

# Effect of mechanical vs dilute ethanol epithelial removal on keratocyte apoptosis and polymorphonuclear leukocyte migration

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## Abstract

**Purpose** To investigate keratocyte apoptosis and polymorphonuclear (PMN) cell infiltration to the corneal stroma after mechanical epithelial scraping and chemical de-epithelialization with 18% ethanol solution.

**Methods** Twelve New Zealand Albino rabbits (24 eyes) were randomly divided into three groups. Group A was the control group with no epithelial removal. Group B underwent a 7.5-mm mechanical epithelial removal with a blunt spatula. Group C underwent 7.5-mm chemical de-epithelialization with 18% ethanol-balanced salt solution. Corneas were stained with terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay after 24 h. Only nuclear staining in keratocytes was counted. Polymorphonuclear (PMN) leukocyte densities were also assessed by light microscopy.

**Results** Mechanical de-epithelialization (group B) and chemical de-epithelialization with 18% ethanol (group C) showed no difference in keratocyte apoptosis compared with the control group. There was also no difference between groups B and C. Group B showed no difference in PMN leukocyte counts compared with the control group. But the number of PMN leukocytes observed in group C was significantly higher than those encountered in the corneas of the control group ( $P < 0.05$ ) and group B ( $P < 0.05$ ).

**Conclusions** Dilute alcohol induces more PMN cell infiltration when compared with mechanical de-epithelialization although there is no difference in the apoptosis rates.

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**Keywords:** epithelial removal; keratocyte apoptosis; polymorphonuclear leukocyte

## Introduction

Removal of corneal epithelium causes loss of superficial stromal keratocytes in rabbits and monkeys.<sup>1,2</sup> Previous studies have demonstrated that anterior corneal keratocytes undergo apoptosis in response to mechanical and chemical deepithelialization with concentrated ethanol solutions.<sup>3–5</sup> In the clinical studies chemical de-epithelialization with dilute ethanol (18% or 20%) has been found to be a simple, safe, fast and effective alternative to mechanical epithelial scraping before photorefractive keratectomy (PRK).<sup>6,7</sup> Smoother Bowman's layer surface and sharper edges have been observed after chemical de-epithelialization with 18% or 20% ethanol solution and the better clinical results in terms of haze and corneal regularity have been explained with these findings.<sup>7,8</sup> But the keratocyte apoptosis has not been investigated after chemical de-epithelialization with dilute ethanol up to now.

In the present study keratocyte apoptosis and polymorphonuclear (PMN) cell infiltration to the corneal stroma is investigated qualitatively and quantitatively after mechanical epithelial scraping and chemical de-epithelialization with 18% ethanol solution.

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## Materials and methods

Twelve New Zealand Albino rabbits (24 eyes) were randomly divided into three groups each containing four rabbits (eight eyes). Accepted principles of animal maintenance and care were conformed to in the present study. A control group is designed in the study to find out if any of the epithelial removal groups differ in keratocyte apoptosis and polymorphonuclear leukocyte migration when compared to normal rabbit corneas, and may suggest one of the epithelial removal methods to be less traumatic to the corneal stroma according to the results.

The rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (40 mg/kg) and xylazine hydrochloride (7 mg/kg). Group A was the control group with no epithelial removal. Group B rabbits underwent a 7.5-mm mechanical epithelial removal with a blunt spatula. Group C rabbits underwent 7.5-mm chemical de-epithelialization with 18% ethanol-balanced salt solution soaked in a synthetic sponge and applied for 20 s. After the application, the corneal surface was dried using synthetic sponges. The eye was then copiously irrigated with balanced salt solution to wash out residual alcohol remnants, and the melted epithelium was removed using dry sponges.

Animals were euthanized with 100 mg/kg intravenous pentobarbital injection, 24 h following corneal surgery. The eyes were enucleated and central corneal tissues were removed by using a 7.5-mm trephine and fixed in 10% formaldehyde solution for 2 h, then embedded vertically in paraffin. Full thickness sections were taken from different levels of 4  $\mu$ m. Following deparaffinisation proteinase K (Applipere, Oncor 130202) was applied for 15 min. The corneas were stained according to the manufacturer's instructions with terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay by using ApopTag plus peroxidase in situ apoptosis detection kit (S-7101, Oncor). Counterstaining of the specimens was made in 0.5% methyl green. Rat breast tissue was used as a positive control material. Only nuclear staining in keratocytes was counted; four sections from each specimen were evaluated by light microscopy at higher magnification fields ( $\times 400$ ). PMN leukocyte densities were also assessed by light microscopy on all sections at higher magnification fields ( $\times 400$ ) with hematoxylin-eosin staining. The mean values of 40 different fields of each specimen were calculated.

Anova analysis was used to compare the results

**Table 1** Results of the keratocyte apoptosis counts in the control group and epithelial removal groups

Groups	Apoptosis (mean $\pm$ SD)	Anova test*	P value**
Group A (control)	0.012 $\pm$ 0.031		
Group B (mechanical)	2.55 $\pm$ 3.05	0.473	$P > 0.05$
Group C (18% ethanol)	6.0 $\pm$ 10.79	0.101	$P > 0.05$

\*Anova test results show comparisons of Group B and C with Group A (control group).

\*\*Significant P value:  $P < 0.05$ .

obtained in all groups and an overall  $P < 0.05$  was considered to be significant.

## Results

The results of the keratocyte apoptosis counts in the control group and epithelial removal groups are summarized in Table 1. Mechanical de-epithelialization (group B) and chemical de-epithelialization with 18% ethanol (group C) showed no difference in keratocyte apoptosis compared with the control group (group A). There was also no difference between groups B and C.

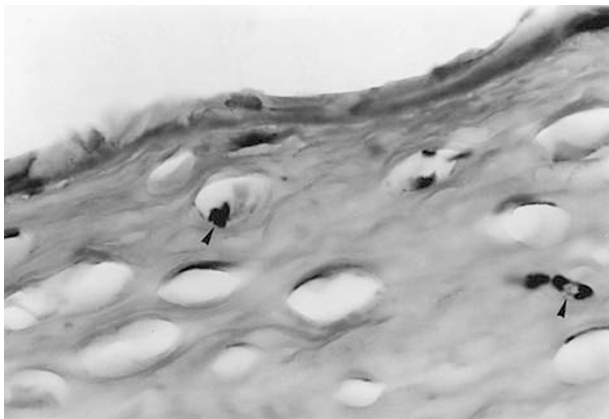
The results of the PMN leukocyte counts in the control group and epithelial removal groups are summarized in Table 2. Mechanical de-epithelialization (group B) showed no difference in PMN leukocyte counts compared with the control group (group A). But the number of PMN leukocytes observed in chemical de-epithelialization with 18% ethanol (group C) was significantly higher than those encountered in the corneas of the control group ( $P < 0.05$ ) and the mechanical de-epithelialization group ( $P < 0.05$ ) (Figures 1 and 2).

**Table 2** Results of the polymorphonuclear leukocyte counts in the control group and epithelial removal groups

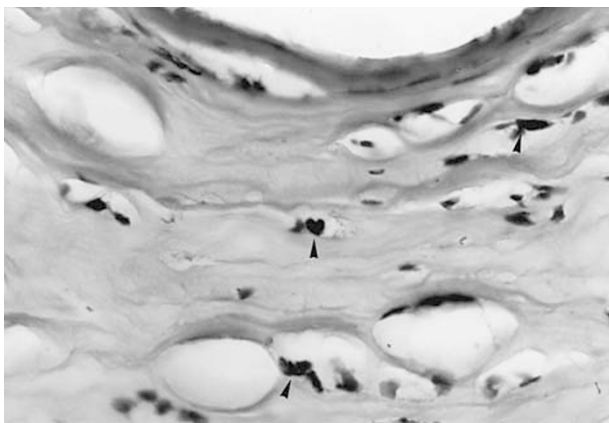
Groups	PMN counts (mean $\pm$ SD)	Anova test*	P value**
Group A (control)	0.33 $\pm$ 0.35		
Group B (mechanical)	1.99 $\pm$ 1.36	0.286	$P > 0.05$
Group C (18% ethanol)	11.71 $\pm$ 4.68	0.00	$P < 0.005$

\*Anova test results show comparisons of Group B and C with Group A (control group).

\*\*Significant P value:  $P < 0.05$ .



**Figure 1** PMN leukocytes in the mechanical epithelial removal group (arrows) by light microscopy (HE 1000 $\times$ ).



**Figure 2** PMN leukocytes in the dilute alcohol epithelial removal group (arrows) by light microscopy (HE 1000 $\times$ ).

## Discussion

A consistent degree of keratocyte loss is observed in rabbit and monkey corneas after de-epithelialization using mechanical means, chemicals or the excimer laser.<sup>1,2</sup> Apoptosis is a different way of cell death from ordinary cell death. It is a programmed, complex and controlled death of a cell without damage to surrounding tissue and, in contrast to ordinary cell death, there is no incitement of inflammatory reaction.<sup>9–14</sup> But the induction of apoptosis by reactive oxygen species has been demonstrated in recent studies.<sup>15,16</sup> PMN leukocyte infiltration of the corneal stroma after PRK has been demonstrated in many studies.<sup>17,18</sup> PMN leukocyte infiltration in the superficial corneal stroma might be responsible for the lipid peroxidation after PRK.<sup>2</sup> Free oxygen radicals are toxic products and may be responsible for some of the complications of the excimer laser corneal surgery.<sup>18–20</sup> Nitric oxide (NO) is a diffusible free radical gas and is formed in large amounts by the inducible nitric oxide

synthase in PMN leukocytes and other cell types stimulated by inflammatory cytokines. NO reacts extremely rapidly with superoxide to form the peroxynitrite.<sup>21</sup> Peroxynitrite, the reaction product of superoxide and NO, may be a major cytotoxic agent produced during inflammation; peroxynitrite is a highly reactive molecule and induces lipid peroxidation<sup>22,23</sup> and antioxidant therapy may have a role in diminishing oxidative damage.<sup>24</sup> Therefore the amount of PMN cell infiltration into the corneal stroma may be an indicator of tissue damage which induces corneal wound healing, haze and regression. The question of whether different de-epithelialization techniques may induce different healing responses and keratocyte apoptosis, and consequently different clinical results is still controversial. Various methods of de-epithelialization induce significant stromal acute inflammation 1 day after surgery compared with normal corneas.<sup>1</sup>

De-epithelialization with 50% and higher concentrations of ethanol causes more keratocyte loss with perhaps more corneal edema, but less stromal influx of neutrophils, than does a mechanical technique 24 h after PRK in rabbit models and clinical trials.<sup>1,3</sup> Corneal de-epithelialization with 20% dilute alcohol was shown to provide sharper edges and a smoother Bowman's layer surface than mechanical debridement which may explain the better results in terms of haze and corneal regularity.<sup>8</sup> Corneal de-epithelialization with dilute ethanol may be a viable option in PRK and that is why dilute alcohol (18% ethanol) was used for chemical de-epithelialization in our study. There was no significant difference in keratocyte apoptosis between mechanical de-epithelialization and 18% ethanol de-epithelialization but the amount of PMN cell infiltration was significantly higher ( $P < 0.05$ ) in 18% ethanol compared with mechanical de-epithelialization in the present study.

We attempted here to evaluate the more common clinically used de-epithelialization methods and examine their effects on keratocyte apoptosis and stromal inflammation which is also related to keratocyte survival. Dilute alcohol (18% ethanol) may be used for corneal de-epithelialization but it seems that it induces more PMN cell infiltration compared with mechanical de-epithelialization although there is no difference in the apoptosis rates.

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