Alterations of Tear Neuromediators in Dry Eye Disease

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Objectives: To evaluate tear levels of neuromediators in patients with dry eye disease and to identify statistical correlations with the clinical findings.

Methods: Nineteen patients with dry eye disease (Sjogren syndrome, n=5 patients; non–Sjogren syndrome, n=10; and ocular cicatricial pemphigoid, n=4) and 12 healthy volunteers were enrolled. The eyes of all participants were evaluated by slitlamp examination, Schirmer testing, fluorescein staining, and tear film break-up time. Grading of dry eye severity was recorded. Tear samples were collected, and substance P, calcitonin gene–related peptide (CGRP), neuropeptide Y (NPY), vasoactive intestinal peptide, and nerve growth factor (NGF) concentrations were evaluated by enzyme-linked immunoassay and correlated with the clinical findings.

Results: Nerve growth factor tear levels were significantly increased in participants with dry eye disease; CGRP and NPY concentrations were significantly decreased when compared with those in healthy participants. Dry eye severity showed a direct correlation with NGF and an inverse correlation with CGRP and NPY tear levels. Nerve growth factor tear levels showed a direct correlation with conjunctival hyperemia and fluorescein staining results, CGRP directly correlated with Schirmer test values, and NPY inversely correlated with tear film break-up time. Subgroup analysis showed that CGRP and NPY but not NGF were changed in autoimmune (ie, Sjogren syndrome and ocular cicatricial pemphigoid) dry eye disease.

Conclusions: The decreased tear levels of NPY and CGRP in dry eye disease are related to impaired lacrimal function, and tear levels of NGF are more closely related to corneal epithelial damage. Our findings suggest that NPY, CGRP, and NGF could become useful markers of dry eye severity.

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The ocular surface is extensively supplied by sensory and autonomic nerve fibers that play a crucial role in maintaining healthy epithelia and represent the main sources of neurogenic inflammation.1-3 Cross-communication between the nervous and immune systems is shown by the release and binding of common neuromediators and cytokines.4-6 Specifically, substance P (SP), calcitonin gene–related peptide (CGRP), vasoactive intestinal peptide (VIP), and neuropeptide Y (NPY) are released from ocular surface epithelial cells, lacrimal gland tissues, and nerve endings at inflammatory sites, modulating the infiltration and activation of the immune cells and triggering the reflex of tearing and ocular discomfort.7-8 Evidence suggests that neuromediators are involved in chronic ocular surface diseases such as dry eye. Dry eye is a complex disease characterized by changes in the ocular surface related to reduced quality and/or quantity of tears, inflammatory reaction, and impairment of ocular surface sensitivity.9,10 Lacrimal gland function is regulated by the nervous system, with autonomic and sensory nerves being capable of influencing acinar gland secretion and inflammatory reactions through the release of neuromediators.8,11,12 Substance P and CGRP promote local inflammation by inducing blood vessel dilatation, leukocyte extravasation, immune cell activation, and synthesis and release of several cytokines.8,13-15 Vasoactive intestinal peptide and NPY exert anti-inflammatory actions by inhibiting T-cell proliferation and helper T-cell type 1 response and modulating the release of cytokines, chemokines, and nitric oxide.16-20 This neuroimmune cross-communication is capable of modulating local inflammation by enhancing sensory nerve excitability and triggering the activation of different immune cell types. Changes in circulating levels of neuropeptides and neurotrophins, as well as the
impairment of salivary gland innervation in patients with Sjögren syndrome, have been hypothesized.21-24 These changes are hypothesized to be a cause of salivary fluid flow decrease during Sjögren syndrome. In fact, a remarkable discrepancy can be observed between the inflammatory involvement of the glands and the decrease in fluid flow. One possible explanation is that this autoimmune disease and/or involvement of the glands and the decrease in fluid flow. One possible explanation is that this autoimmune disease and/or local inflammation may cause vasoneural dysregulation of and injury to the peripheral nerves, leading to decreased fluid flow and atrophy of the acinar cells.25,26 Patients with autoimmune diseases are known to have a decrease in circulating levels of NPY, and the salivary glands of patients with Sjögren syndrome have reduced NPY-containing nerve terminals.27 Of interest, an increase in nerve growth factor (NGF) tear levels also has been reported in patients with dry eye disease due to Sjögren syndrome (n=5), non–Sjögren syndrome (n=10), or ocular cicatricial pemphigoid (n=4), with no history of other ocular diseases.

The diagnosis of dry eye disease was based on clinical history and the results of slitlamp examination, Schirmer testing, ocular surface fluorescein staining, and tear film break-up time. Conjunctival hyperemia was graded as follows: 0, absent; 1, mild; 2, moderate; and 3, severe. Corneal staining was recorded and graded according to the Oxford scheme. Dry eye severity grading from 1 to 4 was recorded according to the Dry Eye Workshop classification.20,21 Evaluation by an immunologist and biochemical investigations were performed to identify systemic autoimmune diseases.

Tear samples were collected without anesthetic from all participants using dry microsponges (Sharp-tip Microsponges; Alcon Laboratory Inc, Fort Worth, Texas) inserted simultaneously in the inferior conjunctival fornix of both eyes, removed after 60 seconds, and then immediately immersed in a 1.5-ml Eppendorf vial containing 50 µL of tissue protein extractor solution (T-PER; Thermo Scientific Pierce Protein Research Products, Rockford, Illinois) with 10 µg/mL of aprotinin and 1 mM of phenyl methyl sulfonyl fluoride. Microsponges were then centrifuged to dryness at 13 000 rpm for 3 minutes to recover tears. The amount of fluid recovered was determined, as previously described.21 Briefly, it was obtained by weighing the sponge and the tube after the absorption and centrifugation steps. The protein profile of the tears was then recorded (A280 program of a NanoDrop ND-1000 UV-Vis Spectrophotometer; NanoDrop Technologies Inc, Wilmington, Delaware).
NEUROPEPTIDES AND NGF DETERMINATION

Specific enzyme-linked immunoassay was performed on extracted proteins to quantify the amount of neuropeptides in tear samples, using specific commercially available kits and following the manufacturer’s instructions (Phoenix Pharmaceuticals Inc, Burlingame, California; detection limits: NPY, 0.15 pg/mL; SP, 0.07 ng/mL; CGRP, 0.28 ng/mL; and VIP, 0.12 ng/mL).

To evaluate NGF concentration in the tear samples, we performed a 2-site NGF enzyme-linked immunosassay (sensitivity, 0.5 pg/mL). In brief, 96-well Maxisorp enzyme-linked immunosassay plates were precoated with monoclonal anti-human βNGF antibodies (0.4 µg/mL; MAB256; R&D Systems Inc, Minneapolis, Minnesota). Standards (0.15 pg/mL to 1 ng/mL βNGF; Alomone Labs, Jerusalem, Israel) and samples were incubated at 4°C for 18 hours. Enzyme-linked immunosassay was developed by using polyclonal biotinylated anti-human NGF antibodies (0.15 µg/mL, 500-P85B; PeproTech, Milan, Italy), horseradish peroxidase streptavidin (R&D Systems) (1:300; DY998, R& D), and the ready-to-use 3,3',5,5'-tetramethylbenzidine substrate (Zymed Laboratories, San Francisco, California).

Optical density was measured at λ450 to λ530 by a microplate enzyme-linked immunosassay reader (Sunrise; Tecan Systems Inc, San Jose, California). All samples were evaluated in duplicate. Data are expressed as neuropeptide concentration per milliliter.

STATISTICAL ANALYSIS

Data are presented as mean (SD), median, minimum, and maximum values. The nonparametric Mann-Whitney test was used to compare tear levels of neuropeptides between patients with dry eye disease and healthy participants and between patients in different dry eye subgroups (Sjögren syndrome, non-Sjögren syndrome, and ocular cicatricial pemphigoid). Spearman ρ correlation analysis was performed to identify relationships between clinical variables and neuromediator tear levels (SPSS 15.0; SPSS Inc, Chicago, Illinois). P < .05 was considered statistically significant.

RESULTS

Nineteen patients with dry eye disease were included in the study. According to the etiology of the disease, patients were further divided: non–Sjögren syndrome (n = 10); Sjögren syndrome (n = 5), and ocular cicatricial pemphigoid (n = 4) (Table 1).

Table 2. Correlation of Neuropeptide Tear Levels With Clinical Variables

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SP</th>
<th>CGRP</th>
<th>NPY</th>
<th>VIP</th>
<th>NGF</th>
</tr>
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<tr>
<td>Conjunctival hyperemia</td>
<td>P = .29</td>
<td>P = .07</td>
<td>P = .54</td>
<td>P = .90</td>
<td>P = .01</td>
</tr>
<tr>
<td>Schirmer test</td>
<td>P = .97</td>
<td>P = .003</td>
<td>R = .574</td>
<td>P = .19</td>
<td>P = .61</td>
</tr>
<tr>
<td>BUT</td>
<td>P = .45</td>
<td>P = .20</td>
<td>P = .006</td>
<td>R = -.741</td>
<td>P = .73</td>
</tr>
<tr>
<td>Oxford score</td>
<td>P = .87</td>
<td>P &lt; .001</td>
<td>R = -.629</td>
<td>P = .005</td>
<td>R = -.526</td>
</tr>
<tr>
<td>Dry eye severity grade</td>
<td>P = .59</td>
<td>P &lt; .001</td>
<td>R = -.674</td>
<td>P = .049</td>
<td>R = -.515</td>
</tr>
</tbody>
</table>

Abbreviations: BUT, tear film break-up time; CGRP, calcitonin gene–related peptide; NGF, nerve growth factor; NPY, neuropeptide Y; SP, substance P; VIP, vasoactive intestinal peptide.

Compared with healthy participants, patients with dry eye disease showed a significant decrease in tear levels of NGF (mean [SD], 3.6 [2.3] ng/mL; median, 3.0 [range, 0.2-9.3] ng/mL vs mean, 6.0 [2.2] ng/mL; median, 5.8 [range, 2.5-10.5] ng/mL; P = .01) and NPY (mean, 3.1 [3.3] ng/mL; median, 1.6 (range, 0.2-9.6) ng/mL vs mean, 4.3 [1.9] ng/mL; median, 4.3 [range, 1.6-33.1] ng/mL; P = .011), but not of SP and VIP (Figure 1A). Figure 1B shows that NGF tear concentration was significantly increased in patients with dry eye disease (mean, 137.7 [79.4] pg/mL; median, 150 [range, 22-327] pg/mL vs mean, 64.7 [48.0] pg/mL; median, 64.7 [range, 7.3-135.2] pg/mL; P = .02).

Neuromediators tear levels showed significant correlations with clinical variables and dry eye severity grade. Dry eye severity grading was inversely correlated with CGRP and NPY (P < .001 and P = .049, respectively) and directly with NGF (P = .009) tear levels (Table 2).

Conjunctival hyperemia was directly correlated with NGF tear levels (P = .01); the Oxford score showed an inverse correlation with CGRP and NPY tear levels (P = .001 and P = .005, respectively) and a direct correlation with NGF tear concentration (P = .006). Schirmer test values showed a direct correlation with CGRP (P = .003), and tear film break-up time values were inversely correlated with NPY tear levels (P = .006).

Also, CGRP tear levels were significantly decreased in patients with dry eye disease who had severe reduction in Schirmer test values (mean, 2.2 [1.3] ng/mL) when compared with those of patients with moderate reduction (mean, 4.0 [1.7] ng/mL; P = .03) (Figure 2A). Neuropeptide Y tear levels were significantly increased in patients with dry eye disease who had lower tear film break-up time values (mean 7.6 [3.0] ng/mL) when compared with patients with moderate reduction (mean, 2.2 [2.4] ng/mL; P = .02) (Figure 2B).

Different neuromediator patterns were observed in the 3 dry eye disease subgroups when compared with the healthy participants (Table 3 and Figure 3). Specifically, in non–Sjögren syndrome, CGRP was significantly decreased (P < .001) and NGF was significantly increased (P = .002). In ocular cicatricial pemphigoid, CGRP was significantly decreased (P < .001). In Sjögren syndrome, only NPY was significantly decreased (P = .006).

These findings in neuromediator tear levels in the dry eye disease subgroups were not related to any difference in clinical severity; clinical signs (ie, conjunctival hyperemia and Oxford score) and tear film variables (ie,
Dry eye is a multifactorial disease characterized by decreased tear flow, conjunctival inflammation, impairment of corneal sensitivity, decrease in goblet cell density, epithelial metaplasia, and corneal damage, frequently leading to visual impairment. It is well known that normal tear flow is regulated by a fine balance between the parasympathetic, sympathetic, and sensory nerves. In fact, the autonomic and sensory nerves locally release several mediators that are able to influence acinar gland secretion as well as the inflammatory reaction at the lacrimal gland and the conjunctiva. The main causes of this neurogenic inflammation are considered to be SP and CGRP, both released by the sensory nerves. In this study, we demonstrated a significant decrease in CGRP in the tears of patients affected by dry eye; although not significant, a trend of decrease in SP also was observed in patients with dry eye. Also, in those patients, we observed a significant decrease in NPY and a significant increase in NGF; changes in VIP levels were not significant. These results suggest that at least CGRP, NPY, and NGF are involved in dry eye disease. However, because the source of these factors was not established in this study, it is unclear whether these alterations in the tears of patients with dry eye represent a pathogenic mechanism of the disease or whether they may result from changes in the ocular surface of dry eyes. We hope that these aspects will be investigated in studies aimed at other subgroups. (Table 4).

Table 3. Tear Neuromediator Levels in Dry Eye Disease Subgroups Compared With Healthy Participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>CGRP, ng/mL</th>
<th>NPY, ng/mL</th>
<th>NGF, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>6.0 (2.2)</td>
<td>4.3 (1.9)</td>
<td>64.7 (48.0)</td>
</tr>
<tr>
<td>Median (range)</td>
<td>5.8 (2.5-10.5)</td>
<td>4.3 (1.6-32.0)</td>
<td>64.7 (7.3-135.2)</td>
</tr>
<tr>
<td>Total dry eye</td>
<td>3.6 (2.3)</td>
<td>3.1 (3.3)</td>
<td>137.7 (79.4)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>3.0 (0.2-9.3)</td>
<td>1.6 (0.2-9.6)</td>
<td>150.0 (22.0-328.0)</td>
</tr>
<tr>
<td>SS (Sjögren syndrome)</td>
<td>6.0 (2.4)</td>
<td>1.5 (0.3)</td>
<td>54.5 (61.8)</td>
</tr>
<tr>
<td>Median (range)</td>
<td>6.3 (2.6-9.3)</td>
<td>1.4 (1.1-1.9)</td>
<td>28.9 (22.0-179.0)</td>
</tr>
<tr>
<td>Non-SS (sialadenitis)</td>
<td>3.0 (1.7)</td>
<td>1.7 (3.9)</td>
<td>185.5 (64.8)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>3.0 (0.2-6.3)</td>
<td>3.2 (0.6-9.6)</td>
<td>167.0 (118.4-328.0)</td>
</tr>
<tr>
<td>OCP</td>
<td>2.3 (1.2)</td>
<td>1.5 (2.0)</td>
<td>120.8 (53.3)</td>
</tr>
<tr>
<td>Median (range)</td>
<td>2.3 (0.6-4.4)</td>
<td>0.5 (0.2-3.9)</td>
<td>144.2 (39.9-169.6)</td>
</tr>
</tbody>
</table>

Abbreviations: CGRP, calcitonin gene–related peptide; NGF, nerve growth factor; NPY, neuropeptide Y; OCP, ocular cicatricial pemphigoid.

Schirmer test and break-up time values) were not significantly different between subgroups (Figure 2).

Figure 2. Tear levels as evaluated by clinical tests. A, Calcitonin gene–related peptide (CGRP) tear levels are significantly lower in patients with severe aqueous deficiency (ie, Schirmer test values <5 mm/5 min). B, Neuropeptide Y (NPY) tear levels are significantly higher in patients with severe evaporative dry eye disease (ie, tear film break-up time [BUT], <4 s).

Figure 3. Changes in neuromediator tear levels in Sjögren syndrome (SS), non-SS, and ocular cicatricial pemphigoid (OCP). A, Calcitonin gene–related peptide (CGRP) was significantly decreased (P<.001) in non-SS and in OCP (P<.001). B, Neuropeptide Y (NPY) was significantly decreased in SS (P=.006). C, Nerve growth factor (NGF) was increased in patients without SS (P=.002). *Indicates a statistically significant difference.
addressing the clinical implications of our results; however, several hypotheses may be created based on our results. The decreased levels of CGRP and NPY may be related to changes in lacrimal gland function. In fact, both of these neuromediators are present in the lacrimal gland, and changes in their concentration have been demonstrated in the salivary glands of patients with Sjögren syndrome. A decrease in the circulating levels of NPY also has been described in patients affected by autoimmune diseases, which is in line with the anti-inflammatory role of this neuropeptide. In fact, NPY treatment is known to inhibit experimental autoimmune encephalomyelitis, a model of helper T-cell type 1–driven disease, by inhibiting helper T-cell type 1–driven inflammatory responses.

The explanation of the decrease in CGRP in dry eye disease is more complex. Apparently in contrast with our results, this neuropeptide is considered to be a major source of neurogenic inflammation. Specifically, CGRP is known to dilate blood vessels, to stimulate leukocyte extravasation, and to induce the synthesis of several cytokines (eg, interleukin 8) by corneal epithelial cells. However, recent evidence also suggests a more complex role of CGRP in modulating inflammatory reactions: in the epidermis, release of CGRP from sensory nerves can inhibit antigen presentation by Langerhan cells and impair contact hypersensitivity by triggering mast cell release of tumor necrosis factor. Moreover, to our knowledge, no published evidence exists of a well-defined proinflammatory or anti-inflammatory role of CGRP at the ocular surface; the decrease in ocular surface sensitivity demonstrated in patients with dry eye may be the origin of the decreased tear concentration of this sensory neuropeptide. One could also speculate that the decrease in CGRP in the tears partly may be responsible for reduced mucin secretion by goblet cells, which is a characteristic of dry eye disease.

Of interest, CGRP and NPY tear levels showed opposite changes compared with NGF; our results suggest that these neuromediators play a different role in the pathophysiologic mechanisms of dry eye disease. Our data showing an increase in NGF tear levels in patients with dry eye are in line with the well-known neural sensitizing role of this neuromediator and with the observations published by Lee et al. However, our results also showed a direct correlation between NGF tear levels and the severity of corneal damage (as assessed by the Oxford test), as well as with conjunctival hyperemia. These observations suggest that NGF may be involved more with local tissue damage than with the disease pathogenesis. To support this hypothesis, when splitting our results in the different dry eye disease subgroups, NGF tear levels are increased only in patients with non–Sjögren syndrome dry eye. Moreover, it has been widely demonstrated that NGF exerts a potent action on corneal healing in physiologic and pathologic conditions, and it has been shown in several animal models that corneal injuries induce an increase in local NGF and NGF receptors capable of stimulating epithelial healing. However, the increase in NGF tear levels that we observed in dry eye also could be the result of an increased release by the lacrimal gland because, in animals and humans, lacrimal glands are known to produce, release, and be responsive to NGF.

The findings of this study provide evidence of an imbalance of neuromediators in the tear film of patients with dry eye disease. Although further studies will be needed to demonstrate the source of each altered neuromediator in patients with dry eye, it is possible to speculate that changes in neuromediators’ tear levels may contribute to the disease pathogenesis rather than being a mere epiphenomenon. In fact, locally released neuropeptides are crucial to conserving the homeostasis of the ocular surface by acting on the epithelia, blood vessels, and immune cells infiltrating the conjunctiva, as well as by modulating inflammatory responses, epithelial cell differentiation and proliferation, and conjunctival mucin secretion. Although several studies highlighted the crucial role of neuropeptides on lacrimal gland inflammation in dry eye, only NGF was shown to be increased in the tears of patients with the disease. Our data suggest that this increase in NGF probably is not related to the pathogenesis of dry eye but is the result of ocular surface damage. Moreover, our study shows that NPY and CGRP tear levels correlate with dry eye severity, opening venues to further investigation regarding their potential use as therapeutic targets and diagnostic markers.

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Table 4. Clinical Characteristics of Dry Eye Disease Subgroups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Conjunctival Hyperemia</th>
<th>Schirmer Test</th>
<th>BUT</th>
<th>Oxford Score</th>
<th>Dry Eye Severity Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sjögren syndrome (n = 5)</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>1.0 (0.9)</td>
<td>5.6 (3.6)</td>
<td>4.3 (0.5)</td>
<td>2.1 (1.8)</td>
<td>2.2 (1.2)</td>
</tr>
<tr>
<td>Median (range)</td>
<td>1.0 (0-2.0)</td>
<td>5.0 (2.0-10.0)</td>
<td>4.0 (4.0-5.0)</td>
<td>3.0 (0-4.0)</td>
<td>2.0 (1-4.0)</td>
</tr>
<tr>
<td>Non-Sjögren syndrome (n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>1.0 (0.5)</td>
<td>3.9 (2.0)</td>
<td>3.7 (0.6)</td>
<td>2.8 (1.0)</td>
<td>3.0 (1.0)</td>
</tr>
<tr>
<td>Median (range)</td>
<td>1.0 (0-2.0)</td>
<td>3.5 (1.0-7.0)</td>
<td>4.0 (3.0-5.0)</td>
<td>3.0 (2.0-5.0)</td>
<td>3.0 (1-4.0)</td>
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<tr>
<td>Ocular cicatricial pemphigoid (n = 4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>0.9 (0.7)</td>
<td>4.5 (3.3)</td>
<td>3.3 (1.7)</td>
<td>2.3 (1.5)</td>
<td>2.5 (1.2)</td>
</tr>
<tr>
<td>Median (range)</td>
<td>1.0 (0-1.0)</td>
<td>3.5 (0-13.0)</td>
<td>2.5 (0-5.0)</td>
<td>2.5 (1-3.0)</td>
<td>1.0 (1-0.3)</td>
</tr>
</tbody>
</table>

Abbreviation: BUT, tear film break-up time.
REFERENCES


