenance of adult epithelia in vertebrate animals and humans.\textsuperscript{52} The isozyme ALDH3A1 is considered a mammalian corneal crystalline, attaining high concentrations in this ocular tissue. Its presence has been identified by immunofluorescence in the epithelial and the stromal matrix layers of the normal human cornea, but not in the endothelial layer. The precise role of this enzyme in corneal function remains to be elucidated. However, recent data suggest that ALDH3A1 has a significant role as an enzymatic antioxidant in the protection of cells from 4HNE-induced cytotoxicity.\textsuperscript{52} While we did not investigate the role of ALDHs in our study, alterations in ALDH1A1 and ALDH3A1 expressions also may explain the ocular surface epithelial damage in patients with Sjögren’s syndrome. It might be that other antioxidant enzymes, including catalases, superoxide dismutases, and glutathione peroxidases, also may be important in relation to the ocular surface disease in Sjögren’s syndrome. Indeed, immunohistochemistry and image analysis of conjunctival impression samples from eyes of patients with SS revealed that the expression of these three enzymes are less pronounced in the conjunctival epithelia of patients with SS.

![Figure 5](image_url)

**Figure 5.** Representative immunohistochemistry staining for oxidative stress and histopathologic alterations in samples from an SS patient and control subject. Note the presence of marked inflammatory cells characterized by lymphocytes in dark-pink (arrows) color stained by hematoxylin and cosin in sample from an SS patient (A) compared to control subject (E). Conjunctival tissues from SS patients presented marked positive staining for oxidative stress damage markers HEL (B) and 4HNE (C) compared to control subjects (F, G, respectively). Note that the dark-brown color represents the oxidative stress damage sites. (D, H) The negative control slides from SS patient and control subject, respectively.

| Table 2. Correlations of Tear Functions and Ocular Surface Vital Stainings between Inflammatory Cell Density, HEL Positively-Stained Cells, and Tear HEL Concentrations |
|---------------------------------|-----------------|-----------------|-----------------|
| **Inflammatory Cell Density**   | **HEL-Stained Cells** | **Tear HEL Concentrations** |
| Pearson Correlation Coefficient| $P$ Value       | Pearson Correlation Coefficient| $P$ Value       | Pearson Correlation Coefficient| $P$ Value       |
| Schirmer 1, mm                  | $-0.4886$       | $0.0008$         | $-0.6607$       | $<0.0001$         | $-0.5960$       | $0.0001$         |
| BUT, s                          | $-0.5591$       | $<0.0001$        | $-0.5107$       | $0.0006$          | $-0.4011$       | $0.0153$         |
| Fluorescein score (points)      | $0.8103$        | $<0.0001$        | $0.6302$        | $<0.0001$        | $0.6801$        | $<0.0001$        |
| Rose Bengal score (points)      | $0.7628$        | $<0.0001$        | $0.6061$        | $<0.0001$        | $0.6520$        | $<0.0001$        |
dry eyes as compared to the conjunctival epithelium of normal eyes.

We suggested that the increased membrane lipid peroxidation may evoke and/or increase the immune and inflammatory response, activate gene expression and cell proliferation, or initiate apoptosis. Thus, ROS production, peroxidative lipid membrane damage, inflammatory pathologic processes, and changes in the ocular surface antioxidant status appear to have a role in the pathogenesis of the ocular surface disease in SS.

Indeed, further analysis showed a significant correlation between tear HEL levels and conjunctival HEL staining in our study. Another strong correlation was observed between inflammatory cell density, and the extent of early and late lipid peroxidation marker staining also showed a strong correlation with corneal epithelial damage.

Peroxidation of membrane lipids might have very well induced perturbation of ocular surface epithelial cellular functions causing breakdown and death of epithelial cells, which might have contributed to increased epithelial fluorescein staining scores. TNF-α, which is known to induce inflammation and cell death, also has shown to be elevated in tears of SS patients. However, the pathways regulating the elevation of lipid oxidation status and linking it to inflammation still are unclear.

Some evidence suggests that in SS dry eyes, the formation of inflammatory lymphocytic foci may be a late-stage event resulting in salivary and lacrimal glandular dysfunction. Gao et al. have shown that secretory glandular dysfunction occurred even in the noninfiltrated parts of accessory lacrimal glands.
glands in a dog model of dry eye disease. It is possible that lipid peroxides and their breakdown products, such as HEL and 4HNE, can be directly or indirectly affecting many ocular surface and lacrimal gland functions integral to cellular and tissue homeostasis in early and/or late stages of SS ocular surface disease.

It also remains the further goal of future studies to determine the short- and long-term changes in conjunctival epithelial cytology staining with oxidative stress markers in patients with dry eye, but without evidence of inflammation in the lacrimal or minor salivary glands. Simultaneous studies investigating the alterations in the ocular surface anti-oxidant status also will provide invaluable information.

In summary, to our knowledge our study provided the first evidence that lipid peroxidation and inflammation coexisted in the conjunctiva of patients with SS, a finding that might hold the keys to future studies and explanations for the pathogenesis of the ocular surface disease in SS.

References


