Final report on OTKA NN106649 research grant entitled „Neurophysiopathological basis of dry eye: ion channels involved in altered nerve activity and abnormal ocular sensations”

The objective of this project was to define the neurophysiopathological basis of dry eye, by determining the contribution of the different functional types of sensory nerve endings at the ocular surface and the alterations in their responsiveness during ocular dryness.

We have shown recently that activity of ocular cold sensitive receptors expressing the cold-sensing channel TRPM8 is responsible for cold and freshness sensations and is also necessary to maintain normal basal tearing in young individuals. Thus, it is possible that such cold receptors contribute to dryness sensations in dry eye. Experiments from our research group have shown that 4 weeks after removal of the main lacrimal gland in the guinea pig (lacrimodeficient dry eye), ocular sensory nerves show an altered response to natural stimuli that may constitute the neurophysiological correlate of the abnormal dryness sensations. Preliminary results suggested that several membrane ion channels (TRPA1, TRPV1 and TRPM8, transduction channels; TTX-R Na^+ channels associated to nerve impulse generation and neuronal excitability) may be involved in this altered responsiveness of ocular sensory receptors.

The present project intended:

- to complete the study of the characteristics of the response of the different functional types of ocular sensory receptors in experimental eye dryness conditions;
- to define what ionic channels participate in the changes of sensory nerve activity and ocular sensations in the dry eye inflamed cornea, using ion channel agonists and antagonists, animals with genetic deletion of TRP channels and dry eye patients;
- to investigate whether the reduction of basal tearing rate associated with aging, that is normally accompanied by discomfort and dryness sensations, is due to a

reduction of the density of cold thermoreceptive nerves and/or disturbances of their activity.

From those results we expect to increase the knowledge on neural disturbances in dry eye, and to contribute to the development of specific and effective drugs that interfere directly on ion channels activity related with the genesis of aberrant sensations experienced in dry eye. Combined, the results obtained will help to identify the neurobiological mechanisms that underlie the development of abnormal ocular sensations in dry eye, particularly in elderly people, a relevant socio-sanitary situation due mainly to the increased life expectancy in developed countries.

Methods and Results

We used behavioral and electrophysiological recording of nerve activity in animal models of dry eye and psychophysical analysis of ocular sensations in patients.

Animal experiments

In the experimental animal model of dry eye developed by our research group we found previously, that chronic tear deficiency alters the activity of corneal sensory nerve fibers, leading to the development of increased spontaneous activity and abnormal responsiveness to natural stimulation.

In this set of animal experiments we measured the participation of sodium and potassium channels in in the changes of sensory nerve activity in our experimental animal model of dry eye. Moreover, we evaluated the effect of sodium channel blockers (lacosamide and lidocaine), as well as TRPM8 (menthol) and TRPV1 (capsaicin) agonists on the abnormal activity of cold receptor sin our dry eye animal model. The purpose of these studies was to identify possible molecules, that can block action potential propagation from hyperexcitable nerve endings (lacosamide), or can modify the sensitivity of cold receptors (menthol and capsaicin).

In our experimental animal model of dry eye, electrical activity of corneal cold thermoreceptors was recorded in tear-deficient guinea pigs according to the following steps:

- Surgical removal of the main lachrymal gland was performed in anesthetized guinea-pigs.
- At 4-8 weeks after surgery, animals were killed with an overdose of anesthetics and both corneas were immediately excised and mounted in a recording chamber continuously perfused with physiological solution maintained at 34ºC with a home-made Peltier device.
- Nerve terminal impulse (NTI) activity from cold nerve terminals (CNT) was recorded from excised corneas using glass electrodes (≈50µM) applied to the corneal surface with slight suction (Figure 1).
- Cooling or heating ramps were made changing the temperature of perfusion solution.
- In a set of experiments sodium channel blockers (lacosamide, lidocaine) were added to the perfusion solution.
- In a set of experiments, TRP agonists (menthol, capsaicin) were added to the perfusion solution.

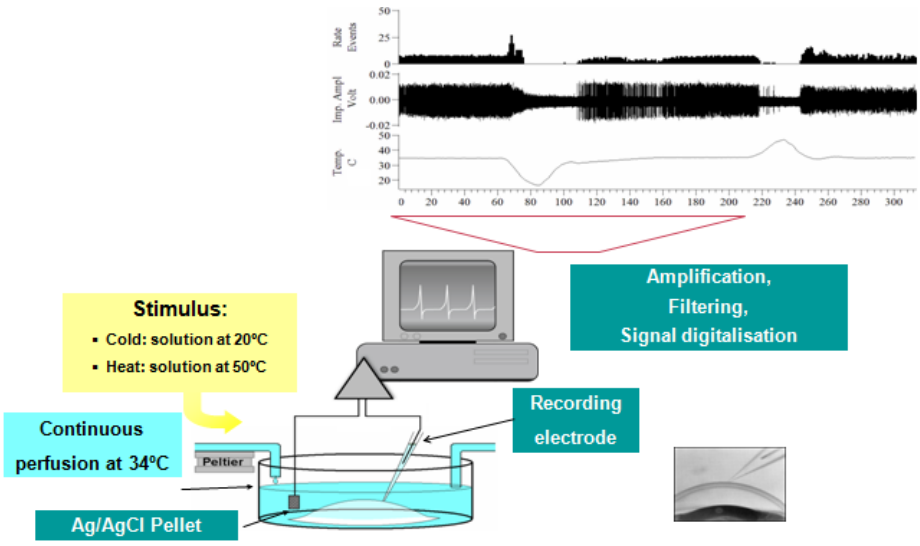


Figure 1. *Schematic representation of the experimental set-up and recordings.*

1. Evaluating the role of ion channels in the changes of sensory nerve activity and ocular sensations in dry eye

Methods

The detailed description of patch-clamp recordings can be found in the reference (Kovacs et al., Pain 2016)

Results

The changes in excitability observed in corneal cold terminals exposed to reduced ocular surface wetness are suggestive of alterations in sodium currents (I_{Na}), similar to those that take place after injury of peripheral sensory nerves. To explore this possibility, we recorded the TTX-r and TTX-s I_{Na} in retrogradely labeled corneal TG neurons from intact (n=8) and operated guinea pigs (n=8) (Fig. 7A). Based on their positive response to 100 mM menthol and their insensitivity to 1 mM capsaicin and 100 mM AITC, 7 of 25 neurons from control animals and 7 of 26 from operated animals were classified as cold-sensitive. Despite the apparently higher current density in neurons of tear-deficient animals, TTX-r current was not significantly different from control guinea pigs (P=0.16, t-test). However, conductance–voltage relationship for TTX-r I_{Na} activation in neurons of operated eyes seemed to have shifted significantly towards more negative voltages without modification in the slope factor (Figs. 7B and C and Table 4). Interestingly, TTX-s I_{Na} current amplitude in coldsensitive corneal neurons was significantly larger at 240 and 230 mV in neurons from the operated animals (Fig. 7D). The mid point of activation was significantly shifted towards more hyperpolarized voltages in tear-deficient animals (Fig. 7E), which implies that TTX-s I_{Na} was activated at more negative membrane potentials. No significant differences were observed between the slope factors of the activation curves (Table 4). From these data, we concluded that the disturbed sodium currents found in the soma of corneal cold neurons of operated animals could be one of the causes of the increased excitability of peripheral terminations in these neurons in tear-deficient eyes. Indeed, hainantoxin-IV, a neurotoxin that selectively inhibits TTX-s Na^+ channels did reduce at 100 nM the spontaneous and coldevoked activity of cold nerve terminals fromboth tear-deficient

and control corneas (Fig. 8), which supports the hypothesis that TTX-s currents are stronger in corneal nerve terminals subjected to chronic dryness, thereby contributing to their enhanced excitability.

We examined the calciumindependent K^+ currents in corneal cold sensory neurons from control and tear-deficient animals (n=7 and 8, respectively). Rapidly inactivating ($K_{A,fast}$) and slowly inactivating ($K_{A,slow}$) K^+ currents were significantly weaker in corneal cold neurons of teardeficient eyes than in those of control eyes, and current–voltage relationships were significantly different for both currents (Figs. 9A and B and Table 5), whereas the noninactivating K^+ currents (K_{dr}, delayed rectifier) were similar in both groups (Fig. 9C). The activation and voltage dependency of the 3 kinetically separate current components were not significantly different between the control and tear-deficient eyes. By contrast, a significant difference was observed between corneal cold neurons of control and teardeficient eyes in terms of the voltage-dependence of inactivation for $K_{A,fast}$ but not for $K_{A,slow}$ (Table 5 and Fig. 10). Collectively, our electrophysiological analysis of corneal cold neurons from intact and tear-deficient animals revealed that the enhancement of sodium currents was accompanied by an impairment of K^+ currents in animals with dry eye, in particular the rapidly and slowly inactivating K^+ currents whereas coldactivated current remained essentially unaffected. These disturbances are likely to at least partially underlie the enhanced excitability exhibited by corneal cold sensory fibers in teardeficient eyes.

We also analyzed the changes in Na^+ and K^+ currents observed in retrogradely labeled corneal TG neurons unresponsive to menthol, but activated by capsaicin and in most cases also by AITC, which were classified as corneal polymodal nociceptor neurons. Four weeks after lachrymal gland removal, TTX-r I_{Na} currents in polymodal nociceptor neurons innervating tear-deficient eyes were not significantly different ($P=0.501$; Fig 11A, Table 4) from those of intact animals. In contrast, a significant increase in TTX-s Na^+ currents ($P<0.001$; 2-way ANOVA) was observed in such polymodal neurons, where significantly larger currents were activated at 230 and 220 mV (n=6) when compared with those found in intact eyes (n=8; Fig. 11B).

Despite a slight leftward shift in the conductance–voltage relationship for TTX-s I_{Na} in neurons of tear-deficient eyes, differences did not reach statistical significance; the same wastrue for the slope factor (Table 4). Likewise, rapidly inactivating, slowly inactivating, and noninactivating K^+ currents were similar in operated (n=15) and control animals

(n=12; data not shown). Finally, we identified a population of retrogradely labeled corneal TG neurons that did not respond to any of the compounds tested (menthol, AITC, or capsaicin) but where depolarized by extracellular K^+ application. By exclusion, we considered these to be corneal mechanonociceptor neurons. The characteristics of Na^+ and K^+ currents in this subgroup of neurons from control and from tear-deficient animals were not statistically different (data not shown).

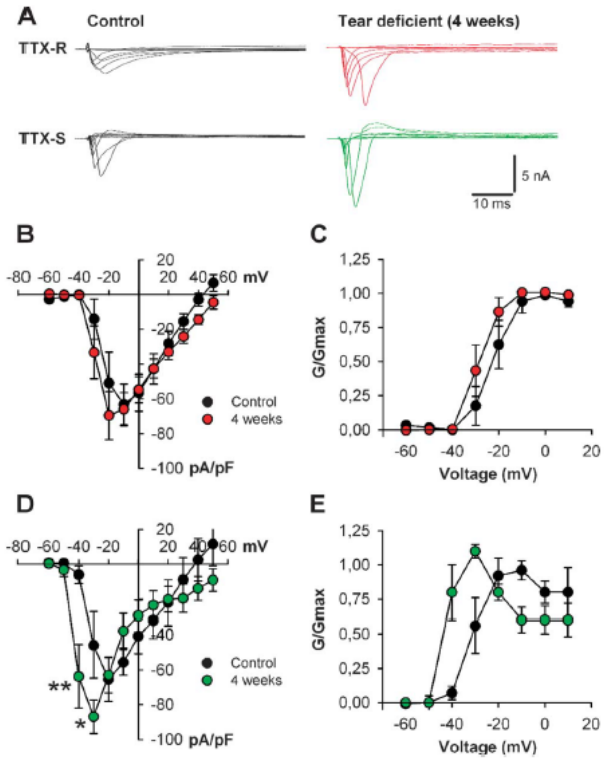


Figure 7. Voltage-gated Na^+ currents in trigeminal corneal cold sensory neurons of control and tear-deficient guinea pigs at 4 weeks after lachrymal gland removal. Corneal neurons were retrogradely labeled with FM 1-43 applied on the cornea 6 days earlier. (A) For each neuron, membrane potential was held at −80 mV; whole-cell sodium currents were evoked after a 500-millisecond prepulse to either −120 or −40 mV with a 100-millisecond step to potentials between −60 and +50 mV in 10 mV increments; current evoked from −40 mV was considered to be TTX-r current, whereas the difference between the current evoked from −120 and −40 mV was considered to be TTX-s; for clarity, only traces from −60 to +20 mV are shown. (B and C) Mean voltage–current relationships and mean relative peak conductance normalized to the maximal conductance (G/G_{max}) and plotted against voltage of TTX-r sodium currents. (D and E) Mean voltage–current relationships and mean relative peak conductance normalized to the maximal conductance (G/G_{max}) and plotted against voltage of TTX-s sodium currents. Data are mean ± SEM; $^{**}P < 0.01$; $^{*}P < 0.05$, *t* test.

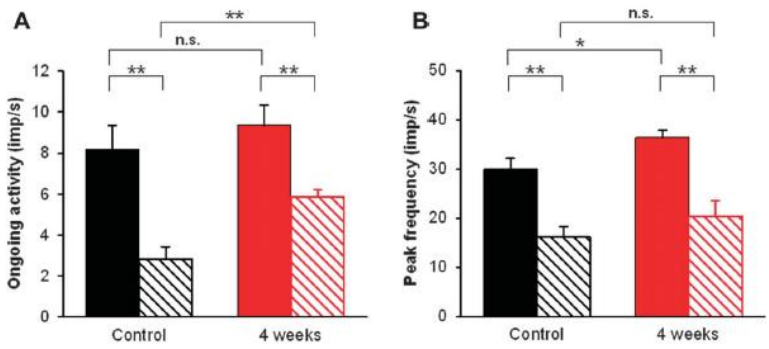


Figure 8. Effect of 100 nM hainantoxin-IV on the spontaneous and cold-evoked activity of corneal cold nerve terminals in control and tear-deficient corneas. (A) Effect of hainantoxin on the ongoing nerve terminal impulse activity of cold nerve terminals at basal temperature in control and tear-deficient corneas. (B) Effect of hainantoxin on the peak frequency of the discharge evoked by a cooling ramp in cold nerve terminals of control and tear-deficient corneas. Data are mean ± SEM, n = 6 in control and n = 4 in tear-deficient corneas; $^{*}P < 0.05$, $^{**}P < 0.001$, paired or unpaired *t* test, as needed.

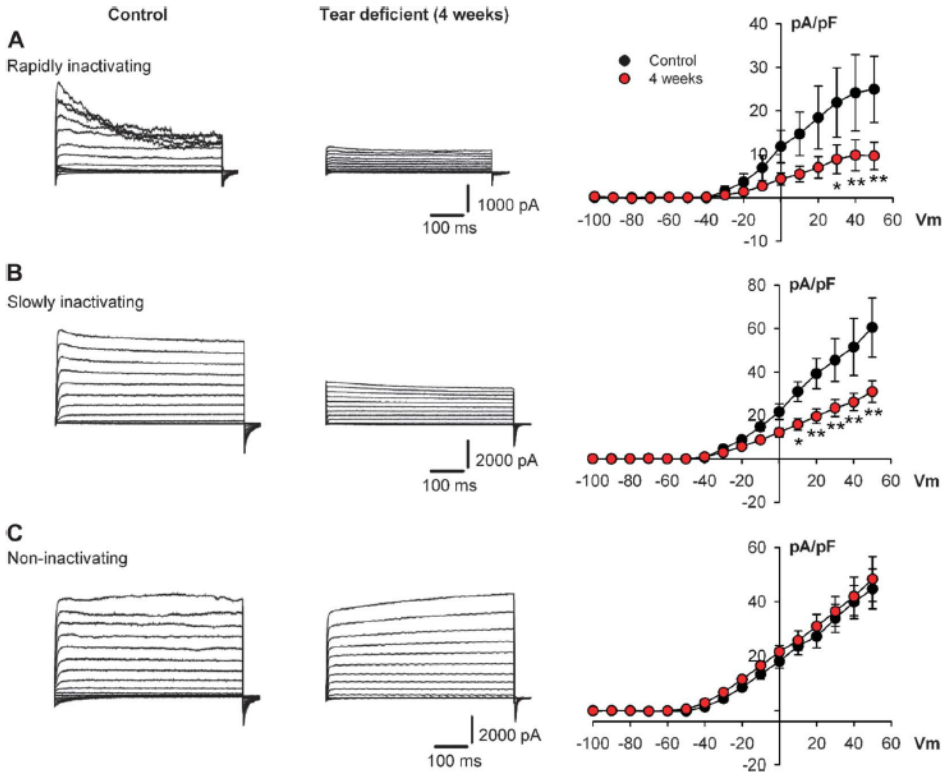


Figure 9. Voltage-gated K^+ currents (rapidly inactivating, slowly inactivating, and noninactivating currents) in control and tear-deficient trigeminal cold-sensitive neurons innervating the cornea. (A) Rapidly inactivating $K_{A,fast}$ currents obtained by subtraction of protocol 2 from protocol 1. Protocol 1: depolarizing voltage steps from −100 to +50 mV (500 milliseconds) made from −100 mV. Protocol 2: similar to protocol 1 but including a short depolarizing prepulse at −10 mV, 60 milliseconds to inactivate the rapidly inactivating component (see Methods). (B) Slowly inactivating $K_{A,slow}$ currents obtained by subtraction of currents elicited by protocol 3 from those obtained by protocol 2. In protocol 3, the same voltage steps were recorded but after a 7-second conditioning step to −10 mV to inactivate both the rapidly and slowly inactivating currents, leaving only the noninactivating and leak components. (C) Noninactivating currents obtained with protocol 3 in which the leak component was removed with a p/8 leak subtraction protocol. On the right side of the figure, the mean voltage–current relationships for the 3 types of K^+ currents (n = 5 for each group) are shown. Rapidly and slowly inactivating V–I curves showed statistically significant differences between control and tear-deficient neurons (repeated-measures analysis of variance, $P < 0.001$; post hoc comparison using a *t* test with Bonferroni corrections; $^{**}P < 0.001$, $^{*}P < 0.05$). Noninactivating currents did not show significant differences between groups.

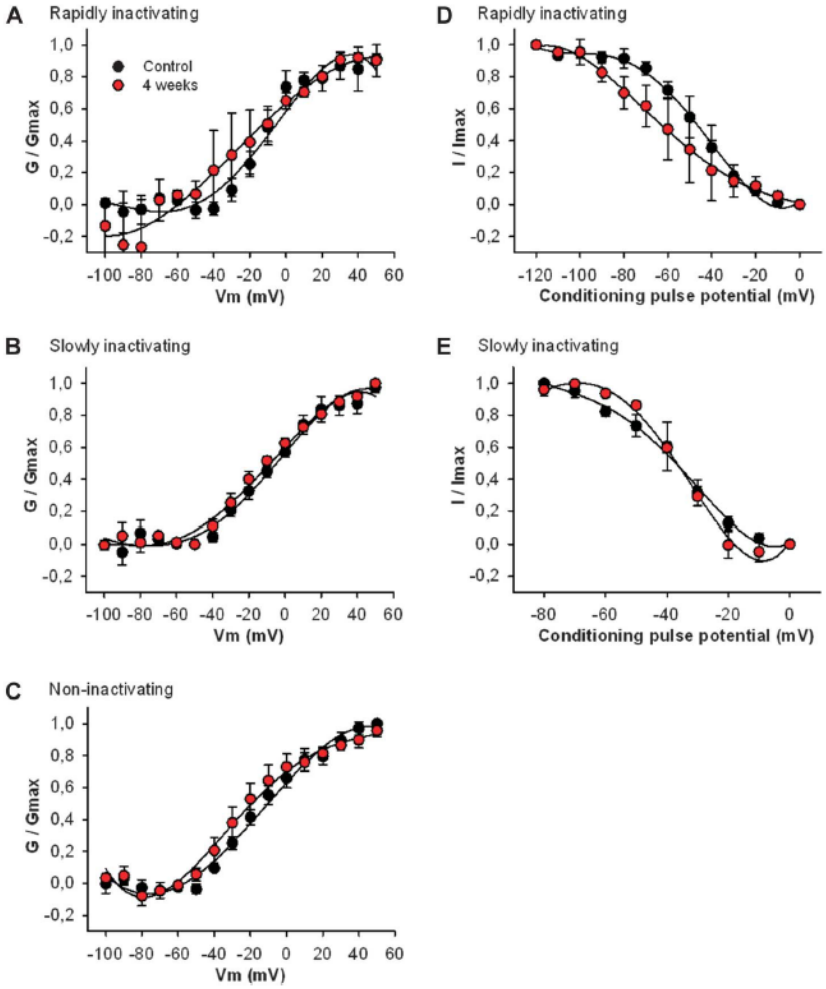


Figure 10. Activation and inactivation curves of voltage-gated K^+ currents in corneal cold sensory neurons from control and 4-week tear-deficient guinea pigs. Activation curves were generated by voltage pulses in 10-mV steps from −100 to +50 mV. Activation voltage dependency was studied by plotting normalized conductance (G/G_{max}) against test pulse voltage, and the data were fitted using a Boltzmann function. (A) Activation curves for rapidly inactivating K^+ current in cold sensory neurons from control and tear-deficient animals (n = 5 neurons in each group). $V_{0.5}$: control −12.5 ± 2.0 mV; tear-deficient −14.5 ± 7.6 mV. Slope factor: control 7.9 ± 1.7; tear-deficient 15.9 ± 6.0. No significant differences were observed. (B) Activation curves for slowly inactivating K^+ current (n = 5 neurons each) $V_{0.5}$: control −8.6 ± 2.4 mV; tear-deficient −10.6 ± 2.1 mV. Slope factor: control 15.1 ± 2.2; tear-deficient 17.2 ± 2.0. (C) Activation curves for noninactivating K^+ current (n = 5 neurons in each group), $V_{0.5}$: control −12.7 ± 1.5 mV; tear-deficient −20.1 ± 2.5 mV. Slope factor: control 16.8 ± 1.5; tear-deficient 20.2 ± 2.5. (D) Inactivation curves for rapidly inactivating K^+ current. Inactivation curves were constructed using a 2-pulse protocol, a 1-second prepulse varying between −120 and 0 mV, followed by a 400-millisecond test pulse of +50 mV. The peak of the transient component was measured. Tear-deficient neurons showed a significant leftward shift in the inactivation curve of $K_{A,fast}$ ($P < 0.05$, 1-way repeated-measures analysis of variance). $V_{0.5}$: control −45.9 ± 2.5 mV, tear-deficient −64.2 ± 7.7 mV. Slope factor: control −12.3 ± 2.2, tear-deficient −20.5 ± 9.0. (E) Inactivation curves for slowly inactivating $K_{A,slow}$ were constructed using a 2-pulse protocol: an 8-second prepulse varying between −80 and 0 mV followed by a 1-second test pulse of +50 mV. No significant effects were observed between groups.

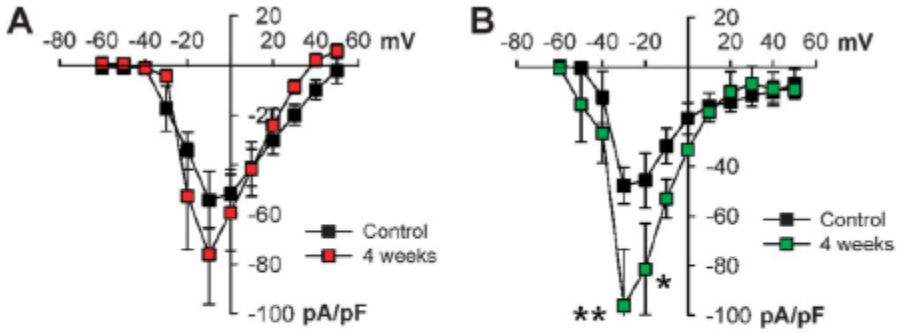


Figure 11. Voltage-gated Na^+ currents in trigeminal polymodal sensory neurons of control and tear-deficient guinea pigs 4 weeks after lachrymal gland removal. Corneal neurons were retrogradely labeled with FM 1-43 applied on the cornea 6 days earlier. Mean voltage–current relationships for TTX-r (A) and TTX-s (B) sodium currents. Data are mean ± SEM; $^{**}P < 0.01$; $^{*}P < 0.05$, *t* test.

Reference: Kovács I, Luna C, Quirce S, Mizerska K, Callejo G, Riestra A, Fernandez-Sanchez L, Meseguer VM, Cuenca N, Merayo-Lloves J, Gasull X, Acosta MC, Belmonte C, Gallar J. Abnormal activity of corneal cold thermoreceptors underlies the unpleasant sensations in dry eye disease. *Pain.* 2016 Feb;157(2):399-417.

2. The effect of lacosamide on dryness-induced hyperexcitability of corneal cold sensitive nerve terminals.

Lacosamide is ananticonvulsant agent, currently used as an antiepileptic drug for adjunctive therapy of partial onset seizures. This drug is believed to enhance the slow inactivation of voltage-gated sodium channels without affecting their fast inactivation, thereby reducing channel opening probability during the action potential. This results in stabilization of hyperexcitable neuronal membrane and inhibition of neuronal firing. Based on these properties, lacosamide has been used also to selectively block the abnormal activity of nociceptive neurons in chronic neuropathies. Lacosamide has shown efficacy in several animal models of neuropathic pain and also in clinical studies on neuropathic pain in diabetes, but its effect on the abnormal impulse activity in corneal nerve fibers, likely associated to unpleasant dryness sensations in DED has not been tested. The purpose of this study was to evaluate the effect of lacosamide on the enhanced activity of corneal cold nerve terminals in an animal model of tear-deficient dryeye. Preliminary results have been presented in abstract form (Kovacs et al.,2013).

Results

In this study, confirming previous reports, corneal cold endings from tear-deficient animals showed a higher ongoing firing frequency and a comparatively larger response to cooling pulses, in comparison with the cold nerve endings of intact corneas. They also showed a significantly lower cooling threshold. Perfusion of tear-deficent corneas with 100 mM lacosamide resulted in a marked decrease of the enhanced spontaneous background activity at basal temperature, so that of nerve terminals from tear-deficent corneas (Table 2), reaching mean frequency values statistically similar to those obtained in intact corneas (see Table1). Perfusion with lacosamide at100 mM also affected the response to cooling ramps of cold nerve terminals from tear-deficient corneas, as evidenced by the significantly reduced peak response frequency values

(Table 2), while the mean cooling threshold temperature was not modified by lacosamide. Despite this significant reduction of peak frequency in response to cooling ramps during perfusion with lacosamide, all cold ther moreceptors increased their firing frequency in response to cold stimulation. When tested, these effects of lacosamide were maintained after 30min of washout. The effects of lacosamide were less prominent in cold thermoreceptors of intact, control corneas. Background ongoing activity at 34 °C and the response to cooling ramp were not significantly affected during perfusion with lacosamide. Lidocaine 100 mM added to the perfusion solution markedly decreased background firing rate of cold nerve terminals at 34 °C. Moreover, increased activity evoked by a cooling ramp was almost abolished in 7 out of 8 cold thermoreceptor units.

Table 1
Spontaneous activity at 34 °C and cold-evoked firing response of cold nerve terminals in control and tear-deficient corneas.

	Control corneas	***Tear-deficient corneas***
	(n=58)	(n=34)
***Spontaneous activity at* 34 °C (imp/s)**	5.2 ± 0.7	8.0 ± 1.1[a]
***Peak firing response* (imp/s)**	16.8 ± 1.3	21.2 ± 1.7[a]
***Cold threshold* (Δ °C)**	−2.80 ± 0.2	−1.5 ± 0.1[a]

[a] $P < 0.05$, Student *t*-test.

Table 2
Effects of lacosamide and lidocaine, both at 100 μM, on the spontaneous activity at the basal temperature (34 °C) and the characteristics of cold-evoked responses of cold nerve terminals in tear-deficient corneas.

	Lacosamide		***Lidocaine***	
	(n =21)		**(n =8)**	
	Before	**During**	**Before**	**During**
***Spontaneous activity at* 34 °C (imp/s)**	8.5 ± 0.9	4.2 ± 0.4[a]	6.1 ± 0.4	1.1 ± 0.3[b]
***Peak response* (imp/s)**	20.8 ± 1.1	14.0 ± 1.0[c]	23.1 ± 2.2	4.7 ± 1.3[d]
***Cold threshold* (Δ °C)**	−1.69 ± 0.08	−1.66 ± 0.14	−1.74 ± 0.1	−2.9 ± 0.3[d]

All are differences between data obtained before and during perfusion with the corresponding drug.

[a] $P < 0.001$, Wilcoxon signed Rank test.
[b] $P < 0.001$, paired *t*-test.
[c] $P < 0.01$.
[d] $P < 0.05$.

Conclusion

In this experimental animal study we have shown that the application of lacosamide results in a significant decrease of the augmented spontaneous activity and responsiveness to cold of corneal sensory nerves from tear-deficient animals. Based on these promising results we speculate that lacosamide might be used to reduce the hyperexcitability of corneal cold receptors caused by prolonged ocular surface dryness due to hyposecretory or evaporative dry eye disease.

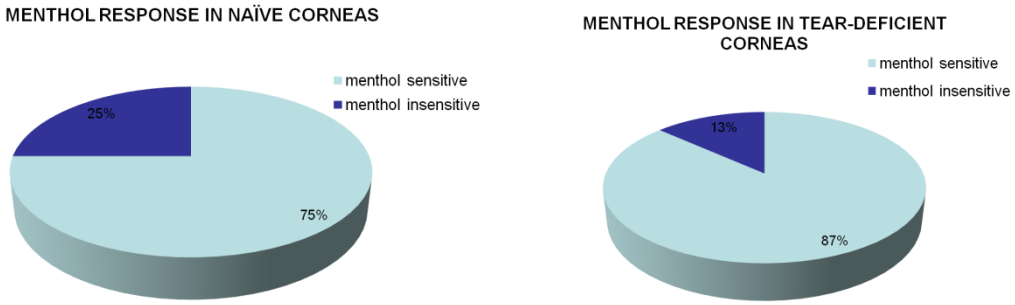
Reference: Kovács I, Dienes L, Perényi K, Quirce S, Luna C, Mizerska K, Acosta MC, Belmonte C, Gallar J. Lacosamide diminishes dryness-induced hyperexcitability of corneal cold sensitive nerve terminals. *Eur J Pharmacol.* 2016;787:2-8.

3. The effect of menthol and capsaicin on the abnormal activity of cold receptors in dry eye model.

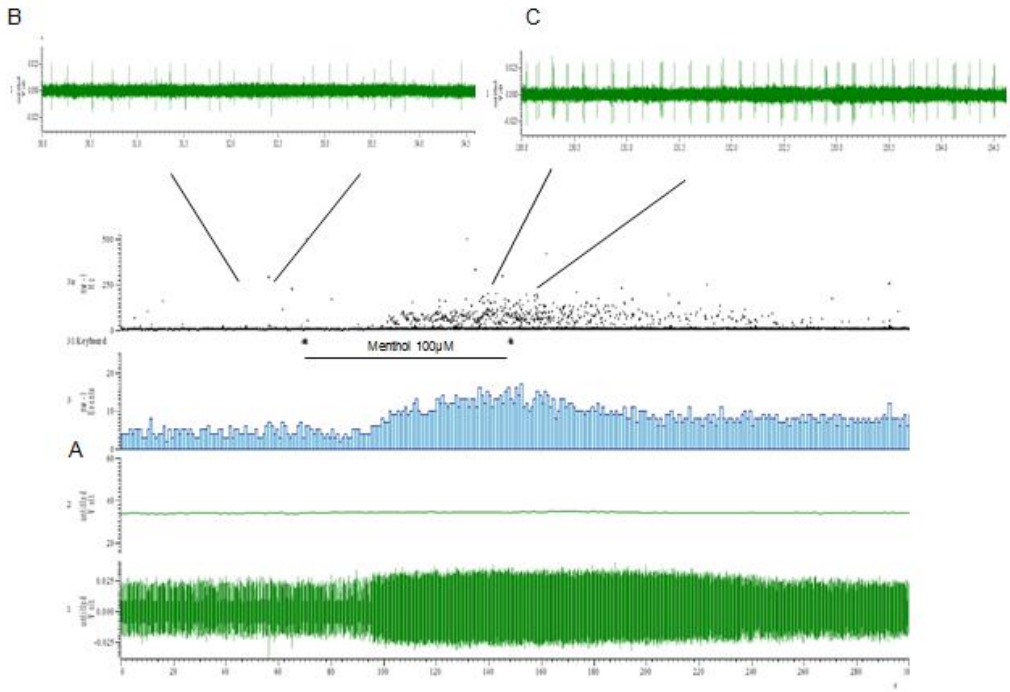
The aim of this study was to characterize the effects of TRP channel agonists menthol and capsaicin on the activity of corneal cold thermoreceptors in tear-deficient guinea pigs.

Results

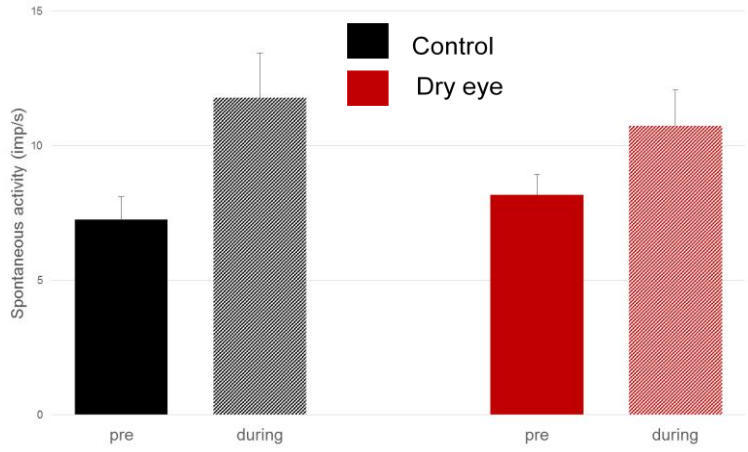
We have shown, that cold thermoreceptors from naive and tear-deficient corneas responded similarly to TRPM8 agonist menthol.



Percentage of cold nerve terminals changing their NTI activity during perfusion at 34ºC with solution containing TRPM8 agonist Menthol (100µM). Naïve corneas, n=16; tear deficient corneas, n=15.

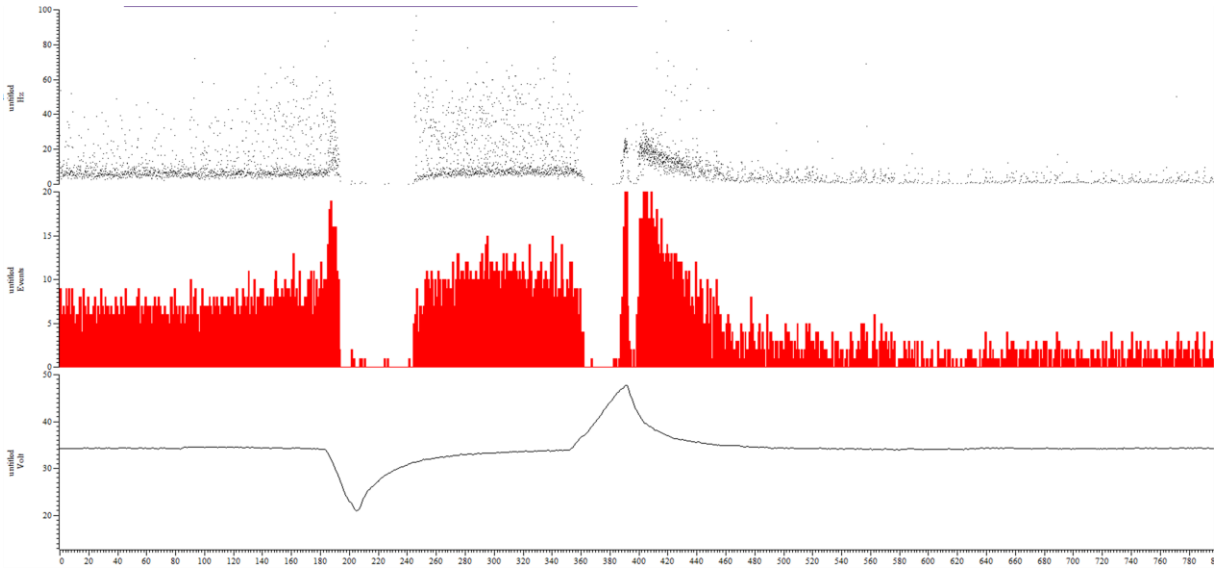


Response of a cold nerve terminal recorded in a naïve cornea in presence of 100µM menthol. (A) Example of NTI activity recorded at 34°C. Traces are instant frequency (Hz), NTI activity (imp/s), temperature (ºC) and electrical recording. Spike firing pattern before (B) and during (C) perfusion with menthol.



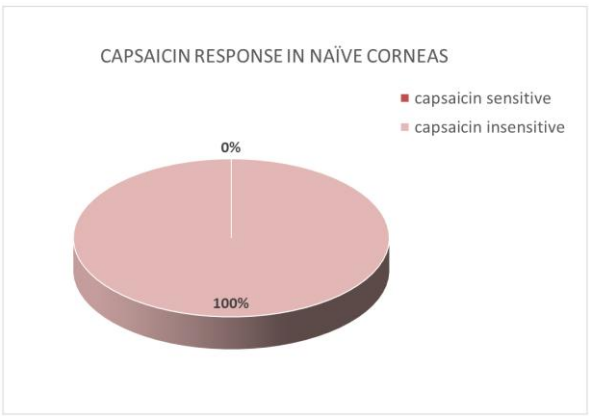
Mean discharge rate (imp/s) of cold nerve terminals in naïve (black bars) and tear-deficient (red bars) corneas before and during perfusion with solution containing 100 μM menthol at 34ºC.

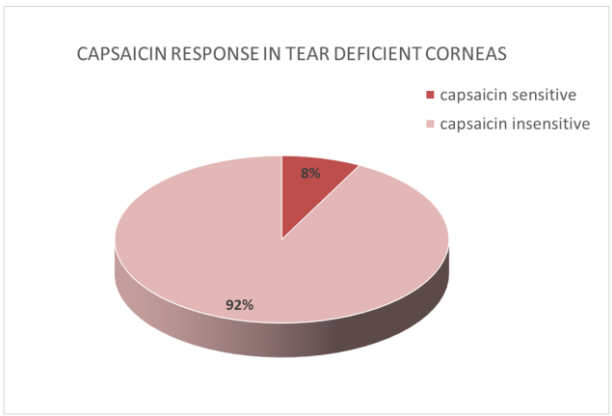
We have shown, that menthol at high concentrations first excited and then inactivated cold thermoreceptors of both naive and tear-deficient corneas



Response of a cold nerve terminal recorded in a tear-deficient cornea in presence of 200μM menthol. (A) Example of NTI activity recorded at 34°C. Traces are instant frequency (Hz), NTI activity (imp/s) and bath temperature (ºC).

We also demonstrated, that only a small proportion of thermoreceptors were activated by the TRPV agonist capsaicin





Percentage of cold nerve terminals responding to perfusion at 34ºC with solution containing TRPV1 agonist Capsaicin (1µM). Naïve corneas, n=15; tear deficient corneas, n=13.

Conclusions

Chronic tear deficiency alters the activity of corneal cold thermoreceptors, leading to the development of increased spontaneous activity and abnormal responsiveness to cold. This neuropathic firing seems to be due to altered expression of Na^+ and K^+ channels involved in impulse generation while the activity of TRP channels involved in sensory transduction is not modified.

Reference: Quirce S, Luna C, Acosta MC, Kovacs I, Belmonte C, Gallar J. Effects of TRPM8 and TRPV1 agonists on the neural activity of corneal cold thermoreceptors in tear-deficient guinea pigs. Acta Ophthalmol. 2016. EVER abstract DOI: 10.1111/j.1755-3768.2016.0690

II. Clinical studies

The aim of our clinical studies was to provide data on ocular surface sensitivity in normal subjects and also in patients with dry eye and to evaluate the change in ocular surface sensations with aging, with corneal nerve pathologies as well as with the treatment of dry eye. All clinical studies were conducted in compliance with the Declaration of Helsinki, applicable national and local requirements regarding the ethics committee and institutional review boards. Ethical approval was obtained from the Institutional Review Board (Semmelweis University Regional and Institutional Committee of Sciences and Research Ethics). A written informed consent was obtained before the examination from each patient or from the parent on behalf of the minors/children.

1. Corneal Sensitivity and Dry Eye Symptoms in Normal Population and in Patients with Keratoconus

Keratoconus is a bilateral, non-inflammatory, progressive disorder characterized by corneal thinning and protrusion that leads to corneal surface distortion. Reduced density and abnormal morphology of the corneal subbasal and stromal nerves has been described in patients with keratoconus using confocal corneal miscroscopy as well as with histological examinations. Based on these morphological deterioration of the corneal nerves the evaluation of the relationship between sensory and tear film impairment might help to understand the pathophysiology of corneal nerve loss not only in this population but in normal subjects also. The aim of this study was to investigate corneal sensitivity to selective mechanical, chemical, and thermal stimulation and to evaluate its relation to keratoconus severity and to dry eye symptoms in patients with keratoconus.

Methods

The original version of the Belmonte noncontact gas esthesiometer was used to explore corneal sensitivity thresholds to selective mechanical, chemical, heat, and cold stimuli in one randomized eye in 19 patients with bilateral mild or moderate keratoconus (KC group) and in 20 healthy controls (control group). The diagnosis of keratoconus was based on classic corneal biomicroscopic and topographic findings in accordance with the criteria of Rabinowitz et al. All patients completed a questionnaire to assess dry-eye disease symptoms (ocular surface disease index - OSDI). Tear film dynamics was assessed by the Schirmer I test without anesthesia and by measuring the non-invasive tear film breakup time with a specific instrument (Keeler Tearscope Plus). Mechanical, chemical, and thermal (hot and cold) thresholds were determined at the center of the cornea using a Belmonte's gas esthesiometer.

Results

KC patients had significatly decreased tear secretion and significantly higher ocular surface disease index (OSDI) scores compared to controls (5.3±2.2 vs. 13.2±2.0 mm and 26.8±15.8 vs. 8.1±2.3; p<0.001). There was no significant difference in NI-BUT between the two groups (KC: 9.8±4.8 vs. control: 10.7±3.8; p>0.05). The mean threshold for selective mechanical (KC: 139.2±25.8 vs. control: 109.1±24.0 ml/min), chemical (KC: 39.4±3.9 vs. control: 35.2±1.9%CO2), heat (KC: 0.91±0.32 vs. control: 0.54±0.26 Δ°C) and cold (KC: 1.28±0.27 vs. control: 0.98±0.25 Δ°C) stimulation in the KC patients were significantly higher than in the control subjects (p<0.001, for all parameters). No correlation was found between age and mechanical, chemical, heat or cold thresholds in the patients with KC (p>0.05), whereas in the control subjects both mechanical (r = 0.52, p = 0.02), chemical (r = 0.47, p = 0.04), heat (r = 0.26, p = 0.04) and cold threshold (r = 0.40, p = 0.03) increased with age. In the KC group, neither corneal thickness nor tear flow, NI-BUT or OSDI correlated significantly with mechanical, chemical, heat or cold thresholds (p>0.05 for all variables).

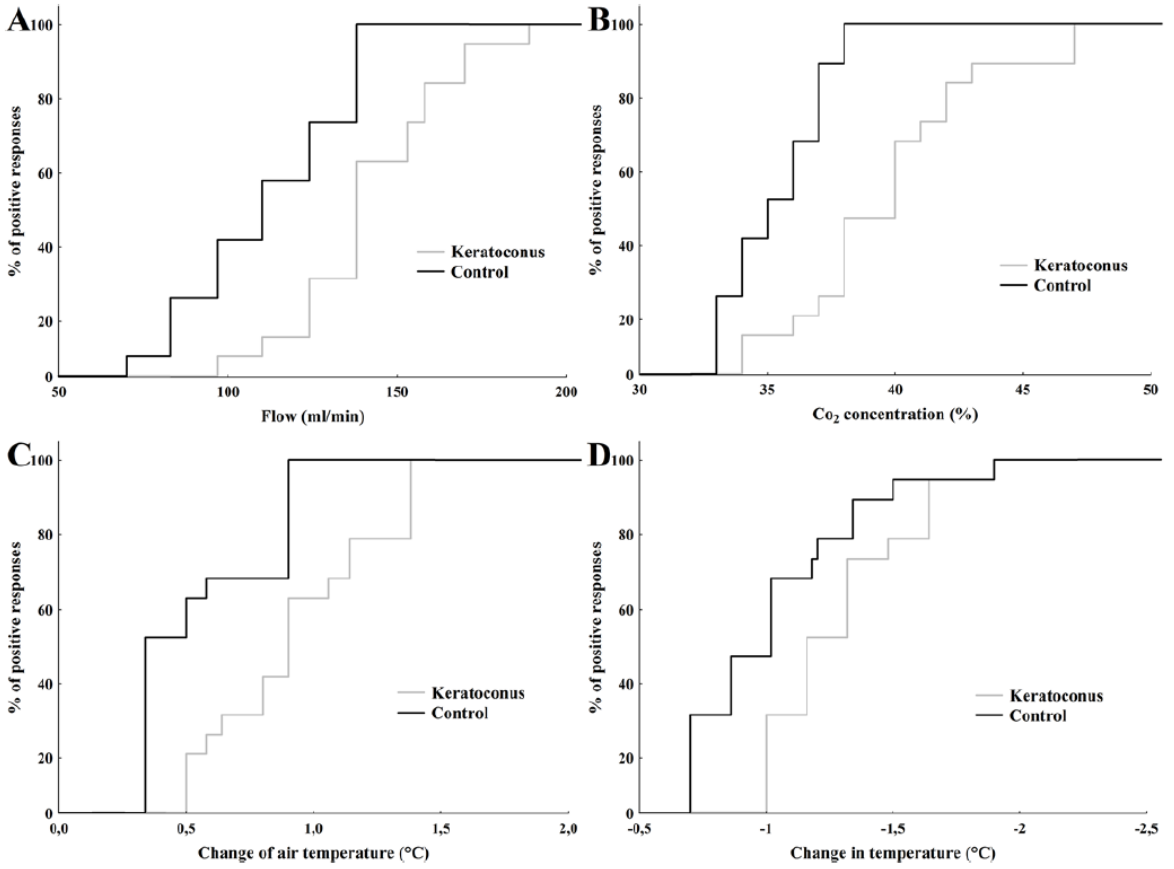


Fig 1. Cumulative distribution of sensation thresholds to selective stimulation of the central cornea in control subjects and keratoconus patients. (A) air pulses of increasing flow (mechanical stimulation), (B) pulses with increasing CO_2 concentration (chemical stimulation), (C) pulses of air at increasing temperatures (hot thermal stimulation), and (D) pulses of air at decreasing temperatures (cold thermal stimulation), in KC patients (gray line) and controls (black line).

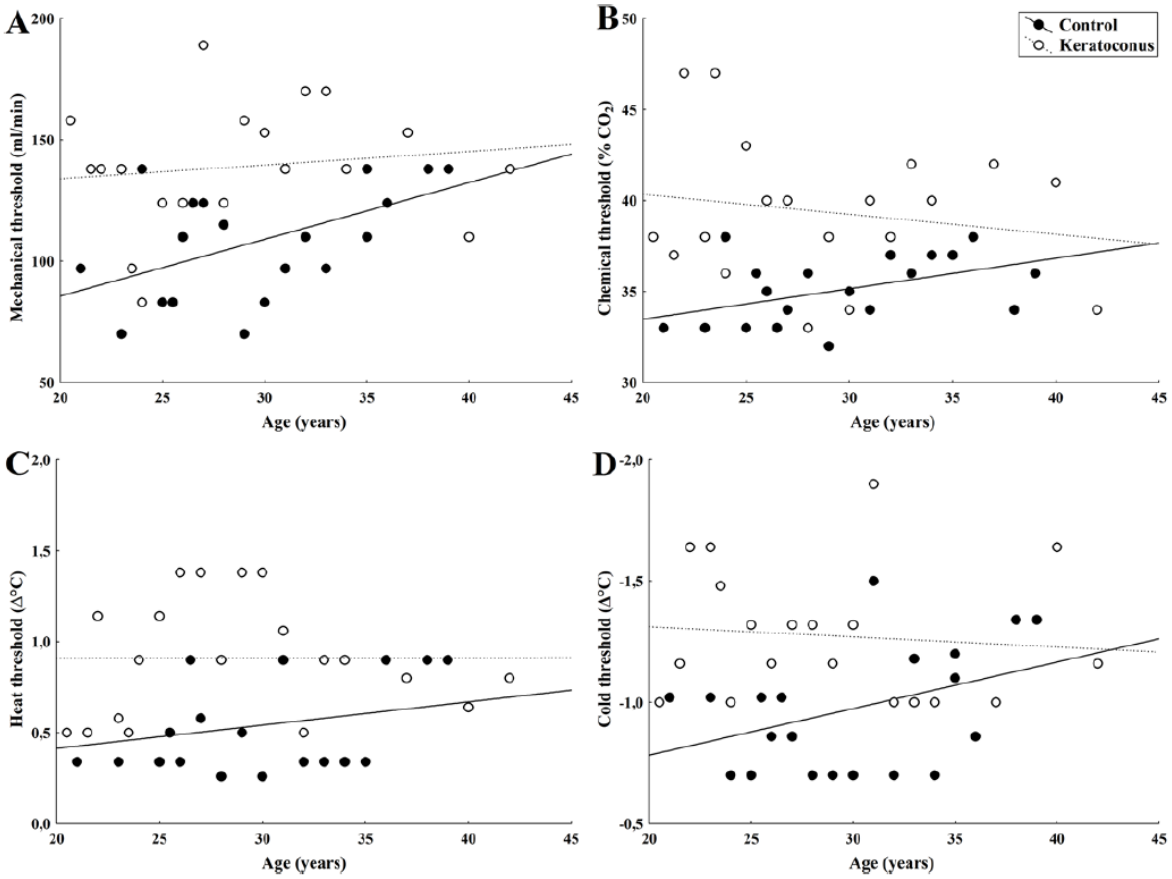


Fig 2. Relationship between age and corneal sensitivity threshold to mechanical (A), chemical (B), heat (C), and cold (D) stimulation in KC patients and in control subjects. Regression lines (solid: control; dotted: KC) are also plotted (see text for details on R coefficients and P-values).

Conclusions

In this study we have demonstrated that corneal sensitivity to different types of stimuli is decreased in patients with keratoconus. The significantly impaired sensitivity suggests that axonal damage and/or altered expression of membrane ion channels involved in transduction and membrane excitability evenly affects the different types of corneal nerve terminals. We have shown that there is a signficant correlation between a decrease in corneal sensitivity and the age in normal subjects for all stimulus modalities. Our finding that changes in corneal sensitivity and tear flow are not related to disease severity or patient's age suggests that there is an early development of impaired corneal nerve function in keratoconus. Although the exact mechanism of corneal nerve damage in keratoconus is still unknown, these structural and neural changes may play a role in the impaired tear secretion as well as in the abnormal ocular sensations experienced by keratoconus patients.

Reference: Dienes L, Kiss JH, Perényi K, Nagy ZZ, Acosta MC, Gallar J, Kovács I. Corneal sensitivity and dry eye symptoms in patients with keratoconus. *PLOS One.* 2015 Oct 23;10(10):e0141621. doi: 10.1371/journal.pone.0141621.

2. The Effect of Tear Supplementation on Ocular Surface Sensations in Dry Eye Patients.

The aim of this study was to investigate the characteristics of ocular surface sensations and corneal sensitivity during the interblink interval in dry eye patients before and shortly after tear supplementation.

Methods

Twenty subjects (41.88±14.37 years) with dry eye symptoms were included in the dry eye group. Fourteen subjects (39.13±11.27 years) without any clinical signs and/or symptoms of dry eye were included in the control group. Tear film dynamics was assessed by noninvasive tear film breakup time (NI-BUT) in parallel with continuous recordings of ocular sensations during forced blinking. Corneal sensitivity to selective stimulation of corneal mechano-, cold and chemical receptors was assessed using a gas esthesiometer. All the measurements were made before and 5 min after saline and hydroxypropyl-guar (HP-guar) drops.

Results

In dry eye patients the intensity of irritation increased rapidly after the last blink during forced blinking, while in controls there was no alteration in the intensity during the first 10 sec followed by an exponential increase. Irritation scores were significantly higher in dry eye patients throughout the entire interblink interval compared to controls ($p<0.004$). NI-BUT significantly increased after HP-guar ($p = 0.003$) but not after saline drops ($p = 0.14$). In both groups, either after saline or HP-guar the shape of symptom intensity curves remained the same with significantly lower irritation scores ($p<0.004$),

however after HP-guar the decrease was significantly more pronounced ($p<0.004$). Corneal sensitivity to selective mechanical, cold and chemical stimulation decreased significantly in both groups after HPguar ($p<0.05$), but not after saline drops ($p>0.05$).

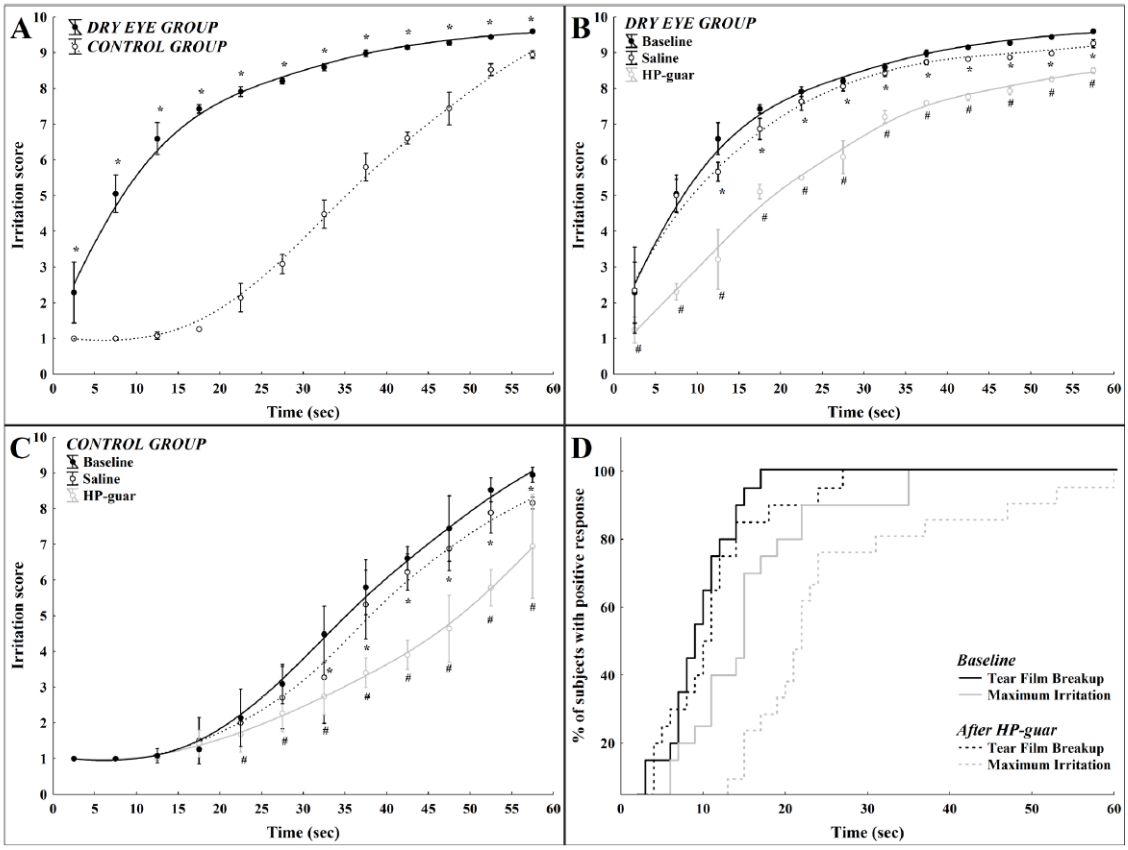


Fig 1. Mean irritation scores as a function of time during forced blinking in dry eye and in normal subjects. (**A**) Mean values of ocular irritation during the interblink interval in the control and in the dry eye group (*: between-group difference $p<0.004$) (**B**) Mean values of ocular irritation scores during the interblink interval after application of saline or HP-guar drops in the control group (*: baseline vs. saline $p<0.004$; #: baseline vs. HP-guar $p<0.004$) (**C**) Mean values of ocular irritation scores during the interblink interval after application of saline or HP-guar drops in the dry eye group (*: baseline vs. saline $p<0.004$; #: baseline vs. HP-guar $p<0.004$) (**D**) Cumulative distribution of NI-BUT and maximum irritation before and after application of HP-guar from both groups. Note: whisker: pooled variance for 5 sec time frames.

Table 1. Change of irritation scores to selective stimulation of corneal nerves after tear supplementation.

	Baseline	After saline		After HP-guar	
	Mean ± SD	*Mean ± SD*	*P*	*Mean ± SD*	*P*
Dry eye group					
Mechanical	4.9 ± 2.9	4.4 ± 1.5	0.23	3.1 ± 2.9	0.001
Chemical	7.1 ± 1.1	6.8 ± 2.7	0.68	5.6 ± 1.7	0.006
Cold	2.1 ± 2.3	1.9 ± 3.0	0.81	0.9 ± 2.4	0.04
Control group					
Mechanical	6.3 ± 2.0	5.6 ± 1.9	0.12	4.3 ± 2.2	0.001
Chemical	7.0 ± 2.4	6.5 ± 2.0	0.28	5.6 ± 2.0	0.006
Cold	2.0 ± 1.6	1.7 ± 1.9	0.49	1.0 ± 1.9	0.02

Mean ± SD values of irritation scores to mechanical, chemical and cold stimulation at baseline and after application of saline or HP-guar drops. Note: P compared to baseline, Wilcoxon signed-rank test.

Conclusions

In this study we have shown, that not only tear film dynamics but the characteristics of ocular surface sensations are also substantially different in dry eye patients compared to normal subjects. We have also shown, that improved tear film dynamics has a beneficial effect on ocular surface protection against environmental stimuli but has no direct effect on the altered characteristics of sensations. Based on these results we may conclude that in spite of the rapid improvement in tear film dynamics after tear supplementation, altered ocular surface sensations are still maintained and might be responsible, at least in part, for the remaining complaints reported by numerous dry eye patients despite treatment.

Reference: Dienes L, Kiss JH, Perényi K, Szepessy Z, Nagy ZZ, Barsi A, Acosta MC, Gallar J, Kovács I. The Effect of Tear Supplementation on Ocular Surface Sensations During the Interblink Interval in Patients with Dry Eye. *PLOS One.* 2015 Aug 24;10(8):e0135629. doi: 10.1371/journal.pone.0135629.

3. The effect of TRPM8 agonist menthol on ocular surface sensations in healthy controls and in dry eye patients

In this clinical study, we explored in humans the quality of the conscious sensations evoked by stimulation of cold sensory receptors of the ocular surface in healthy individuals and in patients with DED. The aim of this study was to add clinical data to our previous animal experiments related to the morphology and impulse firing of corneal nerve fibers, as well as on the sodium and potassium membrane currents in experimental dry eye.

Methods

Eighteen healthy subjects and 9 patients with dry eye were recruited and participated voluntarily in the study. Patients with moderate DED were selected according to the following inclusion criteria: Ocular Surface Disease Index >15 and <40,

Schirmer test <10 mm in 5 minutes, tear break-up time test <10 seconds, absence of ocular pathologies different from DED, and absence of systemic diseases. The participant was aware of a recording camera but unaware of its use for offline counting of blinks. Tear osmolarity was then measured using a lab-on-a-chip system and a the Schirmer test without anesthesia was performed. After a 10-minute rest period, a sterile soft paraffin ointment containing menthol at 1 mM was applied to the malar region of the skin, at a distance of 2.5 cmfrom the lower lid margin to avoid direct spread of menthol or lipids into the tears, using a cotton swab. After 3 minutes, the volunteers rated the magnitude of cooling and unpleasant components of the sensation experienced at the ocular surface produced by menthol, using 2 separate 10-cm continuous VASs. The subjects were also asked to describe in their own words the sensation experienced in the eye after ointment application. A sample of tear was taken thereafter, approximately 4 minutes after ointment application. The Schirmer test and tear break-up time test determination were performed after 3 minutes. After a 10-minute resting period, the procedure was repeated using an ointment containing 10 mM menthol. Menthol concentration in tear samples was quantified using gas chromatography with a flame ionization detector.

Results

The low-concentration (1 mM) menthol ointment evoked weak conscious sensations of cold and no significant unpleasantness or changes in the tearing rate in healthy volunteers, although their blink rate rose significantly. By contrast, the high-concentration (10 mM) menthol ointment significantly increased the blink rate and the perception of discomfort and coolness measured with a VAS scale, reflecting the unpleasant nature of the sensation. No significant changes in the tearing rate were observed with any of these concentrations. A similar yet less pronounced response was obtained after exposing the eyes of healthy subjects and patients with DED to a room temperature air current. Of note, in the group of patients with DED, the basal blink rate at rest was significantly higher than in healthy subjects ($P>0.002$, t-test) and was augmented by menthol. The most striking observation of menthol ointment application in patients with DED at both concentrations was that unpleasantness, which under basal conditions was rated to be higher than in healthy individuals, decreased. The sensation

evoked by menthol in healthy subjects and patients with DED also included a component of coolness. Finally, the higher background discomfort in patients with DED was not further increased by exposure to an air current.

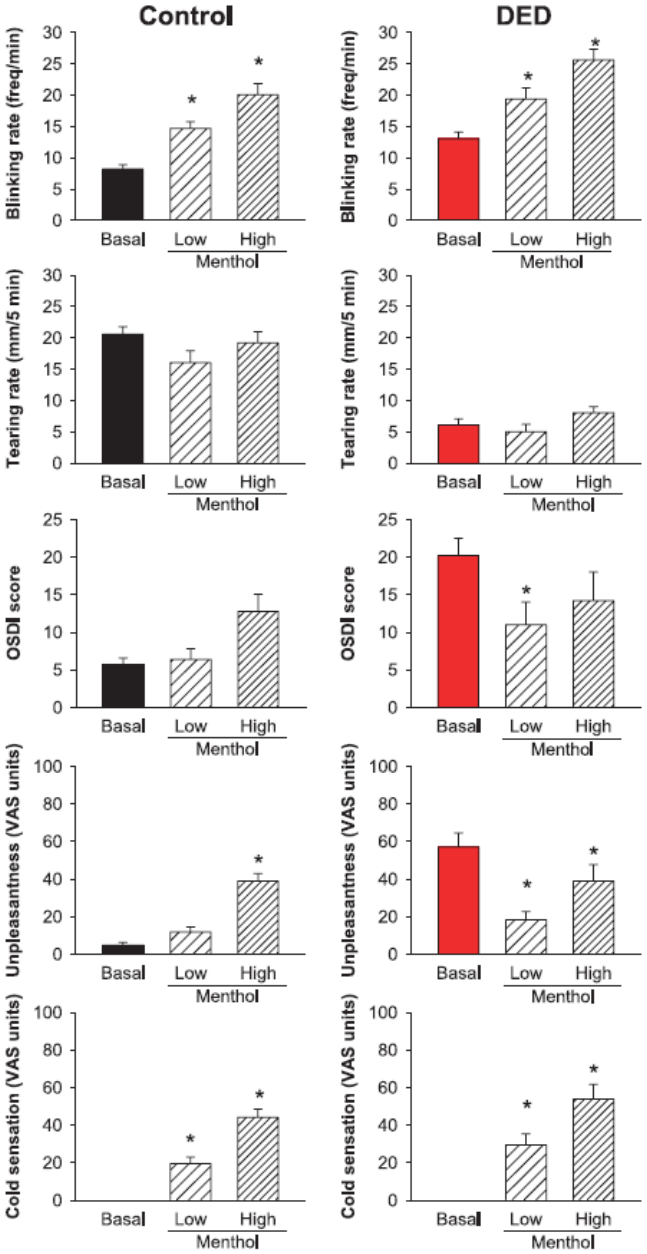


Figure 13. Effects of menthol on blinking frequency, tearing rate, and ocular sensation parameters in control and patients with dry eye disease (DED). Menthol was applied as an ointment onto the cheekbone skin at 1 and 10 mM concentrations (leading respectively to 5 and 39.5 μM menthol concentrations in tears). Data from control subjects (n = 18) and patients with DED (n = 9) are shown. From top to bottom: Mean blinking frequency measured off-line from webcam recordings performed during 3 minutes; tearing rate measured with commercial Schirmer strips without anesthesia during 5 minutes; Ocular Surface Disease Index (OSDI) score measured with a questionnaire approved for Spanish-speaking people; unpleasantness sensation measured with a 0-to-100 visual analog scale (VAS); cold sensation intensity measured with a 0-to-100 VAS. $P < 0.001$, repeated-measures Analysis of variance with post hoc analysis using the Bonferroni t test (*$P < 0.001$).

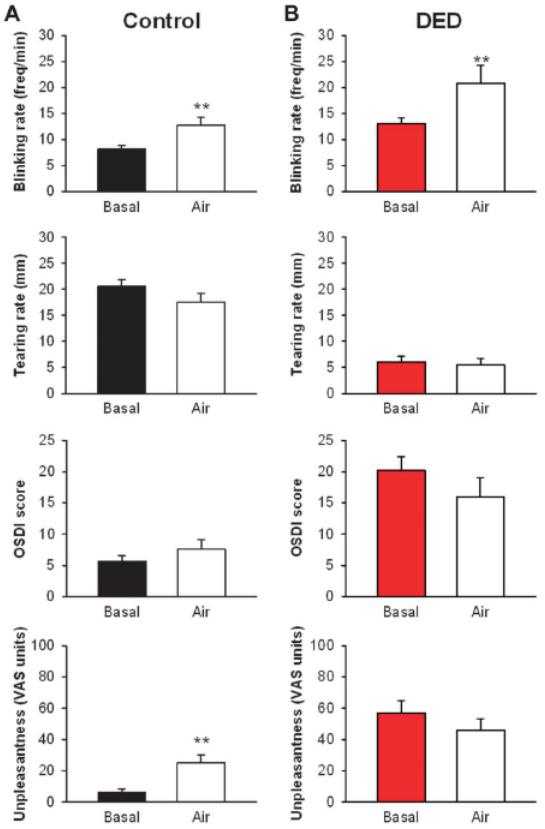


Figure 14. Effect of ocular surface dryness induced by an air stream directed to the face on blinking frequency, tearing rate, and ocular sensations in (A) control subjects and (B) patients with dry eye disease (DED). The following parameters were measured: mean blinking frequency measured off-line from video recordings of 3-minute duration; tearing rate, measured with Schirmer strips without anesthesia during 5 minutes; Ocular Surface Disease Index (OSDI) score determined with a questionnaire validated for Spanish-speaking people; unpleasantness sensation intensity scored with a 0-to-100 visual analog scale (VAS). Data are mean ± SEM, n = 18 control and n = 9 patients with DED; **$P < 0.001$, paired t test.

Reference: Kovács I, Luna C, Quirce S, Mizerska K, Callejo G, Riestra A, Fernandez-Sanchez L, Meseguer VM, Cuenca N, Merayo-Lloves J, Gasull X, Acosta MC, Belmonte C, Gallar J. Abnormal activity of corneal cold thermoreceptors underlies the unpleasant sensations in dry eye disease. *Pain.* 2016 Feb;157(2):399-417.

4. The Effect of Long Term Tear Supplementation with Zinc-Hyaluronate on Ocular Surface Sensations in Dry Eye Patients.

Hyaluronic acid, a natural polymer, helps to maintain ocular surface hydration and can already be found in several artificial tears recommended to alleviate symptoms of dry eye. A recent hyaluronate modification involves zinc-hyaluronate complex formation by adding zinc-chloride to an aqueous sodium-hyaluronate resulting in a very stable molecular structure, which functions as both a mechanical barrier and a biocompatible film on the ocular surface. Apart from its beneficial elastoviscous characteristics, previous results indicate that hyaluronate can also reduce the excitability of the peripheral nociceptor endings underlying pain. Although hyaluronate is widely used in artificial tears to improve tear film stability, its effect on ocular surface sensitivity was not evaluated in patients with dry eye. The aim of this study was to investigate the characteristics of ocular surface sensations and corneal sensitivity in dry eye patients before and after long-term tear supplementation with zinc-hyaluronate.

Methods

Patients who had been diagnosed as having dry eye symptoms for at least 3 months, with an OSDI score of ≥13 evaluated by the questionnaire of Ocular Surface Disease Index (OSDI) have been recruited for this study. Subjects who showed significant corneal staining (>Grade 2, Oxford Scale) were excluded because corneal epitheliopathy could potentially be a confounding factor affecting ocular surface sensitivity. Subjects with ophthalmic conditions other than dry eye or systemic disease including blepharitis, meibomitis, lid abnormalities as well as contact lens wearers were also excluded. Tear film dynamics was assessed by non-invasive tear film breakup time (NI-BUT) in parallel with continuous recordings of ocular sensations during forced

blinking. Corneal sensitivity to selective stimulation of corneal mechanonociceptors, cold receptors and chemical nociceptors were assessed using the Belmonte gas esthesiometer. Ocular surface sensations and corneal sensitivity to the different stimuli were explored before and after instillation of a drop of zinc-hyaluronate (Ophylosa®; Richter gedeon Ltd., Hungary) in one eye of each participants. During the experiments, the right eye was used for data collection and the left eye was closed with a patch. All experiments were performed during the morning hours by the same physician with evaluating of NI-BUT and simultaneous assessment of ocular surface sensations at first, immediately followed by measuring corneal sensitivity. All procedures were repeated 5 min after the application of on drop of zinc-hyaluronate drops, as well as 1 month after daily (4/day) use of zinc-hyaluronate drops. At one month, none of the subjects received any drops at least 12 hours before the measurements.

Results

Twenty eyes of twenty subjects (12 men, 8 women) were included in the study with a mean age of 63.92±12.76 years. At baseline the mean OSDI score was 40.42±16.20, the mean non-invasive tear film breakup time was 3.92±1.28 sec. As a result of tear supplementation with zinc-hyaluronate, tear film breakup time significantly increased both after 5 min (9.58±3.61 sec; $p=0.01$) and after one month of treatment (8.53±4.30 sec; $p<0.001$). Tear supplementation with a zinc-hyaluronate for one month resulted in a significant decrease in the mean OSDI score compared to baseline (14.16±11.15; $p<0.001$).

At baseline, the intensity of irritation quickly increased during forced blinking, while one drop of zinc-hyaluronate significantly reduced sensory responses five minutes after its application ($p<0.004$, Fig. 1C). After one month, 4x/d use of zinc-hyaluronate resulted also in a significant reduction of the sensory responses compared to baseline throughout the interblink interval ($p<0.004$, Fig. 1C) even 12 hours after the last application of the eye drop. At one month, sensory responses were statistically significant lower at every 5 sec timeframes during the interblink interval compared to data obtained at 5 min ($p<0.004$, Fig. 1C).

The time to intense (score >5) irritation responses significantly increased after 1 month of treatment compared to baseline and to data obtained 5 minutes after one drop of zinc-hyaluronate (Fig. 1D).

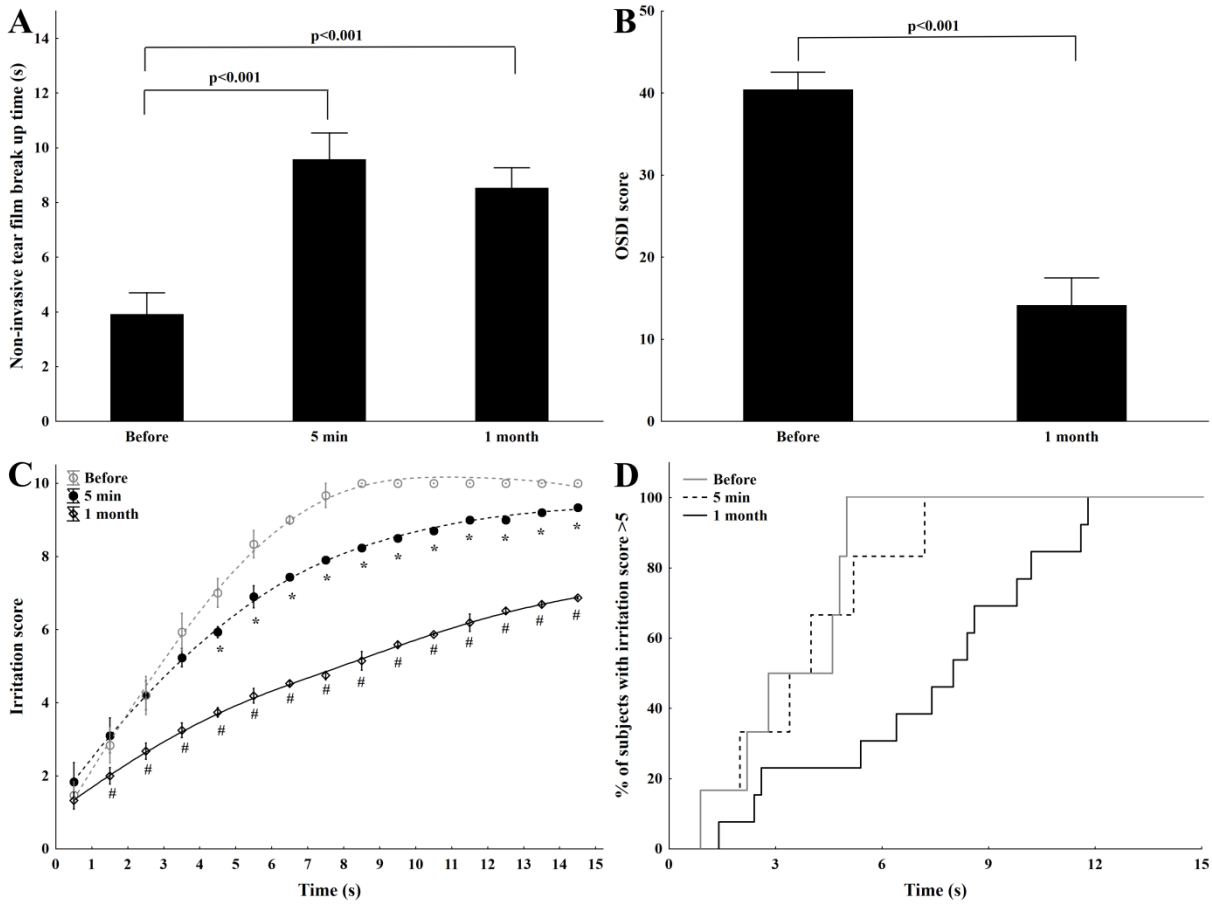
Sensations evoked by mechanical stimuli were defined by subjects as irritating with a predominantly stinging component. The sensation evoked by CO_2 was defined by subjects as irritating, with stinging, burning or pricking components. The irritation after cold stimulation was reported as mildly irritating, occasionally with cooling components.

Five minutes after application of zinc-hyaluronate drop, corneal sensitivity thresholds to mechanical stimulation significantly decreased, however sensitivity thresholds to heat, cold and chemical stimuli remained unchanged (Table 1). Similarly, continuous treatment with zinc-hyaluronate drop for one month resulted in a significant decrease of mechanical sensitivity threshold (Table 1) even 12 hours after the last application of eye drops.

Table 1: Sensitivity thresholds to selective stimulation before and after tear supplementation

	Baseline	**5 min**	**1 month**
Mechanical (ml/min)	97.83 ± 34.96	103.23 ± 36.11*	103.92 ± 41.97*
Cold (Δ°C)	0.16 ± 0.14	0.16 ± 0.21	0.18 ± 0.15
Heat (Δ°C)	0.33 ± 0.18	0.37 ±0.21	0.38 ± 0.16
Chemical (%CO_2)	33.14 ± 0.51	33.17 ± 0.55	33.12 ± 0.55

Note: *: compared to baseline



Conclusions

In this study we demonstrated, that in dry eye patients unpleasant ocular surface sensations were significantly decreased after prolonged use of zinc-hyaluronate containing eye drops even 12 hours after the last application of the eye drop. In a previous study we have shown that tear supplementation significantly decreased ocular surface irritation shortly after its application, and this effect was attributed to an improvement in the structure of the protective tear film layer. That study also has shown that the characteristics of ocular surface symptoms are substantially different in dry eye patients, suggesting altered excitability of corneal receptors, which did not change after short term tear supplementation. Here we describe for the first time that prolonged tear supplementation with zinc-hyaluronate results in a decrease in ocular surface irritation responses even after 12 hours of the application of the last eye drop. Our results suggest, that this beneficial effect might be the results of not only an improvement in tear film dynamics but also a decrease in the sensitivity of corneal sensory nerve endings. The concept of a decrease in the excitability of the corneal nerve

endings is supported by our findings, as one month of treatment with zinc-hyaluronate resulted in a significant drop of symptom intensity curves compared to results 5 min after tear supplementation despite similar mean value of tear film break-up time. It has to be emphasized, that this further reduction in ocular surface irritation was measured 12 hours after the last eye drop and tear film quality at this time was comparable to that was 5 min after tear substitution suggesting decreased sensitivity of corneal sensory receptors after long-term treatment.

Our results showed that mechanical sensitivity threshold increased after 1 month of tear supplementation, but neither chemical nor thermal thresholds were changed. Taken together these results it is reasonable to conclude, that after 1 month of treatment the decrease in ocular surface sensations might be the result of an improvement in tear film dynamics as well as an increase in the sensitivity threshold of corneal mechanoreceptors. However, the exact mechanism of the decreased mechanoreceptor excitability after prolonged use of zinc-hyaluronate remains to be elucidated. Previous results indicate that after intra-articular injections of hyaluronan solutions, the reducing action on joint nociceptor activity appeared to depend on their role as an elastoviscous filter associated with their rheological properties. Moreover, it has already been demonstrated, that extracellular hyaluronate can reduce the excitability of the transient receptor potential vanilloid subtype 1 (TRPV1) channel, thereby lowering impulse activity in the peripheral nociceptor endings underlying pain. We may conclude from our results, that the beneficial effect of long term tear supplementation with hyaluronate might be based on both effects.

Reference: Perényi K, Dienes L, Kornafeld A, Kovács B, Kiss HJ, Szepessy Z, Nagy ZZ, Barsi A, Acosta MC, Gallar J, Kovács I: The Effect of Long Term Tear Supplementation with Zinc-Hyaluronate on Ocular Surface Sensations in Patients with Dry Eye. *Prepared for submission*

Final conclusions

Our results suggest that under comfortable environmental conditions, background activity in low-threshold cold thermoreceptors, the only afferent tonic input produced by corneal nerves, helps to maintain basal tearing and spontaneous blinking, but it is probably too weak to evoke conscious sensations of cooling or dryness. This background sensory input builds up critically with ocular surface temperatures decreases, as those occurring under exposure to extreme low temperatures, cold air, or evaporation in very dry environments. Augmented firing and recruitment of higher threshold cold thermoreceptors would evoke an unpleasant and qualitatively distinct sensation, consciously defined as unpleasant irritating dryness. Conceivably, a similar response is already triggered by less drastic cooling when the tear film is too thin or abnormal, as occurs in patients with aqueous-deficient and evaporative DED. In such cases, ongoing cold thermoreceptor activity is expected to be high and further potentiated by the rise in tear fluid osmolality that accompanies enhanced evaporation and is developed in experimental dry eye models reducing tear volume. Damage to corneal nerve terminals due to long-term reduction of ocular surface wetness, as observed in guinea pig dry eye corneas, possibly confers neuropathic characteristics to the augmented activity of cold thermoreceptors, thereby perpetuating the initial sensations of discomfort. Recruitment of nociceptors by ocular surface injury and inflammation possibly aggravates the unpleasant and distressing nature of the final sensation experienced by patients with DED.

We have also shown in an experimental animal study for the first time, that the application of lacosamide results in a significant decrease of the augmented spontaneous activity and responsiveness to cold of corneal sensory nerves from tear-deficient animals. Based on these promising results we speculate that lacosamide might be used to reduce the hyperexcitability of corneal cold receptors caused by prolonged ocular surface dryness due to hyposecretory or evaporative dry eye disease.

We have also shown for the first time, that not only tear film dynamics but the characteristics of ocular surface sensations are also substantially different in dry eye patients compared to normal subjects. We have shown, that improved tear film dynamics has a beneficial effect on ocular surface protection against environmental stimuli but has no direct effect on the altered characteristics of sensations. Based on

these results we may conclude that in spite of the rapid improvement in tear film dynamics after tear supplementation, altered ocular surface sensations are still maintained and might be responsible, at least in part, for the remaining complaints reported by numerous dry eye patients despite treatment.

We have also shown that there is a signficant correlation between a decrease in corneal sensitivity and the age in normal subjects for all stimulus modalities in contrast to keratoconus, where changes in corneal sensitivity and tear flow are not related to disease severity or patient's age. These results suggests that impaired corneal nerve function can precede the onset of visible morphological deterioration of corneal nerves in keratoconus patients.

Other publications, in which financial support from OTKA NN106649 grant was indicated:

Kovács I, Miháltz K, Kránitz K, Juhász É, Takács Á, Dienes L, Gergely R, Nagy ZZ. The accuracy of machine learning classifiers using bilateral tomography and topography data from Scheimpflug camera for identifying eyes with preclinical signs of keratoconus. *J Cat Refr Surg.* 2016;42:275-283.

Dienes L, Kránitz K, Juhász É, Gyenes A, Takács Á, Miháltz K, Nagy ZZ, Kovács I. Evaluation of intereye corneal asymmetry in patients with keratoconus. A Scheimpflug imaging study. *PLOS One*. 2014 Oct 8;9(10):e108882.