

Evaluation for Safety of Cultured Corneal Fibroblasts with Cotreatment of Alcohol and Mitomycin C

Tae-im Kim, Hungwon Tchah, Eun Hee Cho, and Michael S. Kook

PURPOSE. To investigate the effects of alcohol and mitomycin C (MMC) on cultured corneal fibroblast of the rabbit to determine the safety of this compound for clinical use.

METHOD. Corneal fibroblasts of New Zealand rabbits were cultured. Various concentrations (0%, 10%, 20%, 30%, 40%, and 60%) of ethanol were applied for 10, 20, 30, and 40 seconds to estimate dose- and time-dependent responses of cultured corneal fibroblasts. Cell viability was assessed using the MTT assay. Treated cells were additionally stained with Hoechst and annexin V for the identification of apoptosis. To investigate the effects of ethanol and MMC, dose and time dependency were evaluated after treatment with various concentrations of ethanol and MMC at different exposure times, and cell viability was established. To determine the latent effects of ethanol and MMC, cultured corneal fibroblasts were cotreated with various concentrations of ethanol and 0.02% MMC for various periods and washed out, and then one group was incubated for 24 hours and another group was not incubated. Cell viability was estimated, and Hoechst and annexin V staining were performed before and after incubation. To establish the pathway of cell death, caspase-3 activities were measured in cultured corneal fibroblasts treated with ethanol or MMC.

RESULTS. Cell viability after ethanol treatment was dose and time dependent. After application of ethanol for 10 seconds, cell viability was significantly reduced with 20% ethanol ($P = 0.001$). At 20, 30, and 40 seconds of treatment with 10% ethanol, cell viability was significantly reduced ($P < 0.01$). Hoechst and annexin V staining revealed typical characteristics of apoptosis, such as bright fluorescent chromatin condensation, low fluorescence of nuclear fragmentation, and cell membrane shrinkage. Cell viability was more significantly reduced after cotreatment with alcohol and MMC, compared with treatment with alcohol alone. Moreover, cell viability was considerably decreased in the incubated group, compared with the nonincubated group. After 24 hours of incubation, cultured corneal fibroblasts cotreated with 10% ethanol and 0.02% MMC were stained with Hoechst and annexin V. Results were similar to data obtained with ethanol-treated cells. However, after application of 20% alcohol and MMC, a significant number of cells were not viable and were detached from the well walls. Caspase-3 activity significantly increased after treatment with

30% ethanol only and 30% ethanol in conjunction with 0.02% MMC.

CONCLUSIONS. Alcohol and MMC reduced cell viability in cultured corneal fibroblasts in a dose- and time-dependent manner and had synergistic effects. This is related to the caspase-3 pathway, especially with concentrations of ethanol over 30%. Cotreatment with these reagents may significantly damage cultured corneal fibroblasts. (*Invest Ophthalmol Vis Sci.* 2004;45:86-92) DOI:10.1167/iovs.03-0520

Excimer laser technology provides a safe and effective approach for the correction of refractive errors.¹⁻⁷ Photorefractive keratectomy (PRK)¹⁻⁴ and laser in situ keratomileusis (LASIK)⁴⁻⁵ are the most frequently performed surgical procedures for refractive error treatment. Recently, laser subepithelial keratomileusis (LASEK) has been introduced, in which concentrations of 18% to 30% alcohol are used to create an epithelial flap, followed by excimer laser ablation and repositioning of the flap.⁶⁻⁷ LASEK is an alternative to these former two refractive surgical procedures because of the decrease in the pain and corneal haze of conventional PRK⁸⁻¹¹ and the absence of the flap complications of LASIK.¹²⁻¹⁷ However, in LASEK for high myopia, some degree of corneal haziness and regression is inevitable. Currently, mitomycin C (MMC) is used clinically to prevent corneal haze in treatments requiring high-risk LASEK, such as high myopia, second LASEK, and removal of subepithelial fibrosis.¹⁸ MMC, an alkylating antibiotic agent derived from *Streptomyces caespitosus*, blocks DNA and RNA replication and protein synthesis.¹⁹ MMC inhibits mitosis and proliferation of capillary and corneal endothelial cells, corneal epithelial cells, and conjunctival, Tenon's capsule, and other fibroblasts.^{20,21} However, the long-term safety of this compound in LASEK is not guaranteed.

After exposure to 20% ethanol for 20 to 30 seconds, more than the half the corneal epithelial cells remain viable^{6,22-25} but safety in keratocytes has not been assessed. Alcohol induces cell death or severe inflammatory reactions in keratocytes.²⁶⁻²⁸ No significant differences in keratocyte apoptosis were observed after de-epithelialization, either mechanical or induced with 18% alcohol. However, higher numbers of polymorphonuclear leukocyte cells were detected after de-epithelialization with 18% ethanol.²⁹

Although refractive surgery is an accurate and ideal method for the correction of refractive errors based on the development of equipment, such as the flying-spot beam, eye-tracker system, and wavefront-guided ablation for the treatment of high-order aberration, steroid treatment is currently the mainstay for modulating corneal wound healing. MMC has been introduced as a superior candidate for the modulation of corneal wound healing at the molecular level. Accordingly, we evaluated the effects and safety of alcohol and MMC on cultured keratocytes in vitro.

METHODS

Culture of Corneal Fibroblasts

This study was performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Corneal fibroblasts

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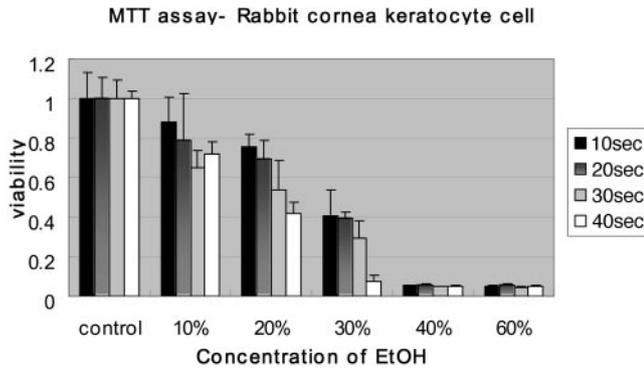


FIGURE 1. Time- and dose-dependent cell death induced by ethanol. Cell death was evaluated using the MTT assay. After treatment for 10 seconds, cell viability was significantly reduced at 20% ethanol ($P = 0.001$). At 20, 30, and 40 seconds of ethanol treatment, cell viability was significantly reduced at 10% ethanol ($P < 0.01$).

were cultured from New Zealand White rabbit cornea. Stromal explants were prepared by removing the epithelium and endothelium and cultured in a humidified atmosphere containing 5% CO_2 at 37°C with 10% FBS-DMEM using six-well tissue culture plates.

Cultured corneal fibroblasts migrated from explants onto the surface of wells. Cells reached confluence within 15 to 21 days. Next, cells were enzymatically detached by application of 0.05% trypsin at 37°C for 3 minutes. Suspended corneal fibroblasts were centrifuged at 1400 rpm for 5 minutes, and the supernatant was removed. Cells were resuspended in 20 mL of culture medium and cultured in 75-mL flasks at 37°C in 5% CO_2 until confluence. Next, cells were serially trypsinized, centrifuged, resuspended, and cultured until enough cells were available for experiments. We used third-passage cells in all the experiments. Cells were plated at 3×10^3 to 5×10^3 /well in 96-well

tissue culture plates and were incubated in 1 mL of 10% FBS-DMEM containing 5% CO_2 at 37°C for 24 to 48 hours.

The Effect of Alcohol on Corneal Fibroblasts

We evaluated dose- and time-dependent cell death induced by ethanol. Cultured corneal fibroblasts were treated with various concentrations of ethanol (0%, 10%, 20%, 30%, 40%, and 60%) for 10, 20, 30, and 40 seconds. Cell viability was evaluated using the MTT assay (a rapid colorimetric assay for cellular growth and survival: proliferation and cytotoxicity assay). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazoyl blue, Sigma-Aldrich, St. Louis, MO) and DMSO (dimethyl sulfoxide, Sigma-Aldrich) were used. The MTT assay was performed according to the procedure described in the following section.

For evaluation of the cell death mechanism induced by ethanol, we treated cells with 0%, 10%, 20%, and 30% ethanol for 20 seconds. Next, we stained cultured corneal fibroblasts with 1 mg/mL Hoechst 33342 (Hoechst-EthD; Molecular Probes, Leiden, The Netherlands) and photographed them with a fluorescence microscope.

After treatment with 0%, 10%, 20%, and 30% alcohol for 20 seconds, cells were stained with annexin V (Annexin V Binding Assay; Caltag Laboratories, Burlingame, CA) and observed the cells using the avidin-horseradish peroxidase (HRP) complex (1:300) and a 0.05% diaminobenzidine/0.01% H_2O_2 solution. Cells were photographed by a fluorescence microscope equipped with an annexin V filter.

MTT Assay

Cells ($3-5 \times 10^3$ in 100 mL per well) were grown overnight in 96-well plates and treated with ethanol and MMC, using the appropriate methods. After incubation, medium was replaced with fresh complete medium (100 mL). PBS (20 mL) containing MTT (5 mg/mL) was added to each well. In the absence of light, samples were incubated for 4 hours, followed by the removal of the medium. Precipitates were resuspended in 50 μL DMSO. Absorbance was measured on a plate

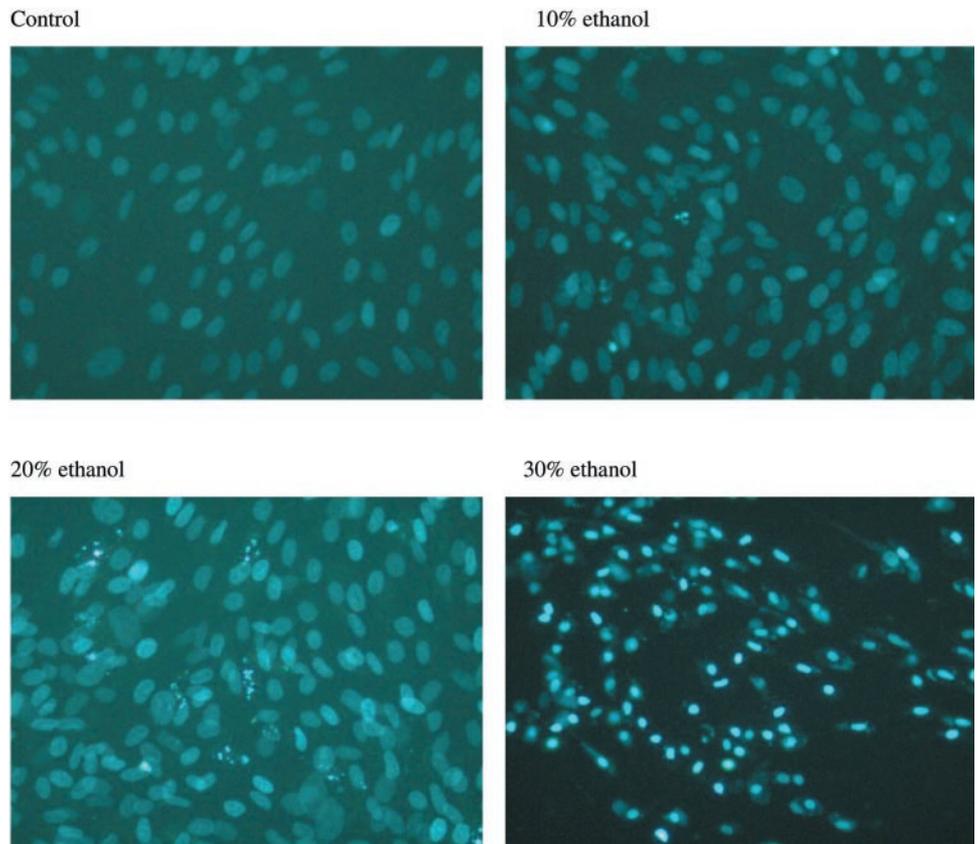


FIGURE 2. Micrographs of Hoechst-stained cells after treatment with concentrations of 0%, 10%, 20%, and 30% ethanol for 20 seconds. Bright fluorescence in faintly stained cell was chromatin condensation. Bright fluorescent small particles were nuclear fragments. These findings were observed in 10% ethanol-treated keratocytes. With increasing ethanol concentrations, fluorescent chromatin condensation and nuclear fragmentation were observed. Magnification, $\times 200$.

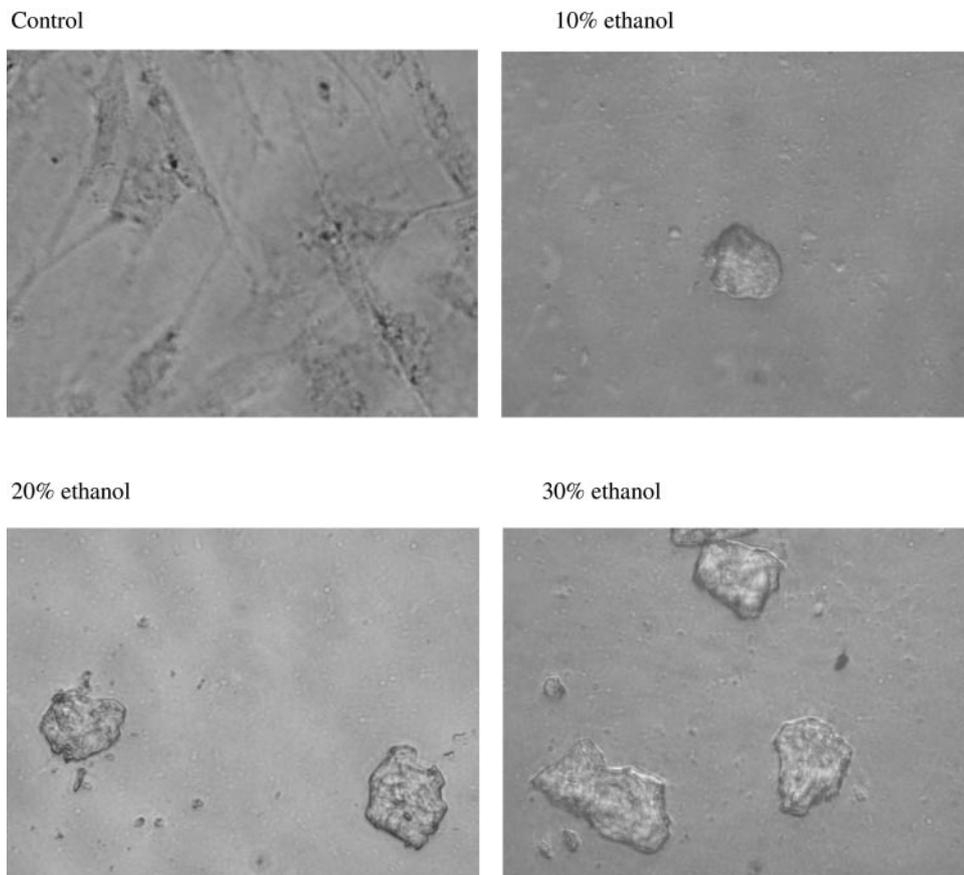


FIGURE 3. Micrograph of annexin V-stained cells after treatment with concentrations of 0%, 10%, 20%, and 30% ethanol for 20 seconds. Cells treated with alcohol were positive for annexin V and displayed typical characteristics of apoptosis, such as shrinkage of cytoplasm and perimembranous stippling. Magnification, $\times 400$.

reader at 570 nm (or 540 nm). Each experiment was performed in triplicate.

Coffect of Alcohol and MMC on Cultured Corneal Fibroblasts

For the evaluation of dose- and time-dependent cell death induced by alcohol at a constant concentration of MMC, cultured corneal fibroblasts were treated with 0%, 10%, 20%, 30%, 40%, and 60% ethanol for 10 and 20 seconds and 0.02% MMC was applied for 5 minutes. After the application, the cultures were washed with PBS, and cell viability was evaluated with the MTT assay. For assessing time-dependent cell death by MMC at a constant concentration of ethanol, cultured corneal fibroblasts were treated with 0.02% MMC for 5, 10, 20, 30, and 60 minutes after treatment with 20% ethanol for 10, 20, and 40 seconds. Cell viability was evaluated with the MTT assay.

To assess the effect of MMC and alcohol between incubated and nonincubated cells, cultured corneal fibroblasts were treated with various concentrations of ethanol and 0.02% MMC for various periods. All treated cultured corneal fibroblasts were washed three times with PBS solution, and fresh medium was added. Cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C with 10% FBS-DMEM for 24 hours. We evaluated cell viability with the MTT assay before and after incubation. For evaluation of the mechanism of cell death induced by alcohol and MMC, cells were stained with Hoechst 33342 and annexin V and photographed by fluorescence microscope.

Quantification of Caspase-3 Activity in Cultured Corneal Fibroblasts Treated with Alcohol and MMC

To evaluate the apoptotic pathway, cultured corneal fibroblasts were treated with various concentrations of alcohol (0%, 10%, 20%, and

30%) for 20 seconds, either with or without 0.02% MMC for 5 minutes. Caspase-3 levels were measured with a Caspase-3/CPP32 colorimetric protease assay using a kit (ApoTarget; BioSource International, Inc., Camarillo, CA). Cells ($3-5 \times 10^6$ in 100 mL per well) were grown overnight in 96-well plates. Apoptosis was induced in cells by the appropriate method. Cells were resuspended in 100 μ L chilled cell lysis buffer and incubated on ice for 10 minutes. After incubation, cells were centrifuged for 1 minute in a microcentrifuge (10,000g). The supernatant (cytosol extraction) was transferred to a fresh tube and left on ice. The standard Bradford method was used to assay protein concentration. Each cytosol extract was diluted to a concentration of 50 to 200 μ g protein per 50 μ L cell lysis buffer (1-4 mg/mL). Samples were measured and aliquoted to provide a 2 \times reaction buffer in a glass tube (assuming 50 μ L of 2 \times reaction buffer per sample). A volume of

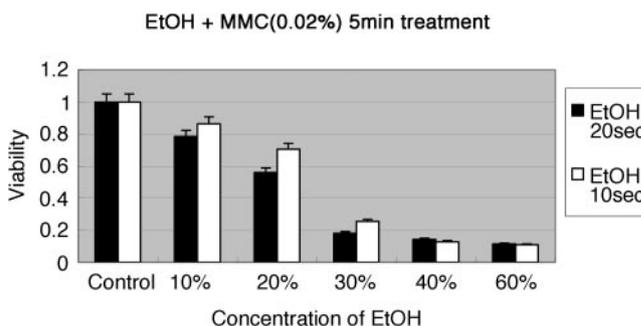
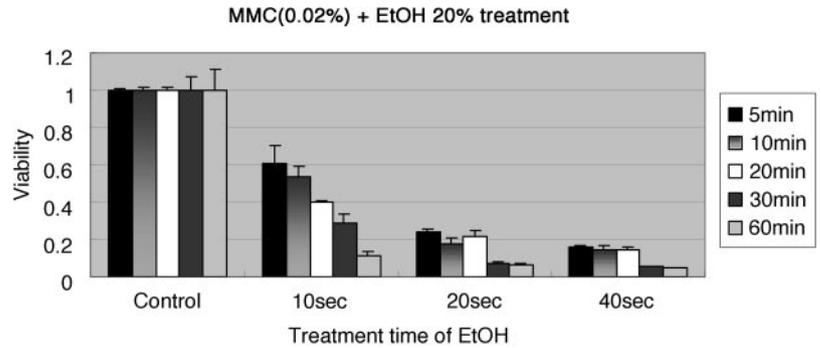


FIGURE 4. Dose- and time-dependent cell death induced by alcohol at a constant 0.02% concentration of MMC for 5 minutes. Cell viability was significantly reduced at 10% and higher ethanol concentrations in cultures treated for 20 seconds ($P < 0.01$) and at 20% and higher concentrations in cultures treated for 10 seconds ($P < 0.01$). After 20% ethanol treatment, fewer viable cells were observed.

FIGURE 5. Time-dependent cell death induced by 0.02% MMC at a constant concentration of alcohol (20%). Significant cell death occurred within 10 seconds in all groups ($P < 0.01$).



2× reaction buffer containing 10 mM DTT (50 μ L) and 5 μ L of the 4 mM DEVD-pNA substrate (200 μ M final concentration) was added to each sample. Samples were incubated for 2 hours in the dark. Absorbance was measured on a plate reader at 400 nm (or 405 nm).

Statistical Analysis

ANOVA was used for comparing cell viabilities. Statistical significance was set at $P < 0.05$. Data were analyzed on computer (SPSS, ver. 10.0 for Windows; SPSS Inc., Chicago, IL).

RESULTS

Cultured Corneal Fibroblasts and Alcohol

Cell viability was significantly reduced after treatment for 10 seconds with 20% ethanol ($P = 0.001$). In addition, cell viability was considerably decreased after treatment with 10% ethanol for 20, 30, and 40 seconds ($P < 0.01$; Fig. 1).

Fluorescent chromatin condensation was observed in 10% ethanol-treated cells stained with Hoechst 33342. As the ethanol concentration increased, more chromatin condensation was observed (Fig. 2). Cells treated with alcohol were positive for annexin V and displayed features typical of apoptosis, such as shrinkage of cytoplasm and perimembranous stippling (see Fig. 3 for details).

Coeffects of Alcohol with MMC on Cultured Corneal Fibroblasts

Cell viability was significantly reduced at 10% ethanol and more in groups treated for 20 seconds ($P < 0.01$) and at 20% and higher in group treated for 10 seconds ($P < 0.01$). With 20% ethanol treatment, fewer viable cells were observed (Fig. 4).

In experiments evaluating the time dependency of MMC, significant cell death occurred within 10 seconds in all groups ($P < 0.01$; Fig. 5). Cell death increased with longer treatment times.

A comparison of viability between incubated and nonincubated groups revealed significant death in all groups treated with 10% and more concentration of ethanol ($P < 0.01$) except for control group. However, profound cell death was observed in the incubation group. In a comparison of cell viability between the incubation and nonincubation groups, more cells were dead at 20% higher concentrations of ethanol in the incubation group than in the nonincubation group ($P < 0.01$). With the 10% ethanol treatment, statistically significant cell deaths were observed with MMC treatment for 60 minutes ($P = 0.008$; Fig. 6).

After a 24-hour incubation, cultured corneal fibroblasts treated with 10% ethanol and 0.02% MMC were stained with Hoechst 33342 and annexin V. Data were similar to results obtained using ethanol-treated cells (Figs. 7, 8). In groups cotreated with 20% and 30% ethanol and MMC, the number of

cells was significantly decreased because of detached nonviable cells.

Caspase-3 Levels in Cultured Corneal Fibroblasts

Caspase-3 activity was significantly increased in the 30% ethanol-treated group ($P < 0.01$) and in cells treated with both 0.02% MMC and 30% ethanol ($P < 0.01$). A more significant increase in caspase-3 activities was observed in the group cotreated with ethanol and MMC (Fig. 9).

DISCUSSION

There are currently two standard techniques for the correction of low to moderate myopia: PRK and LASIK. PRK is a safe and effective procedure.^{1-4,30} However, there are some disadvantages, such as postoperative pain, corneal haze, and slow visual rehabilitation.^{8-11,31} These problems are partially related to removal of the epithelium. LASIK avoids these problems by

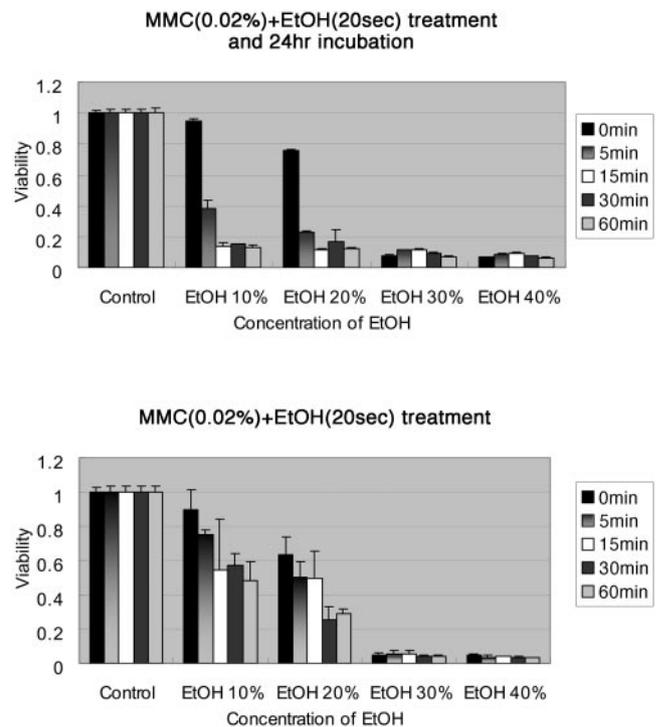


FIGURE 6. Cell viability after a 24-hour incubation, after treatment with 0%, 10%, 20%, 30%, and 40% ethanol and 0.02% MMC. In cases of incubation and nonincubation, significant cell death was observed at 10% and higher concentrations of ethanol in all groups ($P < 0.01$) except for the control group. However, more extensive cell death was observed in the incubation group.

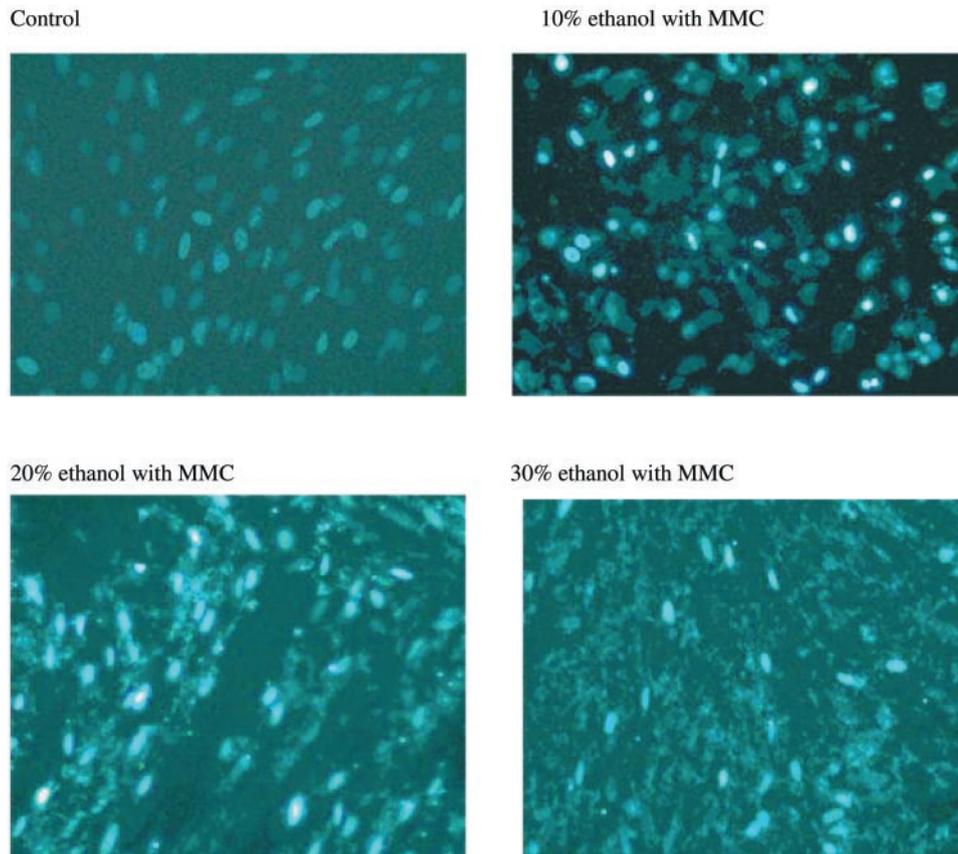


FIGURE 7. Micrographs of Hoechst-stained cells after a 24-hour incubation, after washing out of various concentrations of ethanol and MMC. The bright fluorescence in faintly stained cells was chromatin condensation. The bright fluorescent small particles were nuclear fragments. These findings were observed in 10% ethanol-treated keratocytes. More extensive chromatin condensation was observed with increasing ethanol concentrations. However, in comparison to Figure 2, a decreased number of keratocytes was observed in treated groups because of detached nonviable cells. Magnification, $\times 200$.

using intrastromal ablation.⁵ However, because the LASIK flap cuts through approximately one third of the stromal lamellar thickness and stromal fibers do not cross the LASIK incision after surgery, there is a concern about the long-term biomechanical stability of the cornea.³²⁻³⁵

LASEK is a viable alternative to PRK and LASIK in selected patients, and may reduce the complications associated with these procedures, including postoperative pain, slow visual rehabilitation, and complications with the flap. Moreover, exposure to 20% alcohol for 30 seconds or less has minimal adverse effects on the corneal epithelium.^{6,22-25}

However, adverse effects of alcohol on cultured corneal fibroblasts have been reported, including apoptotic changes in anterior stromal keratocytes, especially around the epithelial flap margin,²⁴ cell loss,²⁶ and inflammation.^{27,28} Alcohol delamination of the corneal epithelium consistently results in very smooth cleavage at the level of hemidesmosomal attachments, including superficial lamina lucida.²³ Thus, alcohol may have a significant effect on keratocytes by breaking intercellular adhesion. We identified one of these cultured corneal fibroblast responses as apoptosis, using Hoechst 33342 and annexin V staining. The response was dose and time dependent. Our results show that 20% ethanol caused significant cell death within 10 seconds. However, these results are not specific for human keratocytes and may therefore not be directly applicable to the human eye.

MMC has been advocated as a potential modulator of the corneal wound-healing process. Talamo et al.³⁶ reported that steroid and MMC inhibits subepithelial collagen synthesis in an additive fashion. Xu et al.³⁷ reported that MMC application after PRK suppresses corneal haze and markedly reduces the number of keratocytes in the anterior stroma. Majmudar et al.¹⁸ clinically used MMC to treat subepithelial fibrosis removal. The prophylactic use of MMC solution applied during surgery in a

single dose after PRK resulted in lower haze rates and more accurate refractive outcomes.³⁸

In an earlier report, we demonstrated that MMC induces apoptosis in keratocytes through apoptosis.³⁹ In the current study, ethanol and MMC had a synergistic effect on apoptosis of cultured corneal fibroblasts in a time- and dose-dependent manner. In addition, a 24-hour treatment with alcohol and MMC caused significant cell death, although MMC was washed out vigorously. A significant number of dead cells and disappearance of cultured corneal fibroblasts was observed with Hoechst staining. MMC blocks DNA and RNA replication and protein synthesis, mainly affecting rapidly proliferating cells. Thus, data from the incubation experiments reveal the more precise effect of MMC on cultured keratocytes than the concept of simple application. Thus, LASEK treatment with MMC application may cause fatal damage to keratocytes and the cornea, particularly considering the latent effect of this reagent. This finding suggests that MMC has residual effects within the intracellular or extracellular environment. These characteristics of MMC explain scleral melting long after pterygium excision with MMC treatment. During pterygium excision, copious irrigation was performed after application of MMC. However, this did not prevent scleral melting. This residual effect of MMC should be investigated in future studies. Thus, the dose and duration of alcohol and MMC treatment should be adjusted for future cases of LASEK with MMC application. Moreover, coeffects of alcohol and MMC on human keratocytes remain to be evaluated in detail.

The increase in caspase-3 activity with cell death progression suggests that the death of cultured corneal fibroblasts is related to the caspase pathway. According to a previous study, MMC induces apoptosis in cultured corneal fibroblasts through the caspase pathway. Thus, our findings are consistent with previous data.³⁹ Statistically significant elevation was detected

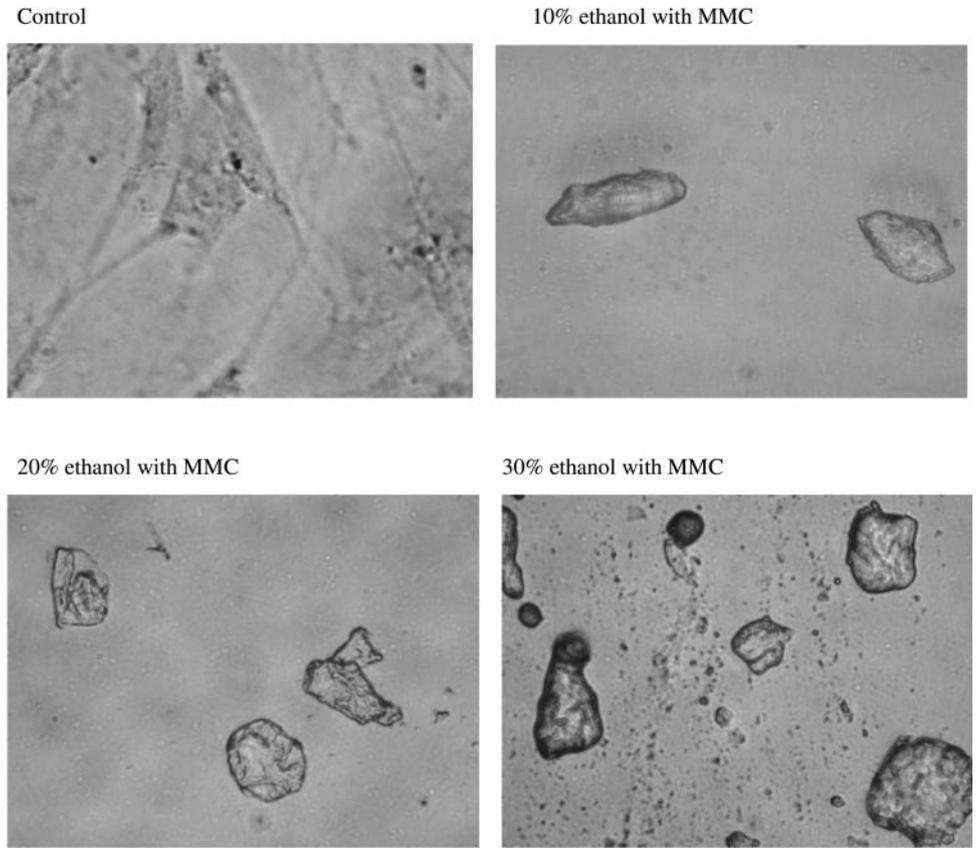


FIGURE 8. Micrographs of annexin V-stained cells after treatment with various concentrations of ethanol and MMC. Cells treated with alcohol and MMC were positive for annexin V and displayed typical characteristics of apoptosis, such as cytoplasm shrinkage and perimembranous stippling. Magnification, $\times 400$.

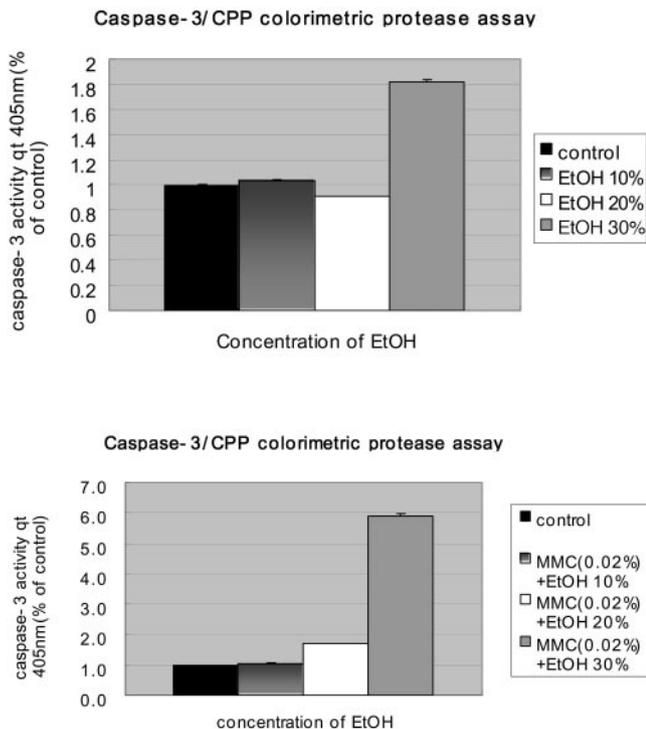


FIGURE 9. Caspase-3 activity after treatment with ethanol or MMC was significantly increased in the groups treated with 30% ethanol ($P = 0.003$) and 0.02% MMC+30% ethanol ($P < 0.01$).

in the 30% ethanol-treated group, suggesting that caspase-3 activity is elevated during significant injury.

In conclusion, alcohol and MMC synergistically induce apoptosis in cultured corneal fibroblasts in a dose- and time-dependent manner, using a mechanism related to the caspase pathway. Experimental and clinical trials on MMC for the modulation of corneal wound healing after LASEK have been initiated. However, the use of this compound must be delayed until the safety of using alcohol and MMC in LASEK is guaranteed.

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