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Nitric oxide levels of aqueous humor after photorefractive keratectomy

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PURPOSE. *To measure the nitric oxide (NO) levels of aqueous humor in rabbits after photorefractive keratectomy (PRK) and to evaluate the alterations of NO levels according to the PRK surgery steps, ablation depth, and time.*
METHODS. *Fifty eyes of 25 New Zealand white rabbits were included in the study. One eye was later randomly excluded from the study in order to equalize the number of eyes in groups. Eyes were divided into seven groups, each comprising seven eyes: unwounded control (Group 1), epithelial scrape (Group 2; aqueous humor samples taken at the 4th hour), superficial PRK (Group 3; samples taken at the 4th hour), deep PRK (Group 4; samples taken at the 4th hour), epithelial scrape (Group 5; samples taken at the 24th hour), superficial PRK (Group 6; samples taken at the 24th hour), and deep PRK (Group 7; samples taken at the 24th hour). The corneal epithelium was mechanically removed in surgical groups. The authors performed superficial corneal ablation (59 μm) in Groups 3 and 6 and deep corneal ablation (99 μm) in Groups 4 and 7. Aqueous humor samples were taken at the 4th hour (Groups 2-4) or 24th hour (Groups 5-7) after corneal surgeries. NO measurements were performed indirectly by using the Griess reaction with a spectrophotometer.*
RESULTS. *Aqueous humor NO levels 4 hours after corneal surgery were statistically significantly lower than the control group ($p<0.05$). However, there was no difference among the surgical groups at the 4th hour ($p>0.05$). At the 24th hour, the deep PRK group had significantly lower NO levels than both the control group and Groups 5 and 6 ($p<0.05$). NO levels were normalized at the 24th hour in epithelial scrape and superficial PRK groups ($p>0.05$) but remained stable at lower levels in deep PRK groups ($p<0.05$).*
CONCLUSIONS. *Corneal surgery caused low NO levels in aqueous humor 4 hours after surgery. However, 24 hours after surgery, NO levels normalized following epithelial scrape and superficial PRK and were stable at lower levels in the deep PRK group. Complications of deep PRK application are possibly induced by low NO existence in the aqueous humor. (Eur J Ophthalmol 2004; 14: 100-5)*

KEY WORDS. *Aqueous humor, Nitric oxide, Photorefractive keratectomy*

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INTRODUCTION

Photorefractive keratectomy (PRK) has emerged as an effective and popular technique for the treatment of myopia and astigmatism in the last decade (1). However, it causes some major complications such as corneal haze and myopic regression, which are thought to be associated with excessive wound healing (2, 3). There are many studies demonstrating formation of free oxygen radicals (4-6) and keratocyte apoptosis (7, 8) fol-

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lowing PRK, which were implicated in the formation of haze and regression.

Nitric oxide (NO) is synthesized by three NO synthetase (NOS) isoforms. NOS I and III are constitutively expressed in cells. In contrast, inducible NOS (NOS II) is typically synthesized in many cells after challenge with immunologic or inflammatory stimuli. Activity of NOS II is independent of calcium and generates large amounts of NO (9). The charge neutrality and its high diffusion capacity are hallmarks that characterize NO bioactivity. It acts as a potent inhibitor of the lipid peroxidation chain reaction by scavenging propatory lipid radicals and peroxidase enzymes. NO has several essential roles in mammals; however, presence of superoxide and unregulated NO production causes peroxynitrite formation and either apoptotic or necrotic cell death (10). The half-life of NO in aqueous humor is short (11) and the stable final oxidative metabolites of NO are nitrite and nitrate (12).

NOS activity was demonstrated in eye and NO has been found to be partly responsible for some ocular inflammatory diseases (uveitis, retinitis) and degenerative diseases (glaucoma, retinal degenerations) (13). Kim et al (14) recently reported that corneal epithelium, fibroblasts, and endothelium were the main sources of NO in ocular surface tissue. NO was also shown to be produced spontaneously in the corneal endothelium by NOS II and III activity and it was thought to be involved in the maintenance of corneal thickness (15-17). NO was also demonstrated to be responsible for corneal endothelial cell and keratocyte death in phototoxicity (18).

In this study, because of the short half-life of NO, we measured nitrite-nitrate level differences as an indirect indicator of NO formation in the aqueous humor of rabbit eyes following epithelial scrape, superficial PRK, and deep PRK.

MATERIALS AND METHODS

Fifty eyes of 25 New Zealand white rabbits, each rabbit weighing between 2.5 and 3.4 kg (3 months old), were included in the study. Institutional guidelines regarding animal experimentation were followed. Anesthesia was induced by an intramuscular injection of 25 mg/kg ketamine HCl, 2.5 mg/kg xylazine, and topical proparacaine hydrochloride.

One eye later was randomly excluded from the study in order to equalize the number of eyes in groups. The remaining 49 eyes were divided into seven groups, each comprising seven eyes. Right eyes of seven rabbits acted as unwounded control group (Group 1) and corneal epithelium of seven left eyes was removed (Group 2). We performed superficial corneal ablation (59 µm or -5.0 D ablation) to right eyes of seven rabbits (Group 3) and deep corneal ablation (99 µm or -9.0 D ablation) to seven left eyes (Group 4) after corneal epithelial removal. These 14 rabbits were killed 4 hours after surgery. Corneal epithelium of seven other rabbits was removed. Right eyes were separated for Group 5 (epithelial scrape, 24 hour), and superficial corneal ablation (59 µm or -5.0 D ablation) was performed on left eyes (Group 6). Deep corneal ablation (99 µm or -9.0 D ablation) was performed to the remaining eight eyes of four rabbits (Group 7) and one eye was randomly excluded from the study to equalize the number of eyes in groups. These 11 rabbits were killed 24 hours after surgery.

Corneal epithelium was removed by a blunt spatula (Visitec, Sarasota, CA) in all surgical groups. A 193-nm argon fluoride excimer laser (Aesculap Meditec MEL 60, Jena, Germany) was used to perform the PRK. The fluence at the cornea was 220 mJ/cm^2, with a repetition rate of 10 Hz, and the diameter of ablation zone was 6 mm.

Topical tobramycin ointment was applied to controls and all surgical groups. We waited for 4 hours (in Groups 1, 2, 3, and 4) or 24 hours (in Groups 5, 6, and 7) following corneal surgeries to kill the rabbits by intravenous injection of pentobarbital sodium (100 mg/kg). Aqueous humor samples were obtained by using a 25-G needle and stored at -28 °C for the assessment of NO levels.

Nitric oxide assay

Because the half-life of NO in aqueous humor is short, NO levels were determined indirectly from the stable end products of NO, nitrite plus nitrate concentrations.

Nitrite was measured by using the Griess reaction (19). We equilibrated to 25 °C the tubes containing 200 µL supernatant, 800 µL of 100 mmol/L potassium phosphate buffer (pH 7.0), and 1000 µL of Griess

reagent (1 g/L sulfanilamide, 25 g/L phosphoric acid, and 0.1 g/L naphthylethylenediamine). After 10 minutes of color development at room temperature, absorbance at 620 nm was measured on a Milton Roy Spectronic Array 3000 spectrophotometer. The results were expressed as µmol/L.

Nitrate was measured by using the enzymatic one-step assay with nitrate reductase (20). The method was based on the reduction of nitrate to nitrite by nitrate reductase in the presence of -NADPH. We equilibrated at 25 °C tubes containing 250 µL of 100 µmol/L potassium phosphate buffer (pH 7.5) and 50 µL of 12 mmol/L -NADPH with 100 µL of sample. To start the enzymatic reaction, we added 40 µL of 500 U/L nitrate reductase. We incubated 45 minutes in the dark. The concomitant oxidation of -NADPH was monitored by the decrease in absorbance at 340 nm. The method of standard addition was used to minimize the effect of interfering substances from serum. The results were expressed as µmol/L.

Statistical analysis

Analysis of variance was used to evaluate the differences between the groups and p values less than 0.05 were considered statistically significant.

RESULTS

Aqueous humor NO levels in all groups are presented in Table I. Aqueous humor NO levels 4 hours after corneal surgery (in Groups 2, 3, and 4) were statistically significantly lower than the control group ($p<0.05$) (Tab. I) (Fig. 1). There was no difference between the surgical groups (in Groups 2, 3, and 4) ($p>0.05$).

Twenty-four hours after surgery, NO levels of Groups 5 and 6 were not significantly different from the NO levels of the control group ($p>0.05$). In the deep PRK group (Group 7), however, NO levels were significantly lower than both the control group and Groups 5 and 6 ($p<0.05$) (Tab. I) (Fig. 1).

When we compared the NO levels measured at the 4th hour (Groups 2, 3, and 4) and at the 24th hour (Groups 5, 6, and 7) in surgical groups, NO levels were statistically significantly higher at the 24th hour in epithelial scrape ($p<0.05$) and superficial PRK groups ($p<0.05$), which had returned to normal at the 24th hour. The NO levels measured at the 24th hour in the deep PRK group (Group 7) were significantly lower than the controls and at the same measured levels at 4th hour (Group 4) ($p>0.05$).

TABLE I - MEAN AQUEOUS HUMOR NITRIC OXIDE LEVELS (µmol/L) AFTER SURGERY

Groups	NO (mean ± SD)	ANOVA p
1 (Control)	366.00 ± 68.36	0.000*
2 (Epithelial scrape, 4 h)	238.43 ± 69.90	0.000†
3 (Epithelial scrape-PRK, 59 µm, 4 h)	225.71 ± 45.52	0.000†
4 (Epithelial scrape-PRK, 99 µm, 4 h)	192.00 ± 42.42	0.000†
5 (Epithelial scrape, 24 h)	381.00 ± 59.53	0.650†
6 (Epithelial scrape-PRK, 59 µm, 24 h)	379.43 ± 72.31	0.685†
7 (Epithelial scrape-PRK, 99 µm, 24 h)	255.29 ± 65.12	0.002†

All groups included seven eyes
*Analysis of variance (ANOVA) test result
†*Post hoc* test result showing the difference from control
NO = Nitric oxide; PRK = Photorefractive keratectomy

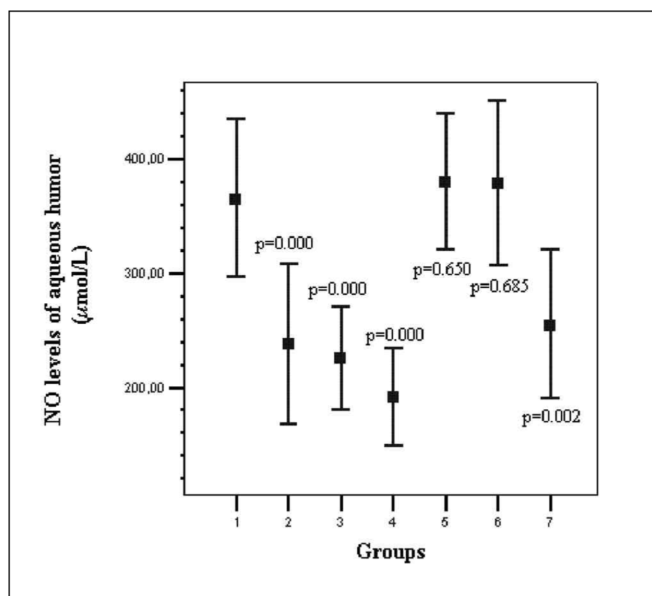

Fig. 1 - *Aqueous nitric oxide (NO) levels after corneal surgery. Error bars represent the standard deviation from mean and p values represent the difference from control.*

DISCUSSION

NO is known to be an important cell-signaling molecule, anti-infective agent, and, as most recently recognized, an antioxidant (21). NO can both promote and inhibit lipid peroxidation. NO is a scavenger of lipid peroxyl radicals, and also an inhibitor of lipid peroxidation initiators, such as peroxidase enzymes in the basal activity of NOS III. However, in the presence of superoxide, NO forms peroxynitrite that initiates lipid peroxidation and oxidizes lipid soluble antioxidants. In contrast, NO is a more potent scavenger than -tocopherol and is able to inhibit the oxidation of -tocopherol (22). On the other hand, NO and peroxynitrite inhibit mitochondrial respiration: inhibition is reversible in low NO levels, and irreversible in high NO levels and/or in the presence of peroxynitrite. The inhibition of NOS III or NO, activation of NOS II, or elevation of superoxide (forms of peroxynitrite) causes cellular and tissue damage. NO is consequently a double-edged sword (23).

In our study, we have demonstrated that NO levels were decreased 4 hours after surgery. Epithelial removal may cause NOS removal in epithelial cells and low NO levels, but the corneas were not completely epithelialized 24 hours after surgery in Groups 5 and 6, in which the NO levels were normalized.

Surgical trauma such as mechanical, thermal, and ultraviolet (UV) radiation can cause low NO levels by inhibiting NOS as a result of direct tissue damage or indirectly by increasing formation of free oxygen radicals, which can react with NO, forming more powerful oxidant peroxynitrite.

Free oxygen radical formation can be initiated with UV radiation (4, 5, 24), thermal increase (6, 25), and accumulation of PMN (26-28) during and after PRK and this causes tissue damage. Several studies have demonstrated that antioxidants such as superoxide dismutase (SOD) (29), ascorbic acid (vitamin C) (28), and -tocopherol (vitamin E) (30) may regulate stromal wound healing, which indirectly suggests radical formation.

Nitration of tyrosine residues in proteins by peroxynitrite to 3-nitrotyrosine is an indication of the presence of peroxynitrite that can be identified using nitrotyrosine antibodies (31). There are several reports suggesting formation of peroxynitrite during inflammatory processes (31-33) and in anterior chamber after experimental uveitis in rabbits (34). Moreover, nitrotyrosine formation was demonstrated in corneal tissue after excimer laser photokeratectomy (14).

The normalized NO levels in epithelial scrape and superficial PRK groups at the 24th hour are caused by reversible damage. The lowest NO levels were obtained in the deep PRK group at the 4th hour, which were stable at the 24th hour. This could possibly be explained by the increased free oxygen radical formation caused by deep PRK application, which resulted in more damage and more conversion of NO to peroxynitrite. In another study by Mirza et al (35), the decreased tear nitrite-nitrate levels in non-Behçet uveitis patients were considered to be the result of rapid transformation of NO to peroxynitrite.

Unregulated NO production and peroxynitrite formation can cause cell death through oxidative stress, disrupted energy metabolism, DNA damage, activation of poly (ADP-ribose) polymerase, or dysregulation of cytosolic calcium. Such disturbances can lead to either apoptotic or necrotic cell death, depending on the severity and context of the damage. Small decreases in cellular ATP levels and mild oxidative stress lead to apoptosis, whereas important decreases in ATP and severe oxidative stress rapidly cause necrosis (10).

Keratocyte apoptosis after refractive corneal surgery is a well-established issue in recent years (7, 8). Shimmura et al (36) have reported that hydroxyl radicals may be partly responsible for keratocyte apoptosis after PRK. We have recently reported keratocyte apoptosis following mechanical epithelial removal, traditional PRK (caused the highest number of apoptotic keratocytes), and LASIK procedures in rabbit corneas (37). We have also shown that topical vitamin E treatment immediately after traditional PRK could prevent apoptosis (37). Necrosis and apoptosis induced by reactive oxygen species were reported in *in vitro* bovine endothelial cells (38). These results also suggest that free oxygen radicals and peroxynitrite may be partly responsible for keratocyte apoptosis after PRK.

In conclusion, this study suggests that corneal surgery caused a decrease in aqueous humor NO levels 4 hours after surgery. This decrease could be due to the inhibition of NOS and peroxynitrite formation from NO in the presence of superoxide radical (induced by corneal surgery). However, 24 hours after surgery, NO levels normalize following epithelial scrape and superficial

PRK, and were still lower than the control group in the deep PRK group. Deep PRK applications possibly induce more tissue damage and the highest amounts of free oxygen radical formation, which led to more peroxynitrite but less NO in the aqueous humor.

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