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14. ABSTRACT
We hypothesize that targeted molecular intervention can preserve vision threatened by battlefield trauma-induced corneal and retinal inflammation, corneal and retina/optic nerve apoptosis, ocular surface dry eye after refractive surgery, and retinal degeneration. We are studying the consequences of trauma-induced (1) corneal inflammation using a gene therapy approach of providing soluble Fas ligand to the cornea to determine if this ligand can suppress corneal inflammation in mice; (2) retinal inflammation by examining if transforming growth factor-beta, thrombospondin, and somatostatin, in subretinal space, can suppress inflammation within retina secondary to autoimmune uveoretinitis and light-induced damage in mice; (3) corneal cell death by apoptosis and promote regeneration by identifying the anti-apoptotic gene with the greatest capacity to suppress corneal cell apoptosis using mice; (4) retinal cell death and regeneration by using mice to determine if systemic treatment with lithium chloride can prevent collateral damage to retinal neurons and promote optic nerve regeneration; (5) dry eye by determining how to minimize dry eye after LASIK refractive surgery by developing new tests to predict pre-disposition to refractive surgery induced dry eye; and (6) retinal injury by generating stem cell polymer composites.

15. SUBJECT TERMS
blindness, trauma, eye, cornea, retina, dry eye, refractive surgery, inflammation, optic nerve, regeneration

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DOD FY03 Interim Report Task #5
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Introduction

An increasing percentage of battlefield injuries occur to the eye in modern warfare. Even treatable battlefield injuries to the eye can lead to blindness because of collateral damage to adjacent tissues. This blindness results from injury-induced inflammation, cell death, failure to regenerate and repair, and development of scar tissue. Task #5 is one portion of a multidisciplinary project that addressed corneal blindness resulting from abrasions, burns, and penetrating wounds acting on normal corneas or exaggerated in corneas that have undergone refractive surgery, as well as retinal blindness resulting from physical trauma, infection, or laser-induced injury that destroy retinal nerve cells. In task 5 our goal is to prevent the consequences of trauma to the cornea after refractive surgery by developing strategies to diagnose dry eye syndromes. Our specific objective was to determine if there are individuals in whom the goblet cells of the conjunctiva do not respond normally to neural and growth factor stimulation and if this abnormal response predisposes these individuals to developing chronic dry eye after laser refractive surgery. Our three subtasks were to 1: determine if the response of conjunctival goblet cells to nerves and growth factors is reduced in a mouse model of dry eye and if loss of corneal nerves (induced by a corneal wound) alters this response. The loss of corneal nerves by a corneal wound mimics the loss of nerves induced in laser refractive surgery. 2: determine if human goblet cells from normal human controls respond to the growth factor EGF, the β-adrenergic agonist isoproterenol, and the cholinergic agonist carbachol. 3: Determine if patients with reduced goblet cell response will have an increased rate of dry eye symptoms and traumatic complications after laser refractive surgery.

Body

I. Research accomplishments for Subtask 1: We have finished our studies on measuring alterations in phosphoprotein levels in conjunctiva of Balb-c mice following stimulation with EGF, carbachol and isoproterenol. We performed corneal wounding (superficial keratectomy) in 12 week-old Balb-c mice in order to mimic laser refractive surgery. Corneal wounding was done in the right eye and left eye was kept unwounded. Mice not wounded in either eye were considered as the controls. The conjunctiva was isolated from both eyes at different time points (2 days, 6 days, 2 weeks and 4 weeks) following wounding and divided into four pieces. Long time points were chosen as our goal is to study chronic dry eye that develops after surgery in a percentage of individuals rather than the acute dry eye that occurs in almost everyone after this surgery. Conjunctival tissue pieces were stimulated with no additions (basal), EGF (10^{-7} M,) carbachol (10^{5} M), and isoproterenol (10^{-5} M) for 5 minutes at 37° C in a water bath. The reaction was stopped in keratinocyte basal medium kept at 4° C. The conjunctival lysates were prepared by homogenizing the tissue in RIPA buffer. BioRad multiplex assay was done to study the levels of various phosphoproteins in the conjunctival lysate samples and to determine alterations in the phosphoprotein levels following corneal wounding. Seven-plex assay kit was used to measure the levels of phosphorylated ERK (p42/p44 mitogen-activated protein kinase), JNK, p38 mitogen activated protein kinase, AKT, IκB alpha, STAT-3 and P70S6 at the same time in each sample. Phosphoproteins levels were standardized to the amount of total ERK (phosphorylated and non-phosphorylated) in each sample. The data were analyzed using Bioplex manager software and fold increase in phosphoprotein levels was determined over the basal levels. Statistical analysis was done using student T test and $P < 0.05$ was considered as significant.

A. Results from non-wounded control mice:

Table 1 summarizes the results from 12 week-old control (n=7) mice. We found:

1. Significant increase in phosphorylated ERK following stimulation with EGF and carbachol.
2. Significant increase in phosphorylated AKT following stimulation with EGF and carbachol.

3. Significant increase in STAT-3 following stimulation with isoproterenol.
4. Decrease in the levels of P70S6 were seen following stimulation with isoproterenol, although it was not statistically significant.

B. Results from the wounded (right) eye 2 days following corneal wounding :

Table 2 summarizes the results in the wounded eye 2 days after superficial keratectomy performed in 12 weeks old Balb-c mice (n=5). We found:
1. Significant increase in phosphorylated ERK following stimulation with EGF and carbachol.
2. Significant increase in phosphorylated JNK following stimulation with EGF and carbachol.
3. Significant increase in phosphorylated P38 MAPK following stimulation with EGF.

C. Results from the unwounded (left) eye 2 days following corneal wounding :

Table 3 summarizes the results in the unwounded eye 2 days after superficial keratectomy performed in 12 weeks old Balb-c mice (n=5). We found:
1. Significant increase in levels of phosphorylated ERK following stimulation with EGF.
2. Significant increase in phosphorylated JNK following stimulation with carbachol.
3. Significant increase in phosphorylated P38 MAPK following stimulation with EGF and carbachol.
4. Significant increase in phosphorylated AKT following stimulation with isoproterenol.
5. Signicant increase in phophorylated IκB alpha, STAT-3 and P70S6 following stimulation with carbachol.

D. Results from the wounded (right) eye 6 days following corneal wounding

Table 4 summarizes the results in the wounded eye 6 days after superficial keratectomy performed in 12 -week old Balb-c mice (n=3). We found:
1. Significant decrease in phosphorylated p38MAPK following stimulation with EGF.
2. Significant decrease in phosphorylated AKT following stimulation with EGF.

E. Results from the unwounded (right) eye 6 days following corneal wounding

Table 5 summarizes the results in the wounded eye 6 days after superficial keratectomy performed in 12 -week old Balb-c mice (n=3). We found:
1. Significant increase in phosphorylated pERK following stimulation with EGF and carbachol.

F. Results from the wounded (right) eye 2 weeks following corneal wounding

Table 6 summarizes the results in the wounded eye 2 weeks after superficial keratectomy performed in 12 -week old Balb-c mice (n=6). We found:
1. Significant increase in phosphorylated pERK following stimulation with EGF.
2. Significant increase in phosphorylated pJNK following stimulation with EGF.
2. Significant increase in phosphorylated pAKT following stimulation with carbachol.

G. Results from the unwounded (right) eye 2 weeks following corneal wounding

Table 7 summarizes the results in the unwounded eye 6 days after superficial keratectomy performed in 12 -week old Balb-c mice (n=6). We found:
1. Significant decrease in phosphorylated p38MAPK following stimulation with carbachol.

H. Results from the wounded (right) eye 4 weeks following corneal wounding

Table 8 summarizes the results in the wounded eye 4 weeks after superficial keratectomy performed in 12 -week old Balb-c mice (n=6). We found:
1. Significant increase in phosphorylated pERK following stimulation with EGF.

I. Results from the unwounded (left) eye 4 weeks following corneal wounding

Table 9 summarizes the results in the unwounded eye 4 weeks after superficial keratectomy performed in 12 -week old Balb-c mice (n=6). We found:

1. Significant increase in phosphorylated pERK following stimulation with EGF.

J, Summary of completed experiments

Table 10 summarizes the number of completed experiments
We have done:

1. Seven stimulation experiments on unwounded mice and analyzed all seven with multiplex.
2. Five stimulation experiments on mice 2 days after wounding and analyzed all five with multiplex.
3. Four stimulation experiments on mice 6 days after wounding and analyzed three with multiplex. Samples from one mouse were unable to be analyzed.
4. Six stimulation experiments on mice 2 weeks after wounding and analyzed six with multiplex.
5. Six stimulation experiments on mice 4 weeks after wounding and analyzed six with multiplex.

K. Future experiments for subtask 1

Experiments for subtask 1 are completed, but we will perform additional statistical analysis to determine if there are differences in the response of phosphoproteins between control and wounded eyes with time and between unwounded and wounded eyes at each time point. With the completion of this statistical analysis we will be able to conclude whether or not corneal wounding cutting the corneal nerves causes chronic changes in the responsiveness of the conjunctiva to neurotransmitters and growth factors.

II. Research Accomplishments for Subtask 2: As Dr. Dimitri Azar our initial collaborator moved from Massachusetts Eye and Ear Infirmary, we revised our IRB protocol to remove him as the doctor to whom adverse advents would be reported and replaced him with Dr. Reza Dana. Impression cytology samples were removed from 14 volunteers. Four samples were obtained from one eye. In the first patients we developed the method for collecting cells from the nitrocellulose membrane and found that placing the membrane in a glass centrifuge tube and centrifuging resulted in the best yield of cells and the most responsive cells as determined by western blotting analysis. Unfortunately not enough protein was recovered in each sample to perform the multiplex analysis. We thus decided to combine samples resulting in two final samples. These samples were stimulated by no additions and carbachol. Using this method we were not be able to study the effect of EGF and isoproterenol. This change was acceptable, as carbachol works via EGF and isoproterenol did not stimulate any phosphoprotein activity in the mouse model. We tried this in samples from one volunteer. Enough protein was obtained in each sample and carbachol increased pERK and p38 MAPK activity. Impression cytology samples were obtained from 10 volunteers at Walter Reed Army Medical Center, stimulated at that location, frozen on dry ice, and transported to the Schepens Eye Research Institute. In most samples there was not enough protein to measure by the Bradford Assay. In spite of this we performed the multiplex analysis. However, for most of the samples there was no consistent stimulation of phosphoprotein activity by carbachol and in most cases there was no stimulation. We felt that for the assay to work, the samples could not be frozen before analysis. As this was not possible, we terminated these experiments and changed the protocol for Subtask 3.

III. Research Accomplishments for Subtask 3: As Dr. Dimitri Azar our initial collaborator moved from Massachusetts Eye and Ear Infirmary, we enlisted COL Kraig S. Bower, LTC Charles Coe, and Ms. Denise Sediq from Walter Reed Army Medical Center as new collaborators. Our IRB documents were approved and the details of the clinical study specified. However, with the failure of Subtask 2, we changed portions of the clinical study. We decided to continue collecting impression cytology specimens, but instead of stimulating them, we would analyze them by

quantifying the number of filled goblet cells per total number of goblet cells using immunofluorescence microscopy. In addition, we began to collaborate with Dr. Robert Sack of SUNY State College of Optometry, New York, NY. We will send Dr. Sack the Schirmer strips that we are already using to determine the volume of tears. He will analyze the tears collected on them by microarray for inflammatory mediators. We have new, approved protocols for these changes. Our patient recruitment has now started at Walter Reed Army Medical Center. To date 22 subjects have been enrolled and treated. Of these 12 have received LASIK and 10 PRK. Six additional patients have been scheduled for enrollment/preop/treatment in the next two weeks. Of these individuals 1 will receive LASIK and 5 PRK. There have been no adverse events to report to date.

KEY RESEARCH ACCOMPLISHMENTS

- Developed a method for measuring multiple second messengers in a single conjunctival sample using bioplex technology.
- EGF and cholinergic agonists cause changes in phosphoproteins ERK and AKT in control, unwounded, mouse conjunctiva, but β-adrenergic agonists alter STAT-3 and perhaps P79S6.
- Two days after wounding the wounded and unwounded conjunctiva respond differently from each other and differently from the control unwounded mice.
- Six days after wounding conjunctival response is lost.
- Two and four weeks after wounding, conjunctival response begins to return.
- Conjunctival cells collected by impression cytology from human subjects do not respond to carbachol and cannot be studied.
- Enrollment has begun for Specific Aim 3 to study the tear film and conjunctiva of patients before and after refractive surgery.
-

REPORTABLE OUTCOMES

Konomi K., Azar D., Dartt D.A. Alteration in Signaling Pathways in Mouse Conjunctiva after Corneal Wounding Mimicking Nerve Loss in LASIK Surgery, Invest. Ophthalmol. Vis. Sci. 2006 47: E-Abstract 4600.

CONCLUSIONS

We conclude that in the normal mouse conjunctiva, the epithelial cells differentially respond to growth factors, cholinergic and beta-adrenergic agonists. Corneal wounding that mimics laser refractive surgery changes this response. Unfortunately, human conjunctival cell function cannot be studied on impression cytology samples.

APPENIDX

1. Konomi K., Azar D., Dartt D.A. Alteration in Signaling Pathways in Mouse Conjunctiva after Corneal Wounding Mimicking Nerve Loss in LASIK Surgery, Invest. Ophthalmol. Vis. Sci. 2006 47: E-Abstract 4600.

Table 1. Alterations in phosphoprotein levels in non-wounded Balb-c control mouse conjunctiva following stimulation with EGF, Carbachol and Isoproterenol using BioRad multiplex assay

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
Phospho ERK	1.35	0.0018 *	1.34	0.0042 *	1.02	0.87
Phospho JNK	1.34	0.053	1.29	0.076	1.14	0.26
Phospho P38	1.06	0.609	0.96	0.73	1.07	0.39
Phospho AKT	1.49	0.044 *	1.22	0.002 *	1.05	0.79
Phospho IkB-alpha	1.41	0.196	1.22	0.07	0.98	0.79
Phospho STAT-3	1.27	0.09	1.32	0.09	1.19	0.012 *
Phospho P70-S6	1.24	0.43	1.30	0.38	0.88	0.18

* P <0.05
N= 7 experiments
Age of mice: 12 weeks old Balb-c mice

Table 2. Alterations in phosphoprotein levels in mouse conjunctiva in the wounded (Rt) eye following stimulation with EGF, Carbachol and Isoproterenol 2 days after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.36	0.0006 *	1.14	0.016 *	0.88	0.07
Phospho JNK	1.44	0.0006 *	1.29	0.0004 *	1.23	0.11
Phospho P38	1.23	0.019 *	1.19	0.22	1.12	0.41
Phospho AKT	1.63	0.39	1.27	0.13	0.87	0.16
Phospho IkB-alpha	1.04	0.31	1.12	0.07	1.03	0.72
Phospho STAT-3	1.23	0.29	1.15	0.21	1.26	0.23
Phospho P70-S6	2.15	0.21	1.53	0.24	1.34	0.39

* P <0.05
N= 5 experiments
Age of mice: 12 weeks old Balb-c mice

Table 3. Alterations in phosphoprotein levels in mouse conjunctiva in the unwounded (left) eye following stimulation with EGF, Carbachol and Isoproterenol 2 days after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.59	0.0001 *	1.23	0.06	1.08	0.46
Phospho JNK	1.18	0.17	1.54	0.02*	1.12	0.16
Phospho P38	1.29	0.04 *	1.28	0.007 *	1.12	0.14
Phospho AKT	1.35	0.21	1.52	0.50	1.66	0.03*
Phospho IkB-alpha	1.17	0.11	1.13	0.01 *	1.08	0.19
Phospho STAT-3	1.13	0.32	1.33	0.04 *	0.92	0.49
Phospho P70-S6	1.24	0.19	1.49	0.009 *	0.89	0.47

* P <0.05
N= 5 experiments
Age of mice: 12 weeks old Balb-c mice

Table 4. Alterations in phosphoprotein levels in mouse conjunctiva in the wounded (right) eye following stimulation with EGF, Carbachol and Isoproterenol 6 days after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.13	0.30	0.84	0.22	1.01	0.86
Phospho JNK	0.97	0.66	0.81	0.40	0.79	0.39
Phospho P38	0.81	0.007 *	0.82	0.10	0.87	0.16
Phospho AKT	0.55	0.04*	0.62	0.20	0.94	0.90
Phospho IkB-alpha	0.89	0.12	1.04	0.38	1.08	0.17
Phospho STAT-3	1.12	0.52	1.01	0.97	1.08	0.46
Phospho P70-S6	0.80	0.53	0.93	0.51	0.72	0.08

* P <0.05
N= 3 experiments
Age of mice: 12-week old Balb-c mice

Table 5. Alterations in phosphoprotein levels in mouse conjunctiva in the unwounded (left) eye following stimulation with EGF, Carbachol and Isoproterenol 6 days after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.74	0.0005*	1.30	0.06	1.13	0.29
Phospho JNK	1.60	0.04*	1.38	0.07	1.07	0.81
Phospho P38	1.60	0.12	1.41	0.10	1.18	0.52
Phospho AKT	1.54	0.15	1.43	0.02*	1.45	0.45
Phospho IkB-alpha	1.63	0.05*	1.69	0.08	1.57	0.36
Phospho STAT-3	1.23	0.09	1.16	0.10	0.90	0.73
Phospho P70-S6	1.50	0.11	1.42	0.27	1.28	0.45

* P <0.05
N= 3 experiments
Age of mice: 12-week old Balb-c mice

Table 6. Alterations in phosphoprotein levels in mouse conjunctiva in the wounded (right) eye following stimulation with EGF, Carbachol and Isoproterenol 2 weeks after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.56	0.03*	1.42	0.002*	1.02	0.89
Phospho JNK	0.97	0.66	1.48	0.08	1.63	0.45
Phospho P38	0.86	0.34	1.22	0.10	0.95	0.68
Phospho AKT	1.36	0.40	1.65	0.38	1.25	0.07
Phospho IkB-alpha	1.06	0.80	1.37	0.26	1.02	0.82
Phospho STAT-3	0.79	0.30	0.86	0.39	0.98	0.93
Phospho P70-S6	1.97	0.54	2.37	0.16	1.11	0.55

* P <0.05
N= 6 experiments
Age of mice: 12-week old Balb-c mice

Table 7. Alterations in phosphoprotein levels in mouse conjunctiva in the unwounded (left) eye following stimulation with EGF, Carbachol and Isoproterenol 2 weeks after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.33	0.17	0.98	0.45	1.10	0.22
Phospho JNK	1.77	0.17	0.72	0.09	0.88	0.45
Phospho P38	1.29	0.09	0.70	0.02*	0.94	0.51
Phospho AKT	1.35	0.40	0.91	0.55	0.97	0.62
Phospho IkB-alpha	1.17	0.06	0.80	0.23	1.12	0.34
Phospho STAT-3	1.17	0.41	0.73	0.08	0.97	0.87
Phospho P70-S6	1.94	0.11	0.91	0.75	1.01	0.96

* P <0.05
N= 6 experiments
Age of mice: 12-week old Balb-c mice

Table 8. Alterations in phosphoprotein levels in mouse conjunctiva in the wounded (right) eye following stimulation with EGF, Carbachol and Isoproterenol 4 weeks after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.63	0.0006*	1.21	0.11	1.07	0.62
Phospho JNK	1.30	0.39	1.28	0.13	1.21	0.49
Phospho P38	1.14	0.24	1.05	0.66	1.24	0.20
Phospho AKT	1.87	0.33	1.11	0.68	0.97	0.91
Phospho IkB-alpha	1.28	0.23	1.17	0.21	1.16	0.33
Phospho STAT-3	0.94	0.77	1.08	0.38	1.18	0.21
Phospho P70-S6	1.14	0.61	1.05	0.68	0.99	0.96

* P <0.05
N= 6 experiments
Age of mice: 12-week old Balb-c mice

Table 9. Alterations in phosphoprotein levels in mouse conjunctiva in the unwounded (left) eye following stimulation with EGF, Carbachol and Isoproterenol 4 weeks after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.74	0.05*	1.51	0.15	1.24	0.39
Phospho JNK	1.37	0.25	1.34	0.13	1.39	0.11
Phospho P38	1.53	0.15	1.31	0.35	1.48	0.17
Phospho AKT	1.52	0.21	1.72	0.09	1.56	0.23
Phospho IkB-alpha	1.31	0.34	1.34	0.34	1.64	0.21
Phospho STAT-3	1.49	0.19	1.14	0.51	1.51	0.09
Phospho P70-S6	2.76	0.20	1.58	0.36	1.85	0.28

* P <0.05
N= 6 experiments
Age of mice: 12-week old Balb-c mice

Table 10. Mouse conjunctival stimulation experiments for multiplex analysis of phosphoproteins

Experiment type	No. of experiments done	No. of experiments analyzed by multiplex	No. of experiments to be analyzed by multiplex
Unwounded	7	7	0
2 day post-wound	5	5	0
6 day post-wound	4	3	1 (lost)
2 week post-wound	6	6	0
4 week post-wound	6	6	0

Invest Ophthalmol Vis Sci 2006;47: E-Abstract 4600.

4600÷B802

Alteration in Signaling Pathways in Mouse Conjunctiva after Corneal Wounding Mimicking Nerve Loss in LASIK Surgery

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Abstract

Purpose: To determine if the response of conjunctival cells to nerves and growth factors is altered in a mouse model of dry eye and if loss of corneal nerves (induced by a corneal wound mimicking refractive surgery) alters this response.

Methods: Four week old, female BALB/c mice and 12 week old, female MRL/MPJ Faslpr mice were used as nonö dry eye and dry eye models.(n= 9, each strain) Corneal wounds were made through the stromal layer in 6 of 9 mice in each strain by using a trephine, mimicking nerve loss in LASIK surgery. Conjunctival tissue was collected from unwounded eyes and from eyes 2 and 6 days after wounding. Tissue was incubated for 5 minutes with keratinocyte basal media alone, the cholinergic agonist carbachol (Cch) ($10^{ö5}$M), EGF($10^{ö7}$M), and the betaöadrenergic agonist Isoproterenol ($10^{ö5}$M). The amount of the phospho (activated) proteins AKT (known as protein kinase B), extracellular signalöregulated kinase (ERK), cöJun Nöterminal kinase (JNK) and p38 mitogenöactivated protein kinase (MAPK) were measured by multiöplex assay. Results were standardized by measuring the amount of total ERK.

Results: Under basal conditions, the amount of phospho JNK in unwounded eyes and phospho ERK 6 days after wounding was higher in the dry eye model than in the nonödry eye model (p= 0.046, 0.033, respectively). Compared to basal, Cch stimulation of JNK phosphorylation in unwounded eyes was lower in the dry eye model than in the nonödry eye model (p=0.048). In the nonödry eye model Cch increased the

phosphorylation of p38 MAPK above basal in unwounded eyes (p=0.02), but this stimulation was decreased in tissue 6 days after wounding (p=0.004). Cch did not significantly alter AKT activation. EGF and isoproterenol did not significantly alter activation of any phosphoproteins measured.

Conclusions: In a mouse model the response of conjunctival cells to cholinergic agonists appears to be altered by dry eye status and loss of corneal nerves (as could occur in refractive surgery).

Key Words: conjunctiva ð phosphorylation ð cornea: tears/tear film/dry eye