Analysis of Ethanol Effects on Corneal Epithelium

Joo Youn Oh,[1,2] Ji Min Yu,[3] and Jung Hwa Ko[1,2]

[1]Department of Ophthalmology, Seoul National University Hospital, Seoul, Korea
[2]Laboratory of Ocular Regenerative Medicine and Immunology, Biomedical Research Institute, Seoul National University Hospital, Seoul, Korea
[3]Institute for Regenerative Medicine, Texas A&M Health Science Center College of Medicine at Scott & White, Temple, Texas

Correspondence: Joo Youn Oh, Department of Ophthalmology, Seoul National University Hospital, 101 Daehak-ro, Jongno-gu, Seoul, 110-744, Korea;
jooyounoh77@gmail.com.

Submitted: January 23, 2013
Accepted: May 1, 2013

Citation: Oh JY, Yu JM, Ko JH. Analysis of ethanol effects on corneal epithelium. *Invest Ophthalmol Vis Sci*. 2013;54:3852–3856. DOI:10.1167/iovs.13-11717.

Purpose. Ethanol is widely used in ocular surface surgeries and for the treatment of corneal diseases. However, ethanol is a toxic agent that is related to the development of a number of alcohol-related diseases. Despite the common use of ethanol for therapeutic purposes in ophthalmology, effects of ethanol on the ocular surface have been poorly defined. Hence, we performed this study to investigate effects of ethanol on corneal epithelium from various aspects.

Methods. We exposed corneal epithelial cells in culture to different concentrations of ethanol for 30 seconds and evaluated the cells for toxicity, survival, and expression of cell-specific markers and inflammatory cytokines at 24, 48, and 72 hours after ethanol exposure.

Results. We found that ethanol markedly decreased the viability of cells in a concentration-dependent manner by causing cell lysis, suppressing proliferation, and inducing apoptosis. Also, expression of corneal epithelial cell–specific markers, both stem cell and differentiation markers, was significantly reduced by ethanol exposure. Expression of proinflammatory cytokines and chemokines was highly increased in corneal epithelial and stromal cells that were exposed to ethanol.

Conclusions. Together, data suggest that brief exposure of the corneal surface to ethanol may have long-term effects by disrupting the integrity of corneal epithelium and generating inflammation, both of which are precursors to a number of ocular surface diseases.

Keywords: cornea, corneal epithelium, ethanol

Ethanol is widely used in ocular surface surgeries such as photorefractive keratectomy or pterygium excision and to treat a variety of corneal diseases such as recurrent corneal erosion and infectious keratitis.[1–7] The use of ethanol in ophthalmology practice is largely based on its ability to damage corneal epithelium and to make epithelial debridement easier. For instance, application of diluted ethanol (mostly 20% ethanol for 30 seconds) delaminates the corneal epithelium at the level of the hemidesmosomal attachments and creates a smooth stromal surface for further surface ablation by excimer laser.[8] Also, ethanol delamination of the corneal epithelium can be used to obtain specimens for microbial detection in patients with corneal infection.[6] Or ethanol helps to remove abnormal epithelial sheets to allow for firmer adhesion of new epithelial cells in patients with recurrent corneal erosion.[3–5]

However, studies on alcohol-related diseases have demonstrated that ethanol is toxic to cells in the liver or brain by inducing apoptosis and increasing reactive oxygen species formation.[9–11] These harmful effects of ethanol on cells and tissues predispose alcohol drinkers to a number of systemic diseases including cancers, liver diseases, and cardiovascular or cerebrovascular diseases.[12,13]

Despite the common usage of ethanol for therapeutic purposes in ophthalmology, toxic effects of ethanol on the ocular surface epithelium have been poorly defined. Hence, we here investigated the effects of ethanol on corneal epithelium from various aspects: cytotoxicity, apoptosis, proliferation, differentiation, and production of inflammatory cytokines and chemokines.

Methods

Cell Culture and Reagents

Primary human corneal limbal epithelial cells (hCLECs) from one donor at passage 2 were obtained from CELLnTEC (Bern, Switzerland) and cultured in serum-free CnT-20 media (CELLnTEC). Passage 4 cells were used in all experiments. For injury induction, cells were seeded at a density of 25,000 cells/cm^2 and incubated in 20% to 100% ethanol for 30 seconds. After thorough washing with PBS three times, the cells were cultured in media for 24 to 72 hours and subjected to further assays.

Cytotoxicity, Viability, and Proliferation Assays

Cytotoxicity was evaluated by measuring activity of LDH released from the cells damaged by ethanol following the manufacturer's protocol (LDH-Cytotoxicity Assay Kit; Abcam, Cambridge, MA). Cell viability and proliferation were measured using MTT assay according to the manufacturer's protocol (Vybrant MTT Cell Proliferation Assay Kit; Invitrogen, Carlsbad, CA). Cell proliferation was quantitated by measuring bromodeoxyuridine (BrdU) incorporation in cells using colorimetric immunoassay (Cell Proliferation ELISA, BrdU; Roche, Indianapolis, IN).

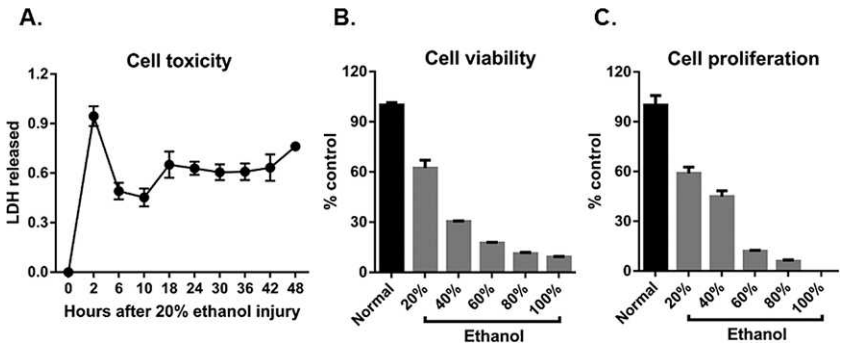

FIGURE 1. Effect of ethanol on cell lysis, viability, and proliferation. (**A**) Analysis of lactate dehydrogenate (LDH) in the supernatant of cultures of human corneal limbal epithelial cells showed that LDH was released from the cells immediately after ethanol exposure, indicating direct toxicity of ethanol. (**B**) MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay showed that the number of metabolically active cells was markedly decreased by ethanol in a concentration-dependent manner, demonstrating that the overall number of viable cells was reduced. (**C**) Bromodeoxyuridine (BrdU) uptake assay revealed that cell proliferation was also decreased by ethanol.

Apoptosis Assay

Apoptosis was measured by two independent methods. First, cells were stained with a combination of Annexin V-FITC (Molecular Probes, Inc., Leiden, The Netherlands) and PI-PE (Molecular Probes, Inc.) and analyzed by flow cytometry. The populations of early apoptotic cells (Annexin V^+/PI^-) and late apoptotic or necrotic cells (Annexin V^+/PI^+) were evaluated. Second, the cells were analyzed for apoptosis by staining with MitoCapture (BioVision, Mountain View, CA). In healthy cells, MitoCapture accumulates and aggregates in the mitochondria, giving off a bright red fluorescence. In apoptotic cells, MitoCapture does not aggregate in the mitochondria due to the altered mitochondrial transmembrane potential; thus it remains in the cytoplasm in its monomer form, fluorescing green. The cells were observed by fluorescence microscopy, and apoptotic cells were counted.

Immunocytochemical Staining

For immunocytochemical staining, the cells that were cultured in eight-well chamber slides (Lab-Tek II Chamber Slide; Nalge Nunc, Rochester, NY) were washed with PBS, fixed with 4% paraformaldehyde for 10 minutes, and incubated in blocking buffer (3% BSA, 0.2% Triton X-100, 0.02% azide in PBS) for 1 hour. The cells were then incubated with primary antibodies in blocking buffer for 1 hour and secondary antibodies for 30 minutes after PBS washing. Goat polyclonal antihuman p63 (sc-25,039; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and goat polyclonal antihuman cytokeratin 3 (sc-49,179; Santa Cruz Biotechnology, Inc.) were used as primary antibodies. Antigoat IgG (1:1000) (Jackson Immunoresearch, West Grove, PA) was used as secondary antibody. The slides were visualized with fluorescent microscopy (Eclipse 80i; Nikon, Melville, NY).

Real-Time RT-PCR

The expression of proinflammatory cytokines or corneal epithelial cell–specific markers was evaluated by real-time RT-PCR. For RNA extraction, cells were lysed in RNA isolation reagent (RNA Bee; Tel-Test, Inc., Friendswood, TX), and total RNA was then extracted using RNeasy Mini kit (Qiagen, Valencia, CA). Double-stranded cDNA was synthesized by reverse transcription (SuperScript III; Invitrogen). Real-time amplification was performed (Taqman Universal PCR Master Mix; Applied Biosystems, Carlsbad, CA) and analyzed on an automated instrument (7900HT Fast Real-Time PCR System; Applied Biosystems). PCR probe sets were commercially purchased (Taqman Gene Expression Assay Kits, Applied Biosystems). For assays, reactions were incubated at 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds followed by 60°C for 1 minute. For normalization of gene expression, 18S rRNA probe was used as an internal control.

Statistical Analysis

Comparisons of parameters among the groups were made by unpaired and two-tailed Student's *t*-tests using SPSS software (SPSS 12.0; SPSS, Inc., Chicago, IL). Differences were considered significant at $P < 0.05$.

RESULTS

Effect of Ethanol on Cell Lysis

In order to test direct toxic effects of ethanol on the cells, hCLECs were incubated in 20% ethanol for 30 seconds and cultured for 48 hours in fresh media. The amounts of lactate dehydrogenate (LDH) in the supernatant of cell cultures were serially measured for 48 hours. Results revealed that LDH amount was abruptly increased upon ethanol exposure and maintained at steady levels thereafter (Fig. 1A). These data indicate that ethanol exerted an immediate toxic effect on hCLECs by damaging the cell membrane and causing cell lysis.

Effect of Ethanol on Cell Viability, Proliferation, and Apoptosis

To evaluate the effect of ethanol on cell survival, hCLECs were exposed to different concentrations of ethanol (20%–100%) for 30 seconds and assayed for viability, proliferation, and apoptosis 24 hours later. We found that the numbers of metabolically active cells representing viable cells were markedly reduced by ethanol exposure in a concentration-dependent manner as determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Fig. 1B). The decrease in cell viability was further explained by a decrease in cell proliferation and an increase in cell apoptosis after ethanol exposure. Bromodeoxyuridine uptake assay showed that cell proliferation was markedly reduced after ethanol exposure in a concentration-dependent manner (Fig. 1C). Also, the percentage of apoptotic cells was significantly increased by ethanol as indicated by flow cytometry for Annexin V^+/PI^+ cells (late apoptotic cells) or Annexin V^+/PI^- (early apoptotic cells) (Fig. 2A). Time course demonstrated that the percentage of Annexin V^+/PI^+ cells remained significantly increased until 72 hours

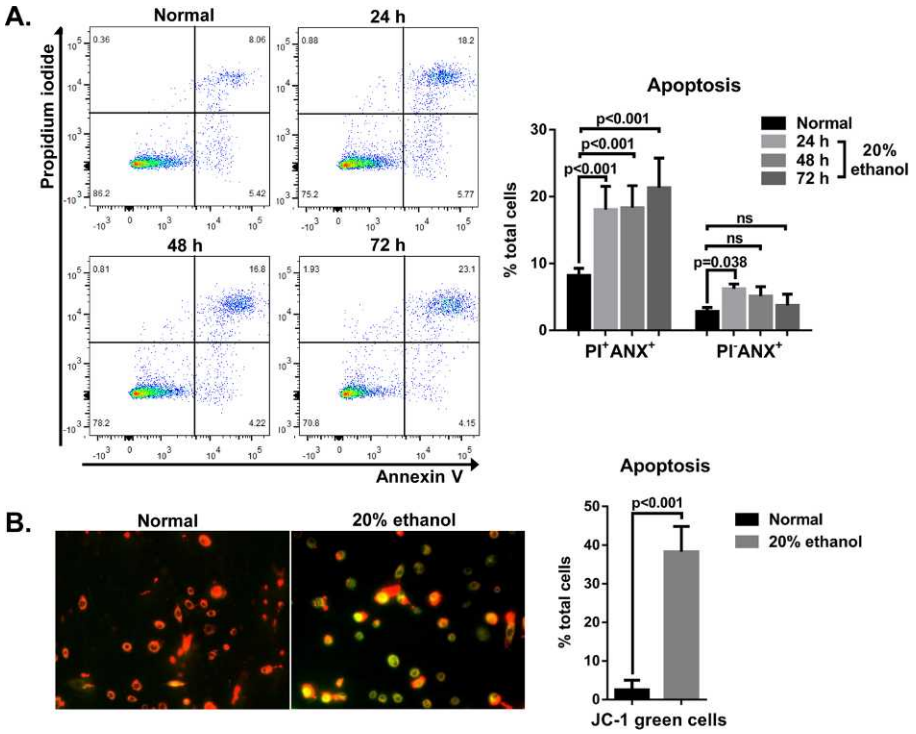

FIGURE 2. Effect of ethanol on apoptosis. (**A**) Flow cytometry showed that the percentages of either Annexin V^+/PI^- (early apoptotic cells) or Annexin V^+/PI^+ cells (late apoptotic cells) were significantly increased in human corneal limbal epithelial cells at 24 hours after ethanol exposure. While the percentage of Annexin V^+/PI^+ cells remained increased until 72 hours after ethanol exposure, the percentage of Annexin V^+/PI^- cells gradually decreased to normal levels until 72 hours. (**B**) Immunostaining of the cells with JC-1 dye (MitoCapture) showed that the number of cells stained with fluorescence green, indicating apoptotic or necrotic cells with altered mitochondrial transmembrane potential cells, was significantly increased 24 hours after ethanol exposure.

after ethanol exposure ($P < 0.001$), while the percentage of Annexin V^+/PI^- cells gradually decreased to normal levels until 72 hours (Fig. 2A). Induction of apoptosis by ethanol was further confirmed by assays of hCLECs stained with a JC-1 dye (MitoCapture) that identifies apoptotic and necrotic cells (Fig. 2B). The number of cells with disrupted mitochondrial transmembrane potential that fluoresced green was significantly increased by ethanol ($P < 0.001$, Fig. 2B).

Effect of Ethanol on Expression of Stem Cell–Specific and Differentiation Markers in Corneal Epithelial Cells

The hCLECs include two populations of stem cells and differentiated cells. In order to evaluate the effect of ethanol on the identity and specificity of hCLECs, we next assayed for expression of putative markers for epithelial stem cells, p63 (transformation-related protein 63) and ABCG2 (ATP-binding cassette subfamily G member 2), and for K3/12 (cytokeratin 3/12), a marker for fully differentiated epithelial cells. Immunostaining revealed that the numbers of p63-expressing cells as well as K3/12-expressing cells in cultures of hCLECs were markedly decreased 24 hours after ethanol exposure (Figs. 3A, 3B). As expected, real-time RT-PCR also demonstrated that expression levels of p63, ABCG2, and K12 were markedly decreased in hCLECs 24 hours after ethanol exposure, suggesting that the markers specific for corneal limbal epithelial cells were reduced by ethanol (Fig. 3C). Time course analysis indicated that the levels of p63, ABCG2, and K12 transcripts were significantly decreased until 48 hours after ethanol exposure and normalized at 72 hours, reflecting compensation for damaged cells by healthy cells (Fig. 3C).

Effect of Ethanol on Inflammatory Cytokine Production in Corneal Epithelial and Stromal Cells

Production of proinflammatory cytokines and chemokines by resident cells in the cornea, including corneal epithelial and stromal cells, plays an important role in ocular surface inflammation, which is a major predisposing factor for ocular surface disorders.[14,15] Therefore, we investigated levels of proinflammatory cytokines and chemokines in corneal epithelial or stromal cells that were exposed to ethanol. We found that transcript levels of IL-1β, IL-6, IL-8, chemokine (C-C motif) ligand (CCL) 2, and matrix metalloproteinase (MMP) 9 were significantly upregulated in hCLECs at 24, 48, and 72 hours after ethanol exposure (Fig. 4A). Also, the levels of transcripts for IL-6, IL-8, CCL2, and MMP9 were markedly increased in corneal stromal cells exposed to ethanol compared to untreated controls at all time points tested (Fig. 4B). Data suggest that brief exposure of corneal cells to ethanol may lead to persistent inflammation in the ocular surface.

DISCUSSION

The data demonstrate that ethanol not only exerted an immediate cytotoxic effect on corneal epithelial cells, but significantly interfered with cell viability by reducing proliferation and inducing apoptosis. Also, ethanol decreased the expression of markers specific for corneal epithelial cells and increased the expression of proinflammatory cytokines and chemokines in corneal epithelial and stromal cells. Of note, the harmful effects of ethanol were apparent 72 hours after the cells were treated with 20% ethanol for 30 seconds. These results suggest that brief exposure of corneal epithelium to

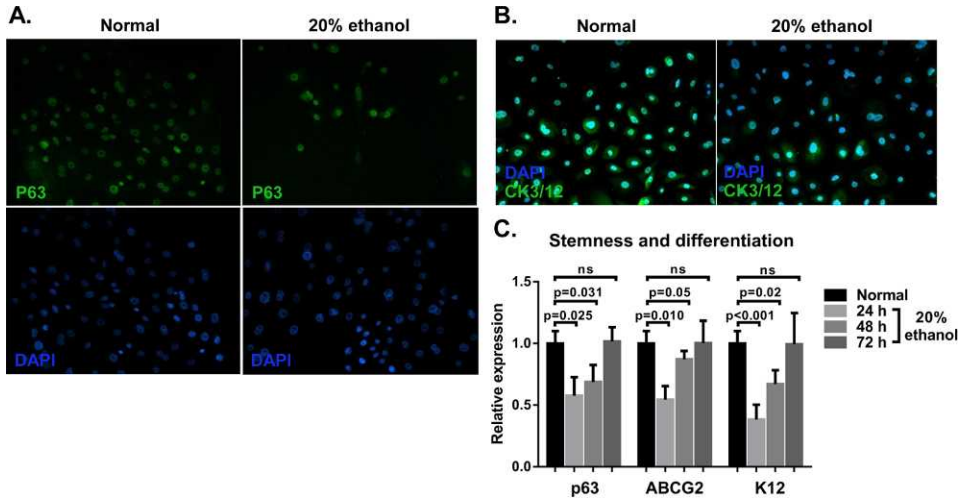

FIGURE 3. Effect of ethanol on corneal epithelial cell marker expression. (**A**, **B**) Immunostaining for p63, an epithelial stem cell marker, and CK3/12, a differentiated corneal epithelial cell marker, showed that the numbers of p63- or CK3/12-expressing cells were markedly decreased in human corneal limbal epithelial cells at 24 hours after ethanol exposure. (**C**) Real-time RT-PCR analysis demonstrated that levels of transcripts for p63, ABCG2, and CK3/12 were significantly reduced at 24 and 48 hours after ethanol exposure and normalized at 72 hours.

ethanol, a commonly performed clinical procedure,[1–7] may have long-term effects on the ocular surface by disrupting the integrity of corneal epithelium and generating inflammation.

One of the critical observations here is that ethanol induced a significant expression of proinflammatory cytokines in corneal epithelial cells and stromal cells. Although alcohol is generally considered immunosuppressive,[16] reports have indicated that ethanol-treated mice had increased levels of proinflammatory cytokines in lung, spleen, and liver[17] and that orally administered ethanol induced microglial activation and expression of proinflammatory cytokines in brains of rats.[18] Ocular surface inflammation is a main pathogenic factor for ocular surface disorders including dry eye syndrome,[14,15] and resident cells in the cornea including corneal epithelial and stromal cells play an important role in ocular surface inflammation by producing proinflammatory cytokines and chemokines. The increased expression of proinflammatory cytokines in corneal cells after ethanol exposure may predispose the ocular surface to diseases.

Another notable finding of this study is that ethanol reduced the proportion of the cells expressing stem cell markers in corneal epithelium and also decreased the total number of viable cells. Therefore, it is possible that exposure of the corneal limbal area to ethanol may deplete epithelial progenitor cells, and cause permanent failure of corneal epithelial regeneration in patients with numbers of epithelial progenitor cells insufficient to compensate for damaged cells. To test this possibility, future studies would be necessary.

Although the mechanism has not been clarified, previous studies have reported that dry eye syndrome is more prevalent and the ocular surface is significantly disrupted in heavy alcohol drinkers.[19–21] Given a high concentration of ethanol detected in tears after oral administration of alcohol,[14] it is possible that increased levels of ethanol in tears may directly affect the corneal epithelium as shown in this study and thus lead to the development of dry eye syndrome, in which integrity of corneal epithelium and inflammation play an important role.

In the present study, we used cultures of corneal epithelial cells because the use of the culture system enabled us to test the direct effects of ethanol on corneal epithelial cells independent of any effects on inflammatory or immune cells. However, the observations obtained from in vitro studies may not exactly reflect the phenomenon in vivo. In an in vivo system, phagocytes such as macrophages are immediately mobilized to the site of injury and clear necrotic or apoptotic cells that reduce the production of danger signals from dying or dead cells, and thereby inhibit the circuit of proinflammatory signals and promote the resolution.[22,23] In fact, in the present study, increases of apoptosis and inflammatory cytokine production in corneal epithelial cells persisted until 72 hours after ethanol injury, suggesting that the proinflamma-

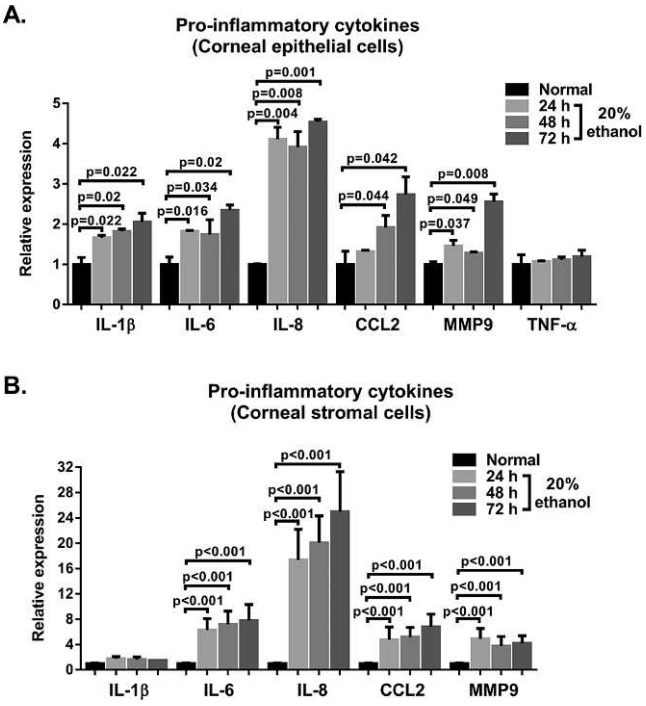

FIGURE 4. Effect of ethanol on inflammatory cytokine production in corneal epithelial and stromal cells. Real-time RT-PCR showed that expression of proinflammatory cytokines and chemokines was significantly increased after ethanol exposure in human corneal limbal epithelial cells (**A**) and human corneal stromal cells (**B**).

tory circuit was not inhibited because of a lack of the clearance system. Hence, in vivo studies would be necessary to further elucidate harmful effects of ethanol on the cornea.

In conclusion, the results demonstrated that ethanol had both direct and indirect effects on corneal epithelial cells by interfering with cell viability and identity and by upregulating expression of proinflammatory cytokines. Both disintegrity of corneal epithelium and inflammation in the ocular surface are hallmarks of and precursors to a number of ocular surface diseases such as severe dry eye syndrome. Given that 20% ethanol is commonly used for ocular surface surgeries, care should be taken with the use of ethanol in clinical practice.

Acknowledgments

Supported by a grant from the Korean Health Technology R&D Project, Ministry of Health and Welfare, Republic of Korea (A120239).

Disclosure: **J.Y. Oh**, None; **J.M. Yu**, None; **J.H. Ko**, None

References

1. Sia RK, Ryan DS, Stutzman RD, et al. Alcohol versus brush PRK: visual outcomes and adverse effects. *Lasers Surg Med*. 2012;44:475–481.
2. Luger MH, Ewering T, Arba-Mosquera S. Consecutive myopia correction with transepithelial versus alcohol-assisted photorefractive keratectomy in contralateral eyes: one-year results. *J Cataract Refract Surg*. 2012;38:1414–1423.
3. Singh RP, Raj D, Pherwani A, et al. Alcohol delamination of the corneal epithelium for recalcitrant recurrent corneal erosion syndrome: a prospective study of efficacy and safety. *Br J Ophthalmol*. 2007;91:908–911.
4. Dua HS, Lagnado R, Raj D, et al. Alcohol delamination of the corneal epithelium: an alternative in the management of recurrent corneal erosions. *Ophthalmology*. 2006;113:404–411.
5. Singh RP, Raj D, Pherwani A, et al. Alcohol delamination of the corneal epithelium for recalcitrant recurrent corneal erosion syndrome: a prospective study of efficacy and safety. *Br J Ophthalmol*. 2007;91:908–911.
6. Li ML, Shih MH, Huang FC, Tseng SH, Chen CC. Treatment of early Acanthamoeba keratitis with alcohol-assisted epithelial debridement. *Cornea*. 2012;31:442–446.
7. Tsumi E, Levy J, Pitchkhadze A, Baidousi A, Lifshitz T. New approach for pterygium removal using 20 % ethanol. *Int Ophthalmol*. 2012;32:443–448.
8. Browning AC, Shah S, Dua HS, et al. Alcohol debridement of the corneal epithelium in PRK and LASEK: an electron microscopic study. *Invest Ophthalmol Vis Sci*. 2003;44:510–513.
9. Cohen JI, Nagy LE. Pathogenesis of alcoholic liver disease: interactions between parenchymal and non-parenchymal cells. *J Dig Dis*. 2011;12:3–9.
10. Luo J. Mechanisms of ethanol-induced death of cerebellar granule cells. *Cerebellum*. 2012;11:145–154.
11. Cherian PP, Schenker S, Henderson GI. Ethanol-mediated DNA damage and PARP-1 apoptotic responses in cultured fetal cortical neurons. *Alcohol Clin Exp Res*. 2008;32:1884–1892.
12. Ezzati M, Riboli E. Can noncommunicable diseases be prevented? Lessons from studies of populations and individuals. *Science*. 2012;337:1482–1487.
13. Room R, Babor T, Rehm J. Alcohol and public health. *Lancet*. 2005;365:519–530.
14. Calonge M, Enríquez-de-Salamanca A, Diebold Y, et al. Dry eye disease as an inflammatory disorder. *Ocul Immunol Inflamm*. 2010;18:244–253.
15. Stevenson W, Chauhan SK, Dana R. Dry eye disease: an immune-mediated ocular surface disorder. *Arch Ophthalmol*. 2012;130:90–100.
16. Cook RT. Alcohol abuse, alcoholism and damage to the immune-system—a review. *Alcohol Clin Exp Res*. 1998;22:1927–1942.
17. Spies CD, Lanzke N, Schlichting U, et al. Effects of ethanol on cytokine production after surgery in a murine model of gram-negative pneumonia. *Alcohol Clin Exp Res*. 2008;32:331–338.
18. Zhao YN, Wang F, Fan YX, Ping GF, Yang JY, Wu CF. Activated microglia are implicated in cognitive deficits, neuronal death, and successful recovery following intermittent ethanol exposure. *Behav Brain Res*. 2013;236:270–282.
19. Moss SE, Klein R, Klein BE. Prevalence of and risk factors for dry eye syndrome. *Arch Ophthalmol*. 2000;118:1264–1268.
20. Cumurcu T, Gunduz A, Cumurcu BE, Gül IG, Akpolat N, Karlidag R. The changes in tear film parameters and impression cytology in heavily drinking men. *Cornea*. 2013;32:237–241.
21. Kim JH, Kim JH, Nam WH, et al. Oral alcohol administration disturbs tear film and ocular surface. *Ophthalmology*. 2012;119:965–971.
22. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol*. 2008;8:349–361.
23. Gronert K. Resolution, the grail for healthy ocular inflammation. *Exp Eye Res*. 2010;91:478–485.