Tear Inflammatory Cytokines Analysis and Clinical Correlations in Diabetes and Nondiabetes With Dry Eye

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• PURPOSE: To enhance the understanding of dry eye (DE) in diabetes by evaluating the ocular surface characteristics and the levels of tear inflammatory cytokines.
• DESIGN: Cross-sectional study.
• METHODS: Subjects were divided into 4 groups: 32 patients in the diabetes with DE group; 24 patients in the diabetes without DE group; 28 patients in the nondiabetes with DE group; and 29 volunteers in the normal group. Ocular surface disease index (OSDI) was self-answered and ocular surface characteristics including tear film break-up time (BUT), Schirmer I test, corneal fluorescein staining (CFS), and corneal sensitivity were evaluated. Concentrations of epidermal growth factor (EGF), IL-17A, IL-1β, and tumor necrosis factor alpha (TNF-α) were measured by mutiplex bead analysis. Spearman correlations between cytokines and ocular surface parameters were calculated.
• RESULTS: The level of EGF in tears significantly increased in the diabetes with DE group and positively correlated with the CFS and negatively correlated with the Schirmer I test in this group ($P < .05$). No differences were found in the levels of IL-17A, IL-1β, and TNF-α in the diabetes with DE and diabetes without DE groups compared to the normal group ($P > .05$). The nondiabetes with DE group showed increased levels of IL-17A, IL-1β, and TNF-α in tears compared to the normal group and the levels of IL-1β and TNF-α in tears positively correlated with CFS ($P < .05$).
• CONCLUSIONS: Our study showed that levels of EGF in tears have potential to be the diagnostic biomarker of DE in diabetes. No differences of IL-17A, IL-1β, and TNF-α in tears were found between the diabetes with DE and normal group, suggesting different pathogenesis of diabetes DE vs nondiabetes DE. (Am J Ophthalmol 2019;200:10–15. © 2018 Elsevier Inc. All rights reserved.)

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ACCORDING TO THE REPORT BY THE INTERNATIONAL Dry Eye Workshop (DEWS) in 2017, dry eye (DE) is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiologic roles.[1] Diabetes has been identified as a risk factor for DE. Studies have indicated 54% prevalence of asymptomatic and symptomatic DE in diabetes.[2,3] The awareness of DE in diabetes rose considerably around the world during these years.

Increasing evidence suggests that inflammation plays an important role in DE.[4] The pathogenesis of DE in diabetes is mainly thought to be the microvascular damage to the lacrimal gland and reduced reflex tearing owing to impairment of corneal sensitivity.[5] Many inflammatory mediators are involved in the pathogenesis of DE, including ubiquitous inflammatory cytokines, Th1- and Th17-related cytokines, chemokines, and their receptors. Among them, interleukin (IL)-1β is known to drive Th17 cell differentiation and their production of IL-17A and tumor necrosis factor (TNF)-α, which play key roles in the pathogenesis of DE.[6] Several studies, including ours, have shown that elevated levels of inflammatory cytokines such as IL-17A, IL-1β, and TNF-α were found in tears of DE patients and the severity of DE was correlated with these cytokines.[7,8] Epidermal growth factor (EGF), an indicator of lacrimal gland function, was detected decreased in tears of DE patients, especially those with Sjögren syndrome.[9] Considering the key role and practicability of evaluation, we chose EGF, IL-17A, IL-1β, and TNF-α to evaluate in this study; also, no documented studies have evaluated these cytokines in diabetes with DE. Besides, the ocular surface characteristics of diabetes with DE might be more complicated than the nondiabetic patients with DE owing to the diabetic neuropathy and impairment of corneal sensitivity.[10] To our knowledge, there are no available data in the literature about the comparison of ocular surface characteristics among diabetes with DE, diabetes without DE, nondiabetes with DE and normal subjects.

The results of the present study may enhance our understanding of ocular surface characteristics of diabetes with DE through comparison to the diabetes without DE, nondiabetes with DE, and normal subjects. In

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addition, analysis of EGF, IL-17A, IL-1β, and TNF-α in tears and their clinical correlation was performed to investigate potential biomarkers for diagnosis of DE in diabetes.

METHODS

THIS CROSS-SECTIONAL STUDY WAS CONDUCTED BETWEEN January 2018 and June 2018 at the Department of Ophthalmology, Peking University Third Hospital. The study was performed in accordance with the principles of the Declaration of Helsinki and study protocols were reviewed and approved by the Ethics Committee of Peking University Third Hospital. Informed consent was obtained from each participant enrolled in this study. The study was registered at ClinicalTrials.gov before initiation, under the registry number NCT03576300.

• **SUBJECTS:** Subjects were divided into 4 groups: diabetes with DE group; diabetes without DE group; nondiabetes with DE group; and normal group. Patients who had been previously diagnosed with type 2 diabetes by a physician were enrolled in the diabetes with and without DE groups. Fasting blood glucose was measured for the subjects who were not diagnosed with diabetes. The diagnostic criteria of DE used in this study was as follows: (1) Ocular Surface Disease Index (OSDI) questionnaire score ≥13; (2) break-up time (BUT) ≤10 s or Schirmer I test ≤5 mm; (3) positive corneal fluorescein staining (CFS).[11] Nondiabetes with DE was diagnosed when conditions 1, 2, and 3 were all met. As the diabetes with DE always appeared as asymptomatic, diabetes with DE was diagnosed when conditions 2 and 3 were met.[12]

Exclusion criteria were as follows: an infection or inflammatory disease not associated with DE; ocular surgical history; laser treatment in the last 3 months; ocular therapies other than artificial tears; contact lens wear; abnormalities in the cornea, conjunctiva, or eyelid; any other major systemic diseases affecting tear secretion, including autoimmune diseases such as Sjögren syndrome, psychiatric diseases, and malignant tumor; or Meibomian gland dysfunction (MGD) over grade 2 (the grade is according to the report of the International Workshop on MGD in 2011[13]).

• **OCULAR SURFACE EVALUATIONS:** All patients answered the OSDI questionnaire and ophthalmologic examinations were performed in both eyes in each subject. The data for the worse eye were taken for analysis. The BUT was calculated after placing a fluorescein strip into the lower conjunctival fornix and the average value of 3 measurements was recorded. The intensity of CFS was graded using the Baylor grading scheme and score ranged from1 to 15.[14] The Schirmer test was performed without anesthesia, and the length of wet filter paper (mm) was recorded. The central corneal mechanical sensitivity was evaluated by the Cochet-Bonnet esthesiometer (Luneau Ophtalmologie, Chartres, France) through light touching with a fine nylon monofilament.[15] The length of the nylon filament varied from 0 to 6 cm in 0.5-cm steps. Two positive responses in 3 attempts at each filament length were regarded as a positive result and the longest filament length resulting in a positive response was considered as the indicator of corneal sensitivity.

• **TEAR SAMPLE COLLECTION:** Tear collection was performed before any other test and with a maximum of 10 minutes. Tear samples were collected nontraumatically from the inferior tear meniscus of 2 eyes. Care was taken to avoid additional tear reflex as much as possible. Glass capillary micropipettes (Drummond Scientific, Broomall, Pennsylvania, USA) were used to collect 5 μL of tears. Tear samples from 2 eyes (5 μL total) were fully eluted into a sterile collection tube (Sigma-Aldrich, St. Louis, Missouri, USA). Tubes with tear samples were kept cold (4 C) during collection and sealed with a cap containing a rubber O-ring to prevent evaporation, then stored at -80 C until activity assays were performed.

• **MUTIPLEX BEAD ANALYSIS:** The levels of EGF, IL-17A, IL-1β, and TNF-α in tears of patients were measured using a Milliplex Map Kit (Human Th17 Magnetic Bead Panel, Millipore, Billerica, Massachusetts, USA). The reactions were detected with the Bio-Plex Luminex 200 XYP instrument (Bio-Rad Laboratories, Hercules, Califonia, USA). The samples were analyzed following the manufacturer's protocol. Briefly, samples were incubated under agitation overnight at 4 C with beads coated with antibodies specific for each molecule. After washing, the beads were incubated with biotinylated human antibodies for 1 hour, followed by incubation with streptavidin-phycoerythrin for 30 minutes. Standard curves were used to convert fluorescence units to concentration units (pg/mL). The minimum detectable concentrations (pg/mL) for molecules analyzed were as follows: 2.8 for EGF, 0.7 for IL-17A, 0.8 for IL-1β, and 0.8 for TNF-α.

• **STATISTICAL ANALYSIS:** Statistical analyses were performed using SPSS for Windows version 23.0 software (SPSS Inc, Chicago, Illinois, USA). For all measurements, results from each group were expressed as mean ± standard deviation. Differences of age among groups were tested using 1-way ANOVA test. Differences of sex among groups were tested using χ^2 test. Other data among groups were tested using Kruskal-Wallis 1-way ANOVA test. Correlations between the expressions of cytokines and ocular surface parameters (BUT, Schirmer I test, CFS, and corneal sensitivity) were analyzed by Spearman correlation coefficient, respectively. $P < .05$ is considered as statistically significant.

TABLE 1. Demographics and Characteristics of Study Subjects in Each Group

	Diabetes With DE	Diabetes Without DE	Nondiabetes With DE	Normal Group
Numbers of patients (N)	32	24	28	29
Sex (M/F)	14/18	7/17	8/20	5/24
Age (y)	61.8 ± 9.8	63.5 ± 10.1	63.5 ± 10.1	62.4 ± 7.5
Diabetes duration (y)	11.3 ± 7.1	12.3 ± 6.8	NA	NA
FBG (mg/dL)	8.8 ± 3.0	9.7 ± 4.3	NA	NA
HbA1c (%)	7.64 ± 1.49	7.99 ± 1.59	NA	NA
OSDI scores	18.5 ± 14.0[c,d]	13.2 ± 13.7[c]	33.9 ± 19.1[a,b,d]	10.0 ± 6.1[a,c]
BUT (S)	4.7 ± 1.7[b,d]	7.4 ± 4.2[a,c]	4.8 ± 2.8[b,d]	8.8 ± 2.2[a,b,c]
CFS scores	4.1 ± 3.5[b,d]	0.8 ± 0.9[a,c]	5.1 ± 3.7[b,d]	0.4 ± 0.7[a,c]
Schirmer I test (mm)	7.4 ± 6.9[d]	9.3 ± 7.5	8.4 ± 7.2[d]	10.1 ± 6.0[a,c]
Cochet-Bonnet score (cm)	5.5 ± 0.7	5.5 ± 0.8	5.6 ± 1.1	5.9 ± 0.2[a,b]

BUT = break-up time; CFS = cornea fluorescent staining; DE = dry eye; FBG = fasting blood glucose; HbA1c = hemoglobin A1C; OSDI = ocular surface disease index; NA = not available.

Kruskal-Wallis 1-way ANOVA test; all data are expressed as mean ± SD.

[a] $P < .05$ vs diabetic DE group.

[b] $P < .05$ vs diabetic without DE group.

[c] $P < .05$ vs nondiabetic without DE group.

[d] $P < .05$ vs normal group.

TABLE 2. Comparisons for the Concentrations of Epidermal Growth Factor, Interleukin-17, Interleukin-1β, and Tumor Necrosis Factor α in Tears Among 4 Groups

Tear Cytokines (pg/mL)	Diabetes With DE	Diabetes Without DE	Nondiabetes With DE	Normal Group
EGF	2345.7 ± 1294.4[c,d]	1960.0 ± 1238.0	1974.2 ± 1797.3[a]	1318.9 ± 835.0[a]
IL-17A	48.9 ± 46.7	41.5 ± 46.1	112.9 ± 185.7[b,d]	35.1 ± 29.1
IL-1β	26.7 ± 23.1	24.3 ± 27.9	48.1 ± 50.6[b,d]	21.1 ± 15.6
TNF-α	84.9 ± 63.9	70.8 ± 67.5	143.9 ± 136.9[b,d]	62.9 ± 45.6

DE = dry eye; EGF = epidermal growth factor; IL = interleukin; TNF = tumor necrosis factor.

Kruskal-Wallis 1-way ANOVA test; all data are expressed as mean ± SD.

[a] $P < .05$ vs diabetic DE group.

[b] $P < .05$ vs diabetic without DE group.

[c] $P < .05$ vs nondiabetic without DE group.

[d] $P < .05$ vs normal group.

RESULTS

• **DEMOGRAPHIC AND OCULAR SURFACE PARAMETERS:** A total of 113 subjects were enrolled in this study, with 32 patients in the diabetes with DE group, 24 in the diabetes without DE group, 28 in the nondiabetes with DE group, and 29 healthy volunteers in the normal group. The demographic data and results of ocular surface parameters are presented in Table 1. Age and sex of each group were matched ($P = .278$ and $P = .161$). No differences of diabetes duration, fasting blood glucose, and hemoglobin A1C were found in diabetes with DE and without DE groups ($P = .567$, $P = .390$ and $P = .501$). The comparison results showed that OSDI score of patients in the nondiabetes with DE group was higher than that in the other 3 groups ($P = .001$ vs diabetes with DE group, $P < .0001$ vs nondiabetes with DE group, $P < .0001$ vs normal group) and the OSDI score of patients in the diabetes with DE group was higher than normal group ($P = .033$). The diabetes with DE, diabetes without DE, and nondiabetes with DE groups showed lower BUT values compared to the normal group ($P < .0001$, $P = .019$, and $P < .0001$, respectively), and the BUT values in the diabetes with DE and nondiabetes with

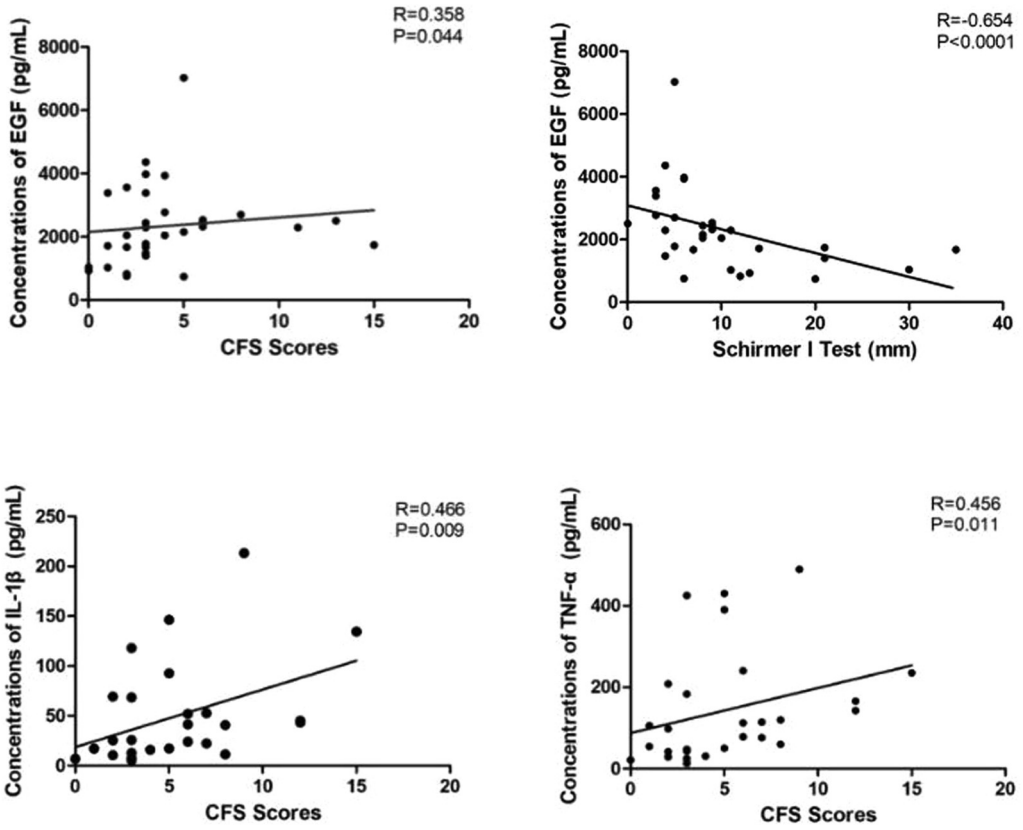

FIGURE. Correlation between levels of epidermal growth factor (EGF), interleukin (IL)-1β, and tumor necrosis factor (TNF)-α in tears and ocular surface parameters including corneal fluorescein staining (CFS) and Schirmer I test. The R and *P* values were determined with Spearman correlation coefficient.

DE groups were lower than diabetes without DE ($P = .006$ and $P = .012$). The CFS scores of patients in the diabetes with DE and nondiabetes with DE groups were higher than the diabetes without DE and normal groups (all $P < .0001$). The diabetes with DE and nondiabetes with DE groups showed lower Schirmer I test scores compared to the normal group ($P = .001$ and $P = .011$). Both the diabetes with DE and diabetes without DE groups showed lower corneal sensitivity compared to the normal group ($P = .007$ and $P = .042$).

• **TEAR CYTOKINE CONCENTRATIONS:** The concentrations of EGF, IL-17A, IL-1β, and TNF-α in tears of each group are showed in Table 2. The diabetes with DE group showed a significantly increased level of EGF in tears compared to the normal group ($P = .001$) and the nondiabetes with DE group showed a decreased level of EGF compared to the diabetes with DE group ($P = .045$). The diabetes without DE group showed significantly decreased levels of IL-17A, IL-1β, and TNF-α compared to the nondiabetes with DE group ($P = .013$, $P = .009$, and $P = .015$, respectively). No differences were found in the levels of IL-17A, IL-1β, and TNF-α in the diabetes with DE ($P = .369$, $P = .579$, and $P = .299$, respectively) and diabetes without DE groups ($P = .869$, $P = .583$, and $P = .944$, respectively) compared to the normal group. The nondiabetes with DE group showed significantly increased levels of IL-17A, IL-1β, and TNF-α in tears compared to the normal group ($P = .015$, $P = .031$, and $P = .013$, respectively).

• **CORRELATIONS BETWEEN CYTOKINES AND OCULAR SURFACE PARAMETERS:** The correlations between the levels of EGF, IL-17A, IL-1β, TNF-α, and ocular surface parameters including BUT, CFS, Schirmer I test, and corneal sensitivity were evaluated in the diabetes with DE and nondiabetes with DE groups. In the diabetes with DE group, the level of EGF positively correlated with the CFS score and negatively correlated with the Schirmer I test and the correlation analysis between the levels of EGF (Figure, Top left and Top right). IL-17A, IL-1β, and TNF-α and other ocular surface parameters showed no statistical significance. In the nondiabetes with DE group, the levels of IL-1β and TNF-α in tears were positively correlated well with CFS score (Figure, Bottom left and Bottom right). The correlation analysis between the levels of EGF, IL-17A, IL-1β, TNF-α, and any other ocular surface parameter showed no statistical significance.

DISCUSSION

CATARACT AND RETINOPATHY HAVE BEEN EXTENSIVELY studied in diabetes in the past. Recently, more and more researchers have focused on ocular surface complications in diabetes.[12,16] Tear film dysfunction, characterized by impairment in tear quantity and quality, can occur in association with abnormal corneal innervations in diabetes.[17] A study by Misra and associates had showed lower BUT and corneal sensitivity in diabetes compared to normal subjects.[18] Decreased Schirmer test and persistent epithelial defects were also found in diabetes.[5,19]

The results of our study are consistent with previous studies. In our study, both lower BUT and corneal sensitivity were found in the diabetes with DE and diabetes without DE groups compared to the normal group. While the corneal sensitivity in the nondiabetes with DE group showed no significant difference from the normal group, the CFS scores in the diabetes with DE and nondiabetes with DE groups are significantly higher than the normal group. The pathogenesis of DE in diabetes is unclear and possibly related to peripheral neuropathy, which is a well-known complication of diabetes. Studies had reported decreased densities of corneal subepithelial nerve plexus in diabetes, corresponding well with reduced corneal sensitivity.[20–22] As the corneal sensitivity is reduced, the tear secretion might decrease accordingly, owing to reduced stimulation from the corneal surface, which finally led to DE.[21] Although most studies reported reduced corneal sensitivity of DE patients,[23–25] no change or even an increased corneal sensitivity were reported in other studies.[14,26] This suggests that reduced corneal sensitivity is more common in diabetes with DE than in nondiabetes with DE. In addition, reduced corneal sensitivity contributed to the DE in diabetes and was secondary to the DE in nondiabetes.

Abundant evidence from animal studies and clinical evaluations has suggested that inflammation plays significant roles in the pathogenesis of DE.[4] Inflammatory cytokines such as EGF, IL-17A, IL-1β, and TNF-α are defined as potential biomarkers for DE.[27] The results of our study showed that elevated levels of IL-17A, IL-1β, and TNF-α in tears were found in nondiabetes with DE and correlated with OSDI score. Levels of IL-1β and TNF-α in tears were also correlated with CFS scores. No documented study had evaluated these mediators in tears of subjects with diabetes with DE. In our study, an elevated level of EGF was only found in the tears of subjects with diabetes with DE and the levels of EGF correlated with CFS score and Schirmer I test in these patients. EGF is constantly present in tears and the lacrimal gland is thought to be the main source of EGF.[28] In the acute phase of corneal wound diseases, including corneal erosion, keratitis, and corneal ulcers, the concentration of EGF was decreased, likely owing to prolonged reflex tearing despite an increased EGF synthesis in the lacrimal gland in response to the corneal disease. The decrease in EGF concentrations in tears would lead to an upregulation of EGF receptors. These EGF/EGF receptor complexes resulted in enhanced corneal wound healing.[28,29] However, our study showed an increased level of EGF in tears in the diabetes with DE group, which might be attributable to a decreased reflex tearing and suppression of the EGFR signaling pathway in wound healing in diabetes.[30] No differences of IL-17A, IL-1β, and TNF-α in tears were found between the diabetes with DE and normal group, suggesting different pathogenesis of diabetes DE vs nondiabetes DE.

In conclusion, our study showed that subjects with diabetes with DE, but not nondiabetes with DE, had a significantly reduced corneal sensitivity compared to the normal subjects. The level of EGF in tears correlated with the ocular surface parameters in diabetes with DE, which could potentially be a diagnostic biomarker for DE in diabetes.

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