Clinical and Molecular Inflammatory Response in Sjögren Syndrome-Associated Dry Eye Patients under Desiccating Stress

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INTRODUCTION

Dry eye disease is characterized by ocular discomfort, visual disturbance, tear film instability, as well as increased tear film osmolarity and lacrimal functional unit inflammation.[1,2] It is considered one of most common eye disorders among adults worldwide. Its prevalence has been estimated to be 4.34% in men[3] and 7.8% in women in the United States of America (USA).[4] It is also one of the main reasons for seeking eye care among older people because its overall risk raises by 1.06 for each increased decade.[5] Sjögren syndrome (SS) is a chronic systemic autoimmune disease characterized by lymphocytic infiltration of the exocrine glands and mucosal epithelia, resulting in dry eye as well as dry mouth.[6] This condition causes one of the most severe types of dry eye disease; the prevalence of its primary type varies greatly, from 0.09% to 2.7% depending on the diagnostic criteria used.[6] However, what is really evident is that SS has a strong female propensity, with ratios (female to male) as high as 20:1 reported in some populations.[7]

Ocular discomfort (including dry eye feeling) is the second most reported symptom in artificially-created environments, where people living in urban areas spend most of their time.[8] These indoor environments tend to alter the tear film because of their low humidity, and high air flow[9] occurring inside conventional buildings, airplanes, and vehicles also tends to produce dry eye. The use of visual display terminals has grown exponentially worldwide, which further impacts dry eye prevalence negatively. The percentage of office workers using these devices and being diagnosed with dry eye has increased up to 10% and 21% in male and female Japanese office workers, respectively.[10] Adverse environmental conditions could trigger the exacerbation of properly managed dry eye patients or borderline subjects. Therefore, our research group and others have studied the clinical and tear changes occurring in dry eye patients after exposing them to several desiccating conditions to evaluate how the lacrimal functional unit responds to a controlled adverse environment.[11-15] However, we were not able to find in the literature reports addressing the variation of tear molecules occurring only in SS-associated dry eye patients when being exposed to controlled adverse conditions in spite of the following two well-known facts: first, inflammation plays a major role in dry eye disease,[2] and second, concentrations of some tear molecules in severe dry eye patients are consistently different from healthy subjects.[16-19]

The analysis of the changes occurring in tear molecules of SS dry eye patients could be interesting from a therapeutic viewpoint,[20] because this biochemical assessment could provide information regarding the mechanism of the acute exacerbations that these severe dry eye patients frequently suffer. Consequently, the purpose of the present study was to analyze how the lacrimal functional unit of SS dry eye patients is affected, from a clinical and biochemical standpoint, after exposing them to desiccating conditions while performing daily living tasks within an environmental chamber.

METHODS

Participants

This prospective cross-over pilot study adhered to the tenets of the Declaration of Helsinki and was approved by the University of Valladolid Ethics Committee. All candidates provided informed consent. The study was double-masked and the same examiner always performed the clinical tests. Dry eye patients were recruited among level 3, as classified by the International Dry Eye Workshop dry eye severity grading scheme.[21]

During a preliminary visit, the recruited SS dry eye patients were screened for the following inclusion criteria: ocular surface disease index[22] score ≥ 33 and a corneal fluorescein staining ≥ grade 2 (Oxford Scale).[3] Patients showing a corneal fluorescein staining grade 2 needed a tear break-up time < 3 seconds to be included in the study following severe dry eye diagnosing criteria recently established by Baudouin et al.[23] The recruited SS dry eye patients had been previously diagnosed of SS following the American European Consensus Group criteria.[24] We included only female patients to prevent misinterpretation of data because of the female-to-male prevalence ratio reported in SS.[7] Exclusion criteria were pregnancy or nursing, contact lens wear, any ocular surgery within the last 6 months, any acute or chronic ocular disease other than dry eye, concomitant allergies (even if mild), and any systemic anomaly (except SS) that contraindicated being subjected to any environmental controlled condition. Patients were not recruited if they were using topical cyclosporine A eyedrops within 3 months prior to the screening visit and/or topical corticosteroids within 1 month prior to that preliminary visit. Any other topical treatment was an exclusion criteria. Only artificial tears and lubricants were allowed to be used as needed. All participants were instructed not to instill any eyedrop within the 4 hours prior to any evaluation. The eye having more severe corneal fluorescein staining was selected.

Environmental Conditions

All patients were exposed for 2 hours to two different environmental conditions in two different sessions while performing near vision tasks (reading, playing cards, etc), within an environmental chamber located inside the Controlled Environmental Research Laboratory (University of Valladolid, Valladolid, Spain).[13] The environmental conditions were the following: (1) Simulated adverse condition of 23ºC, 5% relative humidity, and a low air flow of 0.10 m/s; and (2) simulated normal condition of 23ºC, 45% relative humidity and 0.10 m/s air flow.[25] The order of the exposure to each condition was randomized and separated between 2 to 5 days.

Examination Procedure

All individuals were evaluated twice: immediately before and immediately after the 2-hour exposure. The examinations were performed in the sequence outlined below, with a 2-to-5-minute interval between tests.

Modified Single-Item Score Dry Eye Questionnaire. (Simmons PA. IOVS, 2003;44. ARVO E-Abstract. B287). This questionnaire assesses the ocular discomfort due to symptoms of dryness, ranging from “none” to “severe” (0-4 scale); however, we added a visual analogue scale (0-10 scale) to increase test sensitivity as previously performed,[13,25] and evaluated the following items:

dryness, grittiness, stinging, pain, itching, sensitivity to light, and blurred vision. We averaged all items to compute the total modified Single-Item Score Dry Eye Questionnaire score. Thus, the maximum possible score was 10.

Tear osmolarity. This was assessed using the TearLab osmometer (TearLab Corporation, San Diego, CA, USA).

Phenol red thread test. (Zone Quick Test; Menicon Company Ltd., Nagoya, Japan) The thread was placed over the external canthus, and the length of the wetted thread was read 15 seconds later.

Conjunctival hyperemia. Nasal and temporal bulbar areas were assessed independently based on the Efron scale.[26] The final score was the average of the values.

Tear sample collection. A 2-µl tear sample was collected from the external ocular canthus using a glass capillary tube (Drummond Scientific, Broomall, PA, USA), avoiding reflex tearing as much as possible. Samples were diluted 1/10 in a 0.5 ml microtube (Sarstedt AG&Co, Nümbrecht, Germany) containing ice-cold Cytokine Assay Buffer (Merck Millipore, Millipore Iberica, Madrid, Spain) and frozen at -80°C until analysis.

Tear break-up time. This was defined as the time between the last of 3 blinks and the appearance of the first dry spot after instillation of 5 µl of 2% sodium fluorescein. This procedure was repeated 3 times, and the final tear break-up time value was the average.

Corneal fluorescein staining. This was evaluated using a cobalt-blue filter over the light source slit-lamp biomicroscope (SL-8Z, Topcon Corp., Tokyo, Japan) and a yellow Wratten #12 filter (Eastman Kodak, Rochester, NY, USA) 2 minutes after instillation of 5 µl of 2% sodium fluorescein. The Oxford (0-5 score)[27] and the Baylor[28] schemes were used for assessment.

Conjunctival lissamine green staining. Lissamine green strips (GreenGlo, HUB Pharmaceuticals, LLC, Rancho Cucamonga, CA, USA) were wetted with 25 µl sodium chloride and then gently applied into the inferior fornix. One minute later, staining was evaluated following the Oxford scheme.[27]

Schirmer test without topical anesthesia. One Schirmer sterile strip (Tearflo, HUB Pharmaceuticals, LLC, CA, USA) was placed in the lateral canthus of the inferior lid margin. The length of wetting was measured after 5 minutes, with eyes closed.

Analysis of tear molecules

Two commercial immune-bead based arrays were used to analyze 16 molecules in tear samples with Luminex IS-100 equipment (Luminex Corporation, Austin, TX, USA). The concentrations of epidermal growth factor (EGF), chemokine (C-X3-C motif) ligand 1 (CX3CL1)/Fractalkine, interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-6, chemokine (C-X-C motif) ligand 8 (CXCL8)/IL-8, IL-10, IL-12p70, IL-17A, IL-1 receptor antagonist (IL-1RA), chemokine (C-X-C motif) ligand 10 (CXCL10)/Interferon gamma-induced protein 10 (IP-10), chemokine (C-C motif) ligand 5 (CCL5)/regulated on activation, normal T-cell expressed and secreted (RANTES), tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF) were measured

simultaneously with a 15-plex assay (HCYTO-60K 15X-Milliplex Millipore Iberica, Spain). The matrix metalloproteinase-9 (MMP-9) concentration was measured in a separate assay with a MMP-9 single-plex assay (HMMP2-55K Panel 2, Milliplex, Millipore Iberica, Spain), which recognized both the MMP-9 inactive zymogen and MMP-9 active forms. The samples were analyzed following the manufacturer's protocol, as previously described.[13,25] The minimum detectable concentrations (in pg/ml) for molecules analyzed were as follows: IFN-γ and TNF-α, 0.1; CXCL8/ IL-8 and IL-17A, 0.2; IL-2, IL-6, and IL-10, 0.3; IL-1β and IL-12p70, 0.4; CCL5/RANTES, 1; CXCL10/IP-10, 1.2; EGF, 2.7; IL-1RA, 2.9; VEGF, 5.8; CX3CL1/fractalkine, 6; and MMP-9, 10. Data were stored and analyzed with the "Bead View Software" (Upstate-Millipore Corporation, Watford, UK). In some samples, the assayed molecule was undetectable. To include those samples in the statistical analysis, we assigned each the minimum detectable value provided by the assay manufacturer as previously reported.[13,25] However, molecules that were detected in less than 50% of the samples were not statistically analyzed any further.

Data Analysis

Data were expressed as the mean ± standard error of the mean (SEM) regardless of data normality. Statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS 19.0 for Windows; SPSS Inc., Chicago, IL, USA) and R software by a licensed statistician. For comparisons between clinical dry eye tests performed before and after the environmental exposure, as well as before the exposure during both conditions, the Wilcoxon test was used. For tear molecule concentrations, we performed the same comparisons, but we used a parametric t-test after performing a logarithmic transformation (log 2) as previously performed.[29] Spearman correlation coefficients between parameters were calculated to determine what variables could predict the inflammatory response and to explore relationships between the changes in tear molecule concentrations and clinical variables. Two-sided *P*-values ≤ .05 were considered statistically significant.

RESULTS

Screening Visit

A total of 14 SS dry eye females (58.9±2.8 years-old; range, 40-75) were recruited. Mean SS disease duration was 8.1±3.8 years. At inclusion visit, the mean ocular surface disease index, corneal fluorescein staining (Oxford scheme) and tear break-up time values were 57.0±5.1 units, 2.7±0.3 units, and 1.6± 0.2 seconds, respectively. Four out of the 14 SS patients recruited were also diagnosed of meibomiam gland disease.

Clinical Tests

Clinical test outcomes are summarized in Table 1. There were no significant differences between dry eye test scores obtained before undergoing either exposure. After 2-hour exposure to the adverse condition, SS dry eye patients experienced a significant worsening for tear osmolarity (*P*= .03), conjunctival hyperemia (*P*= .05), and corneal fluorescein staining in the nasal (3.6±0.5 vs

4.5±0.5, *P*= .04) and temporal (*P*= .01) areas (Table 1 & Figure 1). After 2-hour exposure to the normal condition, SS dry eye patients showed only a significant increase of corneal fluorescein staining in the nasal area (*P*= .03) (Table 1). No patients suffered any adverse event throughout the whole study.

Tear Cytokines/Chemokines and MMP-9 levels

Among the 16 molecules analyzed in tears, 8 (EGF, Fractalkine, IL-1RA, IL-6, IL-8, IP-10, VEGF, and MMP-9) had a detection rate above 50% for both conditions. Consequently, these 8 were statistically analyzed; concentrations are summarized in Table 2. Mean concentration values of molecules with a detection rate <50% are also provided (Supplemental Material at AJO.com).

Prior to undergoing both exposures, only IL-1RA showed significantly different values (Table 2). There were no significant (*P*≥ .05) changes in the tear molecule concentrations before and after exposure to the normal condition. On the contrary, after the adverse condition there was a significant increase in concentrations of IL-1RA (*P*= .01), IL-6 (*P*= .02), IL-8 (*P*= .03), and MMP-9 (*P*= .03) (Table 2).

Variables Predicting Inflammatory Response of the Lacrimal Functional Unit

A significant (*P* ≤ .04) inverse relationship was found between nasal (r_s = -0.62) and temporal (r_s = -0.55) baseline corneal staining and the worsening observed in these corneal areas after the adverse exposure. Additionally, a near significant (*P* = .07) inverse (r_s = -0.49) association was also observed between baseline inferior corneal staining and the increase showed in this parameter. Regarding tear molecules, we also found a significant inverse (r_s = -0.55, *P* = .05) association between MMP-9 baseline values and its increase observed after the adverse exposure. The same occurred for IP-10 tear values (r_s = -0.77, *P* = .001).

Relationship between Changes in Tear Molecules and Clinical Variables

We found a consistent relationship between the increase in IL-1RA and the worsening of corneal integrity in terms of corneal staining scores for the central (r_s= 0.62, *P* = .02), temporal (r_s= 0.56, *P* = .04), and inferior (r_s= 0.58, *P* = .03) areas. We also observed a significant inverse association between the increase in IL-6 (r_s= -0.55, *P* = .04), IL-8 (r_s= -0.52, *P* = .05) and MMP-9 (r_s= -0.66, *P* = .02) tear concentration levels and the decrease in Schirmer test scores after the adverse exposure.

DISCUSSION

Although the exact etiopathogenesis of dry eye disease is not yet completely understood, there is global agreement regarding its inflammatory nature.[1,2] With the aim of better understanding the disease and better showing dry eye therapeutics efficacy, customized instruments and facilities where environmental conditions can be controlled have been designed.[11-15] In the present study, we used our own facility (Controlled Environmental Research Laboratory)[13,25] and observed that the lacrimal functional unit status in SS dry

eye patients worsened after undergoing short-term desiccating conditions. This clinical deterioration was accompanied by significant changes in the activity of several tear molecules (IL-1RA, IL-6, IL-8, and MMP-9) previously reported to be elevated in patients with dry eye as compared to healthy subjects.[16-19]

Differences in dry eye test scores between the measurements obtained before each exposure were negligible (Table 1). Likewise, when looking at the changes observed in the clinical dry eye tests after exposure to the normal condition, as expected, there were no significant variations in all tests performed, except for a slight increase in fluorescein staining in the nasal cornea. It has been previously demonstrated that spontaneous blink rate while reading is lower compared to other actions (i.e., conversation),[30] which could explain this mild worsening observed in our SS dry eye patients in terms of nasal corneal staining increase.

Concerning the adverse condition, our SS patients showed a worsening of the lacrimal functional unit (Table 1). This finding agrees with previous studies[12-15] reporting that dry eye patients having diverse etiology can suffer ocular surface deterioration when exposed to desiccating conditions, which also occurs in healthy subjects.[14,30] Most of the dry eye tests tended to worsen in our study, showing significant deterioration in tear osmolarity, conjunctival hyperemia, and corneal staining (Table 1). The increase in tear osmolarity could be associated to tear evaporation increase (low relative humidity environment[31]) and to unconscious blink rate reduction (volunteers were reading[30]). The sum of both factors could have produced a slight decrease in tear volume that would have yielded an increase in the concentration of solutes, leading to tear hyperosmolarity.[32] In our study, we did not observe a significant reduction of tear volume as measured with phenol red thread tear and Schirmer test (Table 1). Nonetheless, these tests might be useful to support diagnosis of dry eye when the scores obtained are below a cut-off value;[33] this does not mean that they are useful to detect minimal changes in tear volume, as has been widely reported, due to their lack of consistency.[34,35]

In our study, the exposure to the adverse condition also produced an increase in corneal fluorescein staining, not only from a global viewpoint (Oxford scheme), but also for each area analyzed (Baylor scheme). However, significant changes were only observed for the nasal and temporal areas (Table 1 & Figure 1). Under desiccating environments, epithelial cells are more likely to be exposed because of tear imbalance, which in our SS patients might have been even higher as a result of the meibomiam gland dysfunction that the vast majority suffered. Hyperosmolarity stimulates the death of the epithelial surface even further.[32] Consequently, a reduction in ocular surface integrity is to be expected, which in our case was also accompanied by an increase in conjunctival hyperemia (Table 1).

In our SS patients, we found an inverse association between the severity of corneal integrity prior to the adverse exposure and the change observed in corneal staining. The same inverse relationship occurred for MMP-9 and IP-10 tear levels. These findings might indicate that those SS patients whose lacrimal functional unit is already highly compromised might not show so much worsening as those SS patients whose lacrimal functional unit is not so greatly

impaired. Alex et al.[14] previously observed just the opposite findings, those individuals having worse dry eye test scores were the ones showing higher worsening after exposing them to adverse conditions using goggles. This discrepancy might arise from the type of participants recruited in each study. They included 15 normal and 10 dry eye patients (only 4 were SS patients) in their study, while all of our individuals were SS patients who showed poorer corneal integrity than the dry eye patients recruited by Alex et al.[14]

We were unable to find previous reports addressing the initial changes in tear molecules occurring only in severe dry eye patients when suffering an acute exacerbation. Consequently, we evaluated the tear concentrations of 16 inflammatory molecules before and after both exposures. In our study, we did not observe significant differences in tear molecule concentrations before each exposure, except for IL-1RA (Table 2). Inter-day differences can be assigned to the inter-day variability reported in these tear molecules.[36] Despite this fact, tear molecule levels could be more consistent than in some clinical dry eye tests.[34] We did not observe any significant changes in tear molecule concentrations after the normal condition exposure. However, there was a significant increase in IL-1RA, IL-6, IL-8, and MMP-9 levels after 2-hour exposure to the adverse condition (Table 2).

IL-1RA is an endogenous inhibitor of IL-1 that impedes the activities of the pro-inflammatory forms of IL-1 by competitively binding to the type 1 IL-1 receptor. Huang et al.[36] have reported that the concentration of this tear molecule is proportional to the severity of the dry eye, correlating with corneal staining. Moreover, our group[37] found that there was an inverse relationship between the levels of IL-1RA and Schirmer and tear break-up time values. Consequently, it seems that this tear molecule is highly involved in the inflammatory process occurring in dry eye patients. In fact, Amparo et al.[38] have reported that Anakinra, a topical IL-1 receptor antagonist, reduced symptoms and corneal epitheliopathy in patients with dry eye after 12-week treatment; they suggested that the administration of an IL-1 antagonist might be a novel therapeutic option in dry eye.

In our study, we also found an increase in IL-6 tear levels after undergoing desiccating conditions (Table 2). The IL-6 molecule is a pro-inflammatory cytokine frequently elevated in tears of dry eye patients,[39,40] moreover, its concentration is increased in SS patients in comparison with non-SS patients.[17,18] It has also been suggested that it is one of the most important tear molecules in dry eye, because it has been found to be one of the factors involved in desiccation-induced cell death in experimental settings.[41] Additionally, increased IL-6 tear levels might be one of the earliest observable changes in patients with dry eye,[16] as we also showed after exposing our SS dry eye patients to adverse conditions. However, its moderate percentage of detection as shown in this study and previous ones,[13,25,37] as well as its moderate inter-day[36] and intra-day[42] variability, might still prevent this tear molecule from being the gold-standard biomarker for dry eye disease.

The desiccating stress exposure also produced an increase in IL-8 tear concentrations (Table 2). This pro-inflammatory molecule has been reported to be elevated in tears of dry eye patients[39,43] and in the conjunctival epithelium of SS dry eye patients.[16] Additionally, previous authors have observed during in-vitro settings that corneal epithelial cells secrete IL-8 when subjected to long-

term desiccation,[41] which might also have occurred in our SS dry eye patients. The increase of IL-8 tear levels might be a signal to recruit inflammatory cells to the ocular surface, which might be responsible for the deterioration of the corneal and conjunctival integrity, as previous authors have hypothesized.[43] In fact, Lam et al.[39] reported a direct relationship between IL-8 concentration and corneal and conjunctival stainings.

We also observed that MMP-9 tear concentration (active and inactive forms), which is usually already elevated in dry eye patients[44] especially in those having SS,[19] also increased after the adverse condition. This finding agrees with the response observed in experimental settings when exposing mice to desiccating stress.[45] Such increased MMP-9 tear levels were also observed in moderate dry eye patients under different adverse conditions.[13,25] This tear molecule has gained popularity in relation to dry eye, as some authors have indicated its potential utility as a biomarker to diagnose dry eye disease using a MMP-9 based point-of-care device.[46] Additionally, other authors have shown in-vivo benefits of using MMP-9 inhibitors for restoring tear production.[47]

In the present study we also found consistent associations between the increase in IL-1RA tear levels and the worsening of the corneal staining, in addition to the relationship between the decrease in Schirmer test and the increase of IL-6, IL-8 and MMP-9 tear levels. These findings provide more evidence (at least in SS patients) to support the relationship existing between the response of the lacrimal functional unit in terms of tear molecule secretion and the clinical signs commonly observed in the slit lamp examination. Thus, it enhances the relevance of performing further research to address the exact mechanism underlying the clinical worsening occurring not only SS patients but in all dry eye patients.

Our research group[13,25] and others[15] have previously showed that not only mild to moderate dry eye patients suffer ocular surface worsening under diverse adverse conditions, but also non-symptomatic subjects. Moreover, this dry eye exacerbation was accompanied by a significant variation in some tear molecules (IL-6 and MMP-9)[13,25] that were also increased in the present study. This means that the acute inflammatory response might be driven by similar mechanisms regardless of the etiological causes already provoking dry eye. Nonetheless, in this study our research group observed for the first time that IL-1RA might play a major role when dealing with SS patients not only based on its increased tear concentration, but also because of its significant association with higher corneal staining. Therefore, the response of SS patients might differ from that of other patients having evaporative-type dry eye (mild to moderate), especially taking into account that SS is a chronic systemic autoimmune disease usually causing severe dry eye, while the origin of mild to moderate evaporative dry eye is not usually systemic.

A limitation of the current study could be the adverse environmental condition that we recreated. The adverse condition might have reproduced a slightly more desiccating environment that the one usually found in shopping malls or office buildings,[48] where dry eye patients usually suffer exacerbations. However, we only exposed volunteers to this environment for 2 hours, which is much shorter than the time that people usually spend within these edifices, thus compensating this problem. Another limitation is that a control group of healthy volunteers is lacking; however, there is already enough evidence showing that

this population also suffers worsening of the ocular surface when exposed to desiccating stress.[14,25,31] Another limitation is that our results do not strictly apply to severe non-SS dry eye patients exposed to adverse conditions, as the origin of the inflammatory process causing dry eye can be different from the one present in SS.[17] However, a similar worsening of the lacrimal functional unit to the one described here or the one previously reported by our group[13,25] and others[14,31] should be expected in terms of ocular surface worsening and increased inflammatory status, Finally, the number of patients included in the present study, although not high, has proved to be enough to show adequate evidence of the increase in the basal inflammatory state occurring in severe dry eye patients subjected to short-term desiccating conditions.

In conclusion, we showed that low humidity conditions can produce an increase in key clinical dry eye signs such as corneal staining or tear osmolarity in female SS dry eye patients. In addition, adverse environments that people are exposed to daily can produce significant changes in tear levels of some inflammatory molecules (IL-1RA, IL-6, IL-8, and MMP-9) in these severe dry eye patients, suggesting an increase in the basal inflammatory state of their lacrimal functional unit. Our study outcomes provide further evidence supporting current industry efforts to develop new therapeutics for ameliorating exacerbation episodes of dry eye patients.[11,29] Moreover, these treatments should also target the increased inflammatory activity previously shown by tear molecule concentrations[29] and supported by these results. Finally, this study supplies additional evidence that artificially controlled environments can be valuable clinical trial tools[25,15] because they permit regulating exposure conditions, thus overcoming habitual clinical trial shortcomings.[49]

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FIGURE CAPTIONS

Figure 1. Images showing corneal fluorescein staining in a Sjögren syndrome-dry eye patient. A representative case of a patient showing corneal fluorescein staining before (Left) and after (Right) 2 hours of exposure to the adverse condition within the Controlled Environmental Research Laboratory (Universidad de Valladolid, Valladolid, Spain).

TABLES

Table 1. Clinical test outcomes (mean ± standard error of the mean) in Sjögren syndrome-dry eye patients before and after 2-hour exposure to each controlled environment.

Dry Eye Disease Test		Exposure	Normal Condition Mean ± SEM	Adverse Condition Mean ± SEM	*P* value**
Modified SIDEQ		**Pre**	4.5 ± 0.5	4.8 ± 0.8	.67
		Post	3.1 ± 0.6	3.6 ± 0.6	
Tear osmolarity		**Pre**	320.9 ± 4.7	**315.7 ± 3.0**	.24
		Post	317.5 ± 5.1	**327.7 ± 5.1***	
Phenol red thread test		**Pre**	13.6 ± 1.8	13.4 ± 1.6	.93
		Post	13.7 ± 2.0	12.3 ± 1.3	
Conjunctival hyperemia (mean)		**Pre**	1.5 ± 0.1	**1.3 ± 0.1**	.25
		Post	1.6 ± 0.1	**1.6 ± 0.1***	
T-BUT		**Pre**	1.3 ± 0.1	1.3 ± 0.1	1.0
		Post	1.5 ± 0.2	1.2 ± 0.1	
Corneal fluorescein staining (Oxford scheme)		**Pre**	2.4 ± 0.2	2.5 ± 0.2	.16
		Post	2.3 ± 0.2	2.7 ± 0.2	
Corneal fluorescein staining (Baylor scheme)	**Central**	**Pre**	2.1 ± 0.4	2.1 ± 0.5	1.0
		Post	2.1 ± 0.4	2.6 ± 0.6	
	Nasal	**Pre**	**2.9 ± 0.5**	**3.6 ± 0.5**	.29
		Post	**3.6 ± 0.5***	**4.5 ± 0.5***	
	Temporal	**Pre**	3.4 ± 0.4	**3.5 ± 0.5**	.83
		Post	3.5 ± 0.5	**4.7 ± 0.4***	
	Superior	**Pre**	0.9 ± 0.4	1.2 ± 0.4	.39
		Post	1.0 ± 0.5	1.6 ± 0.4	
	Inferior	**Pre**	5.1 ± 0.3	4.7 ± 0.4	.30
		Post	4.9 ± 0.5	5.4 ± 0.4	
Lissamine green conjunctival staining		**Pre**	2.0 ± 0.3	1.9 ± 0.3	.83
		Post	2.1 ± 0.3	1.9 ± 0.3	
Schirmer test (no anesthesia)		**Pre**	4.1 ± 0.7	4.1 ± 0.6	.90
		Post	5.2 ± 0.6	5.4 ± 0.7	

SIDEQ: Single-item score dry dye questionnaire; T-BUT: Tear break up time; SEM: standard error of the mean.
*$P \leq .05$: Comparison by Wilcoxon test before (pre) and after (post) 2-hour exposure.
**P value corresponding to comparisons between dry eye disease test scores obtained prior to undergoing both exposures.

Table 2. Tear molecule detection rates and concentrations in Sjögren syndrome-dry eye patients pre- and post-exposure (2 hours) to simulated environments. Only the 8 molecules that had detection rate ≥50% out of the 16 measured are shown.

Tear molecules	Exposure	Normal Condition		Adverse Condition		*P* value**
		Concentration (pg/ml) Mean ± SEM	Detection rate (%)	Concentration (pg/ml) Mean ± SEM	Detection rate (%)	
EGF	Pre	434.9 ± 157.6	78	300.2 ± 75.5	85	.48
	Post	459.7 ± 135.4	71	249.7 ± 77.0	64	
CX3CL1/ Fractalkine	Pre	1701.3 ± 493.1	85	1002.6 ± 225.2	93	.65
	Post	1631.0 ± 447.3	85	1673.2 ± 431.7	93	
IL-1RA	Pre	36395.4 ± 8949.9	93	16557.1 ± 4047.8	100	.01
	Post	39091.7 ± 8810.6	93	**31895.3 ± 5916.5***	100	
IL-6	Pre	157.9 ± 83.8	85	63.8 ± 20.2	57	.08
	Post	235.2 ± 92.5	71	**111.5 ± 29.6***	71	
CXCL8/IL-8	Pre	3567.4 ± 1340.4	93	2196.1 ± 737.9	100	.17
	Post	4400.7 ± 1490.5	93	**3753.2 ± 1106.0***	100	
CXCL10/ IP-10	Pre	51746.1 ± 9410.2	93	50737.1 ± 8831.1	100	.94
	Post	48376.9 ± 11280.1	93	57798.6 ± 7667.5	100	
VEGF	Pre	935.4 ± 299.4	57	677.5 ± 258.1	78	.35
	Post	1079.2 ± 246.0	93	951.5 ± 285.0	85	
MMP-9	Pre	48585.8 ± 21312.8	78	101515.6 ± 37088.4	85	.32
	Post	36423.2 ± 12829.0	78	**145867.1 ± 41651.5***	85	

EGF: epidermal growth factor; CX3CL1: chemokine (C-X3-C motif) ligand 1; IL-1RA: interleukin 1 Receptor antagonist; IL: interleukin; CXCL8: chemokine (C-X-C motif) ligand 8; CXCL10: chemokine (C-X-C motif) ligand 10; IP-10: Interferon γ-induced protein 10; VEGF: vascular endothelial growth factor; MMP-9: matrix metalloproteinase-9; SEM: standard error of the mean.

**P*≤ .05: Comparison by t-test before (pre) and after (post) 2-hour exposure to each environmental condition. ***P* value corresponding to comparisons by t-test between tear molecule concentrations obtained prior to undergoing both exposures.

Figure 1

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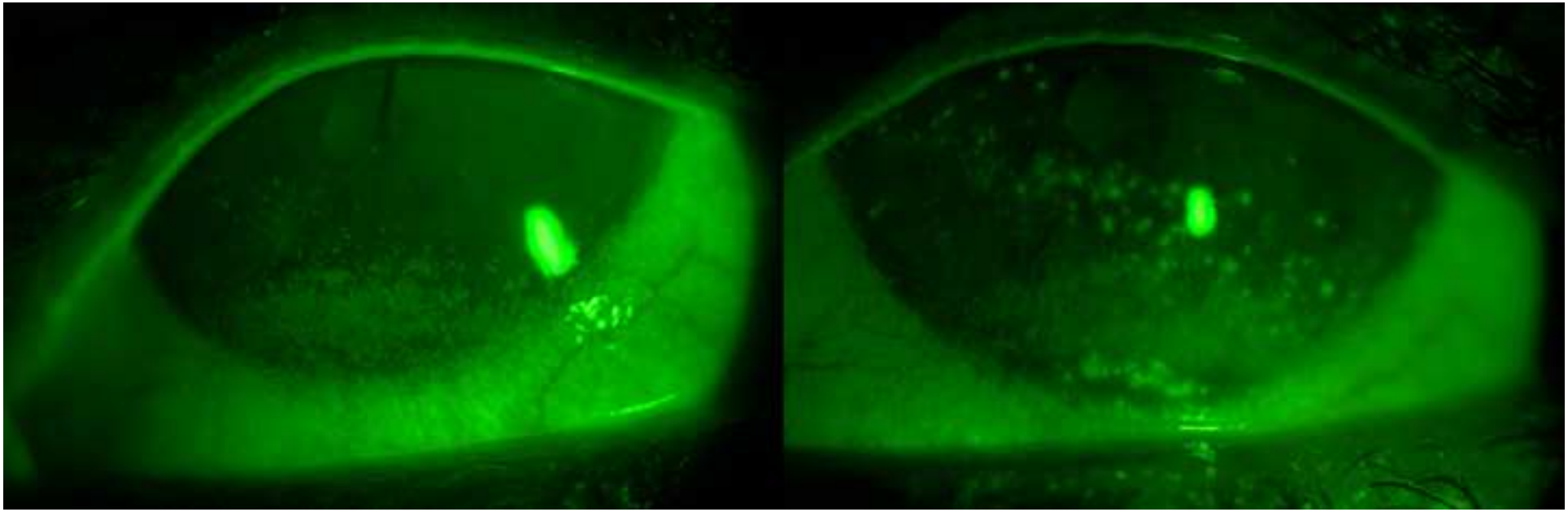

TABLE OF CONTENTS STATEMENT

TITLE:

Clinical and Molecular Inflammatory Response in Sjögren Syndrome-Associated Dry Eye Patients under Desiccating Stress

MANUSCRIPT NUMBER: AJO-15-870

Sjögren syndrome patients suffer from a chronic systemic autoimmune disease that causes dry eye disease. These patients usually experience exacerbations characterized by a reduction of the corneal integrity as well as an increase in some tear inflammatory mediators (interleukin-1 receptor antagonist, interleukin-6, interleukin-8, matrix metallopeptidase-9) when they are exposed to adverse conditions (desiccating stress). This study provides more evidence to target certain inflammatory molecules when designing new therapeutics and also shows that adverse environments can be tightly reproduced to overcome dry eye clinical trial shortcomings.

SUPPLEMENTAL TABLE

Table 1. Tear molecule detection rates and concentrations of molecules whose detection levels were below the limit in less than 50% of the total number of samples (n=14). Sjögren syndrome-dry eye patients were exposed for 2 hours to 2 simulated environments of 23ºC and 0.10 m/s air flow having different relative humidity: normal condition 45%, and adverse condition 5%.

Tear molecules	Exposure	Normal Condition		Adverse Condition	
		Concentration (pg/ml) Mean*	Detection rate N out of 14 (%)	Concentration (pg/ml) Mean*	Detection rate N out of 14 (%)
IFN-γ	Pre	31.2	4 (28)	105.0	1 (7)
	Post	70.9	3 (21)	-	0 (0)
IL -1β	Pre	30.7	2 (14)	31.7	3 (21)
	Post	42.8	6 (43)	19.4	6 (43)
IL -2	Pre	-	0 (0)	57.0	1 (7)
	Post	-	0 (0)	-	0 (0)
IL -10	Pre	53.2	2 (14)	38.4	2 (14)
	Post	152.0	2 (14)	17.0	2 (14)
IL -12p70	Pre	49.5	5 (36)	31.0	1 (7)
	Post	63.4	4 (28)	104.6	3 (21)
IL -17A	Pre	-	0 (0)	18.0	1 (7)
	Post	79.3	1 (7)	-	0 (0)
CCL5/RANTES	Pre	225.5	6 (43)	64.3	6 (43)
	Post	216.4	6 (43)	187.9	5 (36)
TNF-α	Pre	34.3	4 (28)	42.9	1 (7)
	Post	32.3	2 (14)	35.6	1 (7)

IFN-γ: interferon-γ, IL: interleukin; CCL5/RANTES: chemokine (C-C motif) ligand 5 (CCL5)/regulated on activation, normal T-cell expressed and secreted; TNF-α: tumor necrosis factor-α.

*Mean obtained after computing only the real values detected for each molecule. Minimum detectable values were not computed to obtain the mean value for each molecule.