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The Effect of Excimer Laser Keratectomy on Corneal Glutathione-related Enzymes in Rabbits

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Glutathione related enzymes are involved in the metabolism and detoxification of cytotoxic and carcinogenic compounds as well as reactive oxygen species. Excimer laser is a very useful tool for the treatment of refractive errors and removing superficial corneal opacities. Previous studies have shown that excimer laser may initiate free radical formation in the cornea. In the present study, we evaluated the effect of excimer laser keratectomy on corneal glutathione-related enzyme activities in rabbits.

Animals were divided into five groups, and all groups were compared with the controls (group 1), after epithelial scraping (group 2), transepithelial photorefractive keratectomy (PRK) (group 3), traditional PRK (group 4) and deep traditional PRK (group 5). Corneal glutathione peroxidase (GPx), glutathione *S*-transferase (GST) and glutathione reductase (GR) activities were measured after 24 h. Corneal GPx and GR activities significantly decreased only in group 5 ($p < 0.05$) but GST activities significantly decreased in all groups when compared with the control group ($p < 0.05$). In conclusion, excimer laser inhibits the glutathione dependent defense system in the cornea, this effect becomes more prominent after high doses of excimer laser energy and antioxidants may be useful to reduce free radical mediated complications.

Keywords: Excimer laser surgery; Glutathione *S*-transferase; Glutathione reductase; Glutathione peroxidase; Rabbit

INTRODUCTION

Corneal photoablation with 193 nm argon fluoride excimer laser is a technique used for the treatment of refractive errors and for removing corneal opacities and irregularities.[1] Corneal wound healing is an important determinant of the clinical outcome of excimer laser surgery with respect to corneal clarity and regression.[2]

Reactive oxygen species, such as superoxide anion and hydrogen peroxide, may injure the corneal tissues by degrading corneal stromal molecules either directly by scission of covalent bonds or indirectly by enhancing their susceptibility to hydrolytic enzymes. Moreover, reactive oxygen species are proinflammatory because they activate prostaglandin H synthesis through the formation of hydroperoxides and activate polymorphonuclear collagenase.[3] The presence of free oxygen radicals and polymorphonuclear cell infiltration after excimer laser photoablation has been demonstrated in many studies.[4–7]

To defend against free radical attacks, cells have developed, during their evolution, different antioxidant systems; there are low molecular weight antioxidant molecules like α-tocopherol, ascorbic acid, glutathione, etc. and antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase (GPx). In physiological conditions, these defense mechanisms maintain a low steady state concentration of free radicals in the cell and their activities are very precisely regulated.[8] But in pathological states, these enzyme activities have been changed.

GSH (gamma-L-glutamyl-L-cysteinylglycine), present in the cornea in millimolar concentrations,

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functions as an electrophile, radical scavenger, and a redox partner.[9] GSH may also serve as a cofactor for (a) several drug-metabolizing enzymes (i.e. glutathione *S*-transferase, GSTs) where it is consumed, or (b) for antioxidant enzymes (i.e. GPx) where it functions as a redox partner.[10] During oxidation, GSH forms a dimer, glutathione disulfide (GSSG), which, in turn, can be reduced by the enzyme glutathione reductase (GR) at the expense of NADPH.[11] The reaction is essential for the maintenance of glutathione levels.[12] In the cornea, GSH is known to play an important role in protecting cellular membrane integrity, degrading xenobiotic agents and lipid peroxidation products, and maintaining normal corneal hydration.[13] In the present study, we analyzed corneal GPx, GST and GR enzyme activities after superficial (50 μm) and deep (100 μm) corneal photoablation in rabbits.

MATERIAL AND METHODS

Twenty New Zealand white rabbits (average weight 2.5–3.4 kg) were used in the study. Anesthesia was induced by an intramuscular injection of 25 mg/kg ketamin hydrochloride, 2.5 mg/kg xylazine and topical proparacaine hydrochloride.

Study groups were as follows

- Group-1 (epithelial scrape group): In 7 animals, the corneal epithelium of right eyes were removed by a blunt spatula (Visitec, Sarasota, USA).
- Group-2 (transepithelial photorefractive keratectomy (PRK) group): In the same 7 animals used in Group-1, corneal epithelium of the left eyes were removed with excimer laser in PTK mode, then 50 μm photoablation was applied.
- Group-3 (traditional PRK group): In another 7 animals, 50 μm corneal photoablation was applied to right eyes after epithelial removal by a blunt spatula.
- Group-4 (deep traditional PRK group): In the same 7 animals used in Group-3, 100 μm corneal photoablation was applied to left eyes after epithelial removal by a blunt spatula.
- Group-5 (control group): In another 6 animals, right eyes of 5 animals and both eyes of 1 animal were taken as control group.

The eyes were irradiated with 193 nm excimer laser (MEL 60, Aesculap-Meditec, Jena, Germany) under *in-vivo* conditions. The fluence at the cornea was 220 mJ/cm^2, firing rate of 20 Hz, and diameter of ablation zone was 6 mm in all groups.

Animals were euthanized with 100 mg/kg intravenous pentobarbital injection, 24 h following corneal surgery. Central corneal tissues were removed by using a 7.5 mm trephine and were used for enzymatic analysis.

Each corneal tissue was homogenized in 1 ml of 0.1 M Tris/HCl buffer, pH 7.2, containing 154 mM NaCl. Homogenate was centrifuged at 13 000 rpm for 15 min. The protein concentration of the supernatant was measured by the method of Lowry *et al*.[14]

GPx Assay

GPx activity was measured by a modification of the coupled assay procedure of Paglia and Valentine.[15] The assay was performed by incubating 100 μl of corneal supernatant in 1 ml reaction mixture (50 mM potassium phosphate buffer, pH 7.0; 3.6 mM sodium azide, 5 mM GSH, 0.3 mM NADPH, added in 10 μl GR) for 5 min. The absorption was recorded at 340 nm during a 5-min period, after which the reaction was started by addition of 10 μl 0.25 mM H_2O_2. Enzymatic activity was calculated from the decrease in absorption of NADPH, after correction for the volume change, and was expressed as nmoles NADPH oxidized per minute per milligram protein.

GR Assay

The assay was performed according to the method described by Riley.[13] A volume of 10 μl of corneal supernatant (diluted 1:10) was added to cuvettes containing 50 mM Tris–HCl buffer pH 7.4 and 50 μM NADPH in a volume of 0.4 ml, and a reaction temperature of 37°C. The absorption was recorded at 340 nm during a 5-min period, after which the reaction was started by addition of 0.1 ml 3.75 mM oxidized glutathione (GSSG) and was followed for a further 5 min. Enzymatic activity was calculated from the decrease in absorption of NADPH, after correction for the volume change, and was expressed in international units (IU) per milligram of protein.

One international unit of enzyme activity represents the amount that catalyzes the transformation of 1 μmol of substrate per minute.

GST Assay

GST activity was determined according to the method of Habig *et al*.[16] The assay mixture (1 ml) contained 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM GSH in 0.1 M potassium phosphate buffer, pH 6.5. After addition of 10 μl corneal supernatant, the rate of increase of absorption was measured at 340 nm for 3 min at 25°C. The enzyme activity was expressed in international units per milligram of protein.

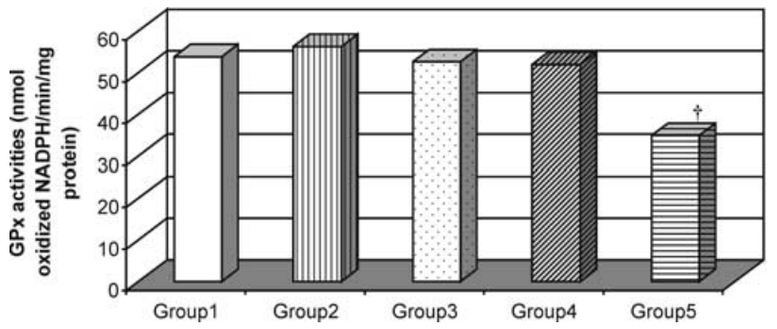

FIGURE 1 Corneal GPx activities in all groups. Group 1; control, Group 2; epithelial scrape, Group 3; transepithelial PRK, Group 4; traditional PRK, Group 5; deep traditional PRK. $^{\dagger}p < 0.05$ when compared with groups 1,2,3,and 4.

Statistical Analysis

As the homogenity of variances was not assumed, Kruskal–Wallis test was used to evaluate the differences between all the groups and p values less than 0.05 were considered statistically significant. Analysis the difference between two groups were evaluted with Mann–Whitney U test and p values less than 0.05 were considered statistically significant. Data were analyzed using SPSS 8.0 for windows (SPSS Inc., Chicago, USA).

RESULTS

Corneal glutathione-related enzyme activities are shown in Figs. 1–3. Corneal GPx activities were found significantly decreased after deep traditional PRK ($p < 0.05$) (Fig. 1). There was no significant difference between other groups when compared with group 1.

Corneal GR activities decreased significantly after deep traditional PRK ($p < 0.05$) (Fig. 2). There was no significant difference between other groups when compared with control. But GR activities of epithelial scrape group and traditional PRK group showed significant difference ($p < 0.05$). Similarly GR activities of transepithelial PRK group and traditional PRK group showed significant difference ($p < 0.05$).

As shown in Fig. 3, corneal GST activities significantly decreased in all groups when compared with the control group ($p < 0.05$).

DISCUSSION

Excimer laser corneal tissue interaction initiates free radical formation in the cornea. There are three possible sources of free oxygen radical production in the cornea during and after excimer laser photoablation. The first one is ultraviolet (UV) radiation (193 nm UV radiation, UV-C) that occurs during excimer laser treatment.[17] The second is excimer laser induced thermal increase in the corneal tissue during photoablation.[5,17] The last one is the accumulation of PMN cells to the laser ablated surface.[6] Free radicals are toxic products and create tissue damage, and may be responsible for some of the complications of the excimer laser corneal surgery.[5–7] We have previously demonstrated that excimer laser keratectomy decreases the superoxide dismutase activity of the aqueous humour[5] and changes the corneal activities of aldehyde dehydrogenase and GST, these two enzymes play an important role in detoxification of aldehydes, which are generated from free radical reactions.[18] In addition ascorbic acid levels of tear fluid significantly decrease after excimer laser surgery in humans.[19]

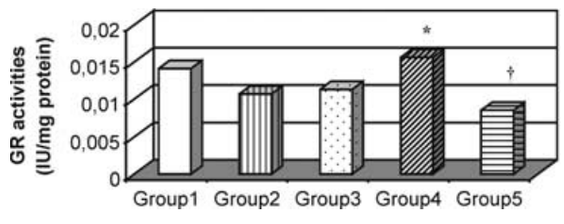

FIGURE 2 Corneal GR activities in all groups. Group 1; control, Group 2; epithelial scrape, Group 3; transepithelial PRK, Group 4; traditional PRK, Group 5; deep traditional PRK. $^{*}p < 0.05$ when compared with groups 2 and 3. $^{\dagger}p < 0.05$ when compared with groups 1,2,3,and 4.

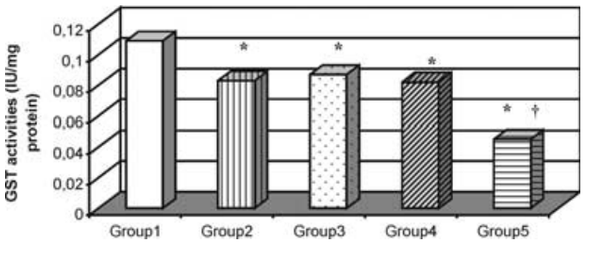

FIGURE 3 Corneal GST activities in all groups. Group 1; control, Group 2; epithelial scrape, Group 3; transepithelial PRK, Group 4; traditional PRK, Group 5; deep traditional PRK. $^{*}p < 0.05$ when compared with group 1. $^{\dagger}p < 0.05$ when compared with groups 2,3,and 4.

In a recent study, excimer laser induced radical formation was demonstrated by electron spin-resonance by Shimmura.[7] In another recent investigation oxidative tissue damage in the form of lipid peroxidation was detected by fluorescent peroxidized carbonyl compounds using a confocal microscope.[20]

The glutathione dependent defense system plays a vital role against lipid peroxidation in mammalian cells. Glutathione directly reacts with reactive oxygen species, and GPx catalyzes the destruction of hydrogen peroxide and lipid hydroperoxide.[21] This catalysis generates GSSG, and finally GSH. In addition, GR also catalyzes the regeneration of GSH from GSSG.[22] Thus, both GR and GPx are the enzymes in the glutathione regenerating pathway, and the changes of both activities are of a similar fashion. Because endogenous glutathione is one of the most abundant and well characterized redox scavenging molecules, glutathione might be one of the first choices for radical scavenging. Goto *et al.*[23] demonstrated that cellular glutathione content is highly correlated with GR activity. In our study after excimer laser photoablation, corneal GPx and GR activities significantly decreased only in group 5 when compared with the control group ($p < 0.05$). The decrease of both GPx and GR activities is indicative of excimer laser-induced slowing of the glutathione redox cycle and, therefore, impairment of hydrogen peroxide-neutralizing mechanisms. Change of these enzymes activities is laser dose-dependent. Because corneal GPx and GR activities significantly decreased only in deep traditional PRK group.

It is reported that toxic aldehyde products of lipid peroxidation are natural substrates for GST.[24] Consequently, a major biological function of GST is to protect the cell from these toxic compounds originating in oxygen metabolism. GST activities were found to be increased in human cornea in some corneal disorders (for example, herpetic keratitis, keratoconus and graft rejection).[25] In addition, we have previously demonstrated that corneal GST activities were increased in guinea pigs 48 h after deep excimer laser keratectomy.[18] In the present study, GST activities were statistically decreased in all groups when compared to controls at 24 h. The differences between animal species and timing may be responsible for this condition. GST was found to be distributed predominantly in the corneal epithelium and endothelium,[24] our results showed paralelism with this finding. We observed a significant decrease in GST activity after mechanical scraping of the epithelium ($p < 0.05$), On the other hand, corneal epithelial scraping induces a significant amount of PMN cell infiltration to the cornea. This is not solely due to the loss of the enzyme activities that was in the epithelium. PMN cells generates reactive oxygen radicals and probably decrease the GST activity of the cornea. The photoablation of the corneal stroma also decreased the corneal GST activity. This finding suggests that stromal photoablation probably inhibits the endothelial GST activity. Cornea become more susceptible to the harmful effects of reactive oxygen radicals after deep traditional PRK.

The surgical outcome of PRK depends on the healing response of the cornea. The action of extracellular matrix proteins, growth factors, cytokines, and free radical damage influence corneal wound healing. Furthermore, the dose of the excimer laser energy may change the corneal wound healing response. Previously, we have demonstrated a positive correlation between the depth of corneal photoablation and aqueous TGF-β1 concentration, corneal ALDH and GST activities, respectively.[18,26] In the present study, we found that high doses of the excimer energy may decrease the corneal glutathione-dependent enzymes activities. These findings support the theory that PRK treatment of high myopia induces a greater healing response in the cornea by reducing antioxidant status. Excimer laser treatment of low and moderate myopia may be safer than high myopia.

Pharmacological treatment to reduce oxidative damage in corneal tissue after PRK is under investigation and ascorbic acid was found effective after PRK.[20] Topical ascorbic acid treatment decreases oxygen radical mediated tissue damage in the form of lipid peroxidation and reduces PMN leukocyte infiltration following excimer laser keratectomy. Recently, we have demonstrated the inhibitory effect of topical vitamin E on corneal wound healing after PRK.[27]

The development of therapeutic modalities directed at the prevention of damage produced by reactive oxygen species may be of benefit in reducing corneal cell damage after excimer laser surgery. In conclusion, the effects of GSH containing solutions on corneal haze and regression may be investigated after excimer laser surgery.

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