

The Effect of Anterior Stromal Puncture Using Q-Switched Nd:YAG Laser on Corneal Wound Healing

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Abstract:

Introduction: Recurrent corneal erosion occurs when the wounded corneal epithelium failed to adhere to the underlying stroma. Therefore, this work aimed to assess the effect of treatment of corneal injury using Q-switched Nd:YAG laser.

Method: Twenty one New Zealand male rabbits weighing 2-2.5 kg and 3 months old were classified into three main groups. The control group: did not received any treatment (n=3 rabbits). The rest of the animals (n= 18 rabbits), corneal epithelium was injured by syringe needle and blade 15 and divided into:(A) Normal healing group: which was divided into three subgroups (n=3 rabbits each), and the animals were left for normal healing for 1 day, 1 week, and 4 weeks respectively, (B) Laser treated group: divided into three subgroups (n=3 rabbits each) and subjected to anterior stromal puncture using Q-switched Nd: YAG laser on corneal sub-epithelium or superficial stroma, and the animals were left for 1 day, 1 week, and 4 weeks respectively. After the demonstrated periods, the corneas were isolated for estimation of total protein content, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), total antioxidative capacity (TAC), total oxidative capacity (TOC) and oxidative stress index (OSI).

Results: The present results of corneal total protein showed increment in the percentage change in normal healed groups after 1 day, 1 week and 4 weeks by values of 93%, 68% and 39%. In Q-switched Nd: YAG laser treated group the results showed better improvement in corneal protein than normal healed group with percentage changes of 58%, 29%, and 7.5% respectively. In SDS- PAGE, a protein band at 110 KD appeared in the migrating epithelium for both normal healed group and Q-switched Nd:YAG laser treated group with changes in the peaks intensities at middle and low molecular weight regions. Moreover, after 4 weeks the peak at 110 KD disappeared in the wounded epithelium treated with Q-switched Nd:YAG. After four weeks, the OSI in laser treated corneas showed pronounced balance between antioxidative capacity and oxidative capacity.

Conclusion: Anterior stromal puncture by Q-switched Nd:YAG laser is an effective, simple, safe and promising procedure to treat recurrent corneal erosion than normal healing.

Keywords: Q-Switched Nd: YAG lasers; wound healing; corneal protein

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Introduction

Recurrent corneal erosion is a condition in which the corneal epithelium fails to establish the normal tight

junctions with Bowman's membrane. This process leads to recurrent loss of the epithelium and it is precipitated by breakdown of hemidesmosomes that play an important role in anchoring the epithelium to the lower layer¹. Failure

of the wounded epithelial cells to adhere to the underlying stroma is probably due to weak hemidesmosomal attachment, reduplication of the basement membrane, action of metalloproteinase, and disruption of type seven collagen fibrils². Trauma is the most common cause of recurrent corneal erosion. Other causes are epithelial basement membrane dystrophy, vitrectomy, alkali burn, post herpetic ulcer, Lasik, diabetes, and dry eye³.

Patient presents with severe pain, lacrimation, photophobia, foreign body sensation, blepharospasm, tearing and blurring of vision specially on waking up or at any time. This may be caused by relative anoxia, hypercapnia or edema of the corneal epithelium when the eyelids are closed during sleep. The symptoms may vary among individuals and with the extent of surface breakdown⁴.

Examination at an early stage reveals localized roughening of the corneal epithelium or corneal abrasion. Epithelial changes may resolve within hours of the onset of symptoms, so the abnormality is difficult to detect when the patient is examined. Immediate management of this condition includes antibiotic eye drops, non-steroidal anti-inflammatory eye drops, cycloplegic eye drops and a lubricating eye ointment. In resistant cases, therapeutic contact lens, corneal stromal micropuncture, epithelial debridement, superficial keratectomy, phototherapeutic keratectomy and Nd:YAG laser micropuncture were used⁵⁻⁸. These procedures aimed to induce firm and permanent adhesion of the surface epithelium to the underlying tissue, which is considered the ultimate goal for resolution.

Recurrent corneal erosion is a challenging condition and may be a difficult disorder to treat, with a number of patients suffering persistent symptoms despite conventional therapy⁹. In addition, corneal clarity and avascularity are critical for maintaining vision, so developing treatments for recurrent corneal erosion is crucial.

Previously, anterior stromal puncture was a well-accepted treatment for recurrent corneal erosions to induce a localized subepithelial fibrosis and to increase adhesion of the epithelium to the underlying layer. Initially, early pioneers of this approach used a microdiathermy needle, a large bore 20 gauge needle, and a specially designed 25 gauge needle to perform anterior stromal puncture⁶. Compared to needle puncture, the laser puncture created less stromal scarring¹⁰. Recently, Nd: YAG laser has the advantages of a minute and uniform wound with less corneal scarring, so the procedure could be repeated whenever necessary. It can ameliorate the frequency of

attacks and the intensity of pain¹¹.

For this reason, this study aimed to evaluate the impact of normal healing and anterior stromal puncture using Q- switched (sometimes called “giant pulses”) Nd:YAG laser treatment of injury on corneal protein after different periods.

Methods

Experimental Animals

Twenty one New Zealand male rabbits weighing 2-2.5 kg and 3 months old were obtained from the animal house of Research Institute of Ophthalmology, Giza, Egypt. The animals were housed in a standard 12hrs light- dark cycle with free access to water and balanced diet. All procedures were conducted according to the principles enunciated in the Guide for Care and Use of Laboratory Animals, approved by the local experimental ethics committee of Research Institute of Ophthalmology, Giza, Egypt.

Slit lamp biomicroscopic examinations of the eye were made following papillary dilation with Mydriacyl eye drop 0.5% (Alcon laboratories, Australia, Pty Ltd.) the results showed no signs of intraocular inflammation and no edema in all eyes.

The rabbits were classified into three main groups, which are: (1) control group (n=3 rabbits). Benoxinate eye drops was used as local anaesthetics and corneal epithelium was injured by 23 syringe needle and blade 15 in the rest of the rabbits (n= 18 rabbits).

(2) Normal healing group: which was divided into three subgroups (n=3 rabbits each), and the animals were left for 1 day, 1 week, and 4 weeks respectively.

(3) Laser treated group: which was divided into three subgroups (n=3 rabbits each), Q-switched Nd: YAG laser was performed on corneal sub-epithelium or superficial stroma, and the animals were left for 1 day, 1 week, and 4 weeks respectively.

Laser treatment parameters

Rabbits were generally anesthetized by using intramuscular ketamine hydrochloride (Ketalar 2.5 mg/ kg). Additionally they received 0.4% Benoxinate eye drops for local anesthesia. Three subgroups of rabbits underwent anterior stromal puncture using Q-switched Nd:YAG laser type “Optimis II Ophthalmic Photodisruptor Nd:YAG 1064 nm LASER CONSOLE SLIT LAMP, Quantel, Inc.,USA”. The energy setting

of Nd: YAG laser was 0.5 mJ per pulse or less, the spot size was 10 microns; the cone angle was 16 degrees; the wavelength was 1064 nm and the duration of the pulse was 4 nanoseconds. After the demonstrated periods, rabbits were sacrificed, eyes were enucleated and the corneas were isolated. Tissue samples from the corneas were accurately weighed and homogenized using cell homogenizer (type Tübingen 7400, Germany), in a 10-fold volume of 20 mM ice-cold tris-HCl buffer, pH 7.4. The homogenate was centrifuged for 20 minutes at 10,000 rpm in a bench centrifuge (Awel centrifuge MS 20, France). The resultant supernatant was used for estimation of total protein content, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), total antioxidant capacity (TAC), Total oxidative capacity (TOC) and oxidative stress index (OSI). All reagents for measurement of total protein, gel electrophoresis, TAC and TOC were of the highest purity available and purchased from Sigma Chemical Co., St. Louis, MO., USA.

Determination of total protein content

Corneal protein content was determined according to the method of Lowry with bovine plasma albumin as the standard¹².

SDS-PAGE for corneal protein

Corneal proteins were separated according to their molecular weights by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the previous method using 5% stacking gel and 12% separating gel¹³. The data were represented graphically with an automatic scanner (model R-112, manufactured by Beckman, Coulter, Brea, CA).

Total antioxidant capacity (TAC)

The supernatants were subjected to biochemical analysis of the total antioxidant capacity using a colorimetric method performed by the reaction of antioxidants in the sample with a defined amount of exogenously provided hydrogen peroxide (H₂O₂). The TAC was determined at 505 nm by an enzymatic reaction and the results were expressed in terms of mM/gm tissue¹⁴.

Total oxidative capacity (TOC)

The TOC levels of the cornea were determined using

a colorimetric method¹⁵. The color intensity measured spectrophotometrically at 510 nm was related to the total number of oxidant molecules present in the sample. The results were expressed in terms of mM/ gm tissue.

Oxidative stress index (OSI)

The ratio of TOC to TAC was accepted as the oxidative stress index (OSI)¹⁶. The OSI values were calculated according to the following formula: OSI =TOC (mM / gm tissue) / TAC (mM/ gm tissue).

Statistical evaluation

Protein content, TAC, TOC and OSI levels were compared between the control group and laser treated groups by student t-test¹⁷. Where “t” is the test of significance, differences were considered significant at P >0.05.

Results

The protein content of the cornea

Figure 1 presented the corneal protein content for the control, normal healing group and treated group with Q-switched Nd:YAG laser after 1 day, 1 week and 4 weeks. The protein content in control cornea was 31.9± 0.3 mg/g tissue but after 1 day of normal healing, the protein content showed a high significant increase (P> 0.001) with a value of 61.4±0.3 mg/g tissue and percentage change of 93% with respect to the control group. Moreover, the protein content was 53±1.9 mg/g tissue (P=0.001) and 44.3±0.5 mg/ g tissue (P=0.01) after one week and 4 weeks respectively. On the other hand, the protein levels were increased with percentage change of 68% and 39% with respect to the control. One day after treatment with Q-switched Nd:YAG laser the protein content was increased with a value of 50.7±1.0

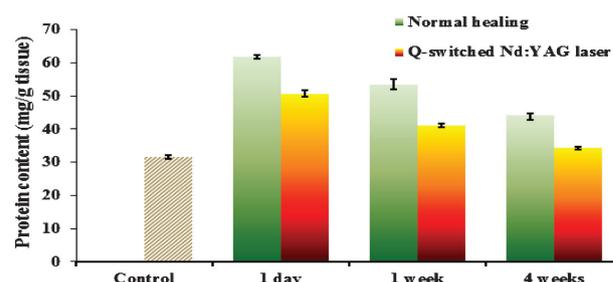


Figure 1. Protein content of the cornea after different periods of normal healing and treatment with Q-switched Nd: YAG laser.

mg/g tissue ($P=0.001$). Moreover, the increase in protein content was less pronounced after 1 week with a value of 41.2 ± 0.6 mg/ g tissue ($P=0.01$). After 4 weeks there was a non -significant change in the protein content ($P>0.05$) with a value of 34.3 ± 0.5 mg/g tissue. Furthermore, the percentage change in protein content showed dramatic reduction after 1 day, 1 week, and 4 weeks with a value of 58%, 29%, and 7.5% respectively.

SDS-PAGE results

Figure 2 showed the SDS-polyacrylamide gel electrophoresis scanning pattern of the corneal protein with normal healing compared with the control group. The control pattern was characterized by presence of 11 peaks which varied in their molecular weights and intensities. After one day for normal healing the protein fraction intensities were increased all over the pattern. In addition, a protein band present at 110 KD appeared in the migrating epithelium that did not appear in control corneal protein. One day after treatment of wound using Q-switched Nd:YAG laser (Figure 3), the change in the peaks intensities was mainly obvious at middle and low

molecular weight regions which was characterized by increases in peaks' intensities than the control. Moreover, the peak at 110 KD was more apparent in the wounded epithelium treated with Q-switched Nd:YAG than the control one. The profile of the peaks was changed in the region between 30-90 KD with respect to the control.

After one week of normal wound healing (Figure 4) the molecular weight at 178KD was slightly improved and the peak at 146 KD still with low intensity and small width. In addition, the peak at 110 KD still persists with the same intensity compared with one day after normal wound healing group. The scanning pattern after one week of wound treated with Q-switched Nd:YAG laser (Figure 5) showed gradual improvement characterized by decrease in the peaks intensities at low molecular weight region between 30 KD and 70 KD. Moreover, there were disappearance of the peak at 110 KD and pronounced improvement in the molecular weight at 146KD.

After 4 weeks for normal wound healing the molecular weight at 178KD and 146 KD were improved and the peak at 110 still persist but with weak intensity. In addition, the intensities of the peaks in the low molecular weight region showed dramatic improvement compared with

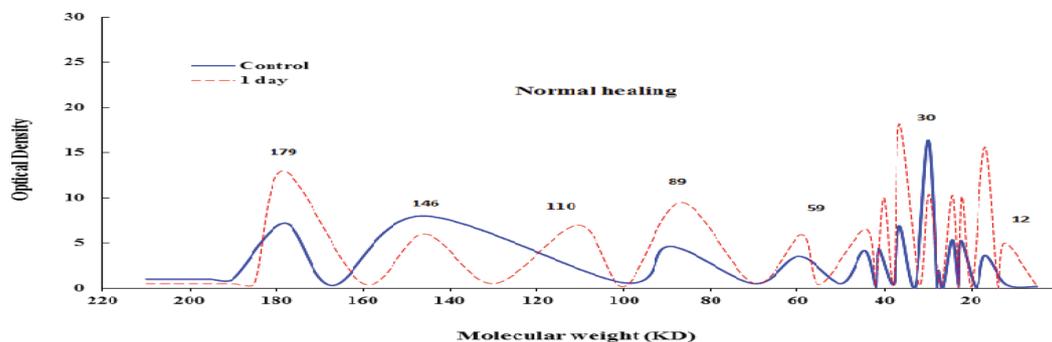


Figure 2. SDS- electrophoresis scanning patterns for control corneal protein and after 1 day of normal wound healing.

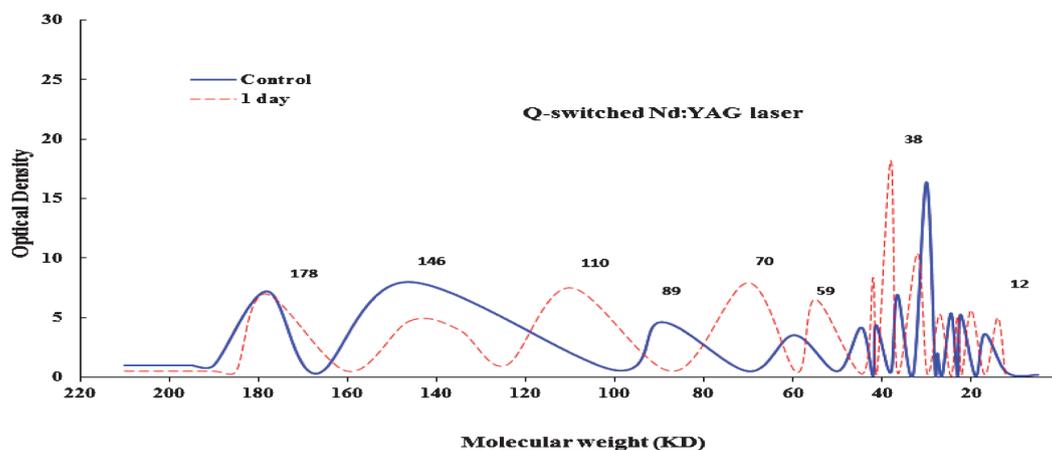


Figure 3. SDS- electrophoresis scanning patterns for control corneal protein and after 1 day of wound healing using Q-switched Nd:YAG laser.

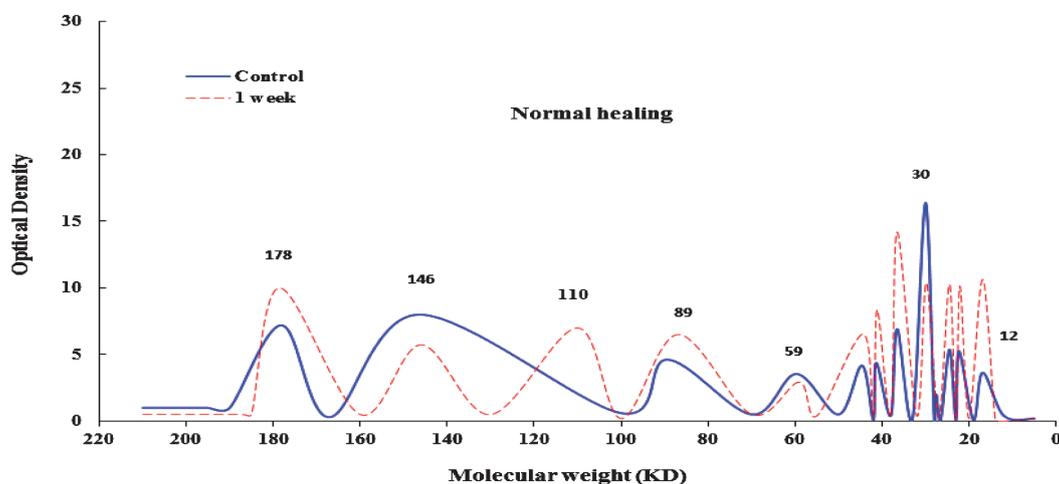


Figure 4. SDS- electrophoresis scanning patterns for control corneal protein and after 1 week of normal wound healing.

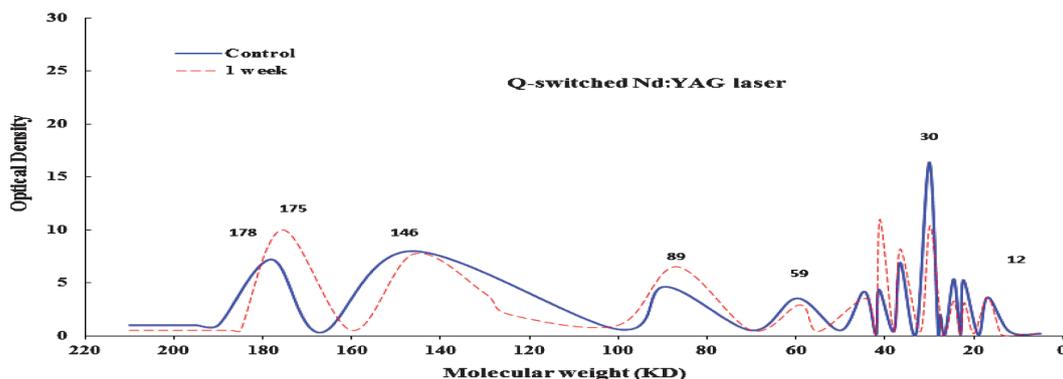


Figure 5. SDS- electrophoresis scanning patterns for control corneal protein and after 1 week of wound healing using Q-switched Nd:YAG laser.

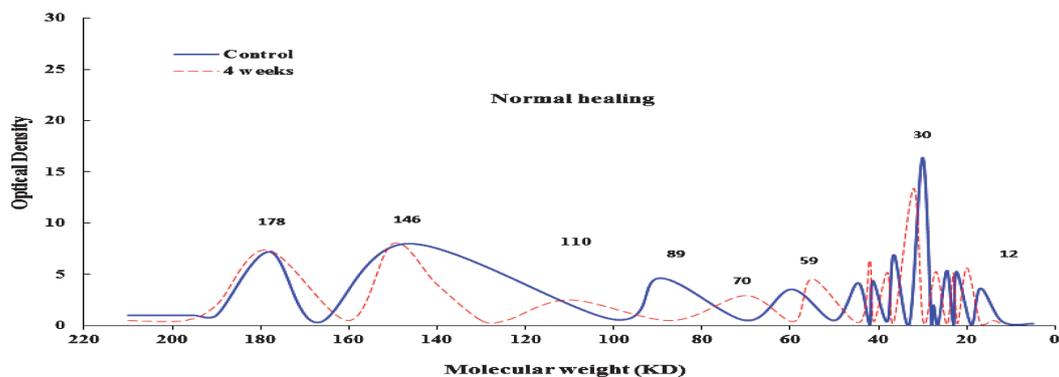


Figure 6. SDS- electrophoresis scanning patterns for control corneal protein and after 4 weeks of normal wound healing.

1 week after normal wound healing group (Figure 6). By contrast, the scanning pattern of SDS-PAGE after four weeks of wound treated with Q-switched Nd:YAG laser (Figure 7) showed better improvement in all protein fractions than in the case of normal healing after 4 weeks.

Total antioxidant capacity (TAC)

In Figure 8 the data showed a significant reduction

in TAC (-49%) from $126 \pm 1.7 \times 10^{-4}$ mM/g tissue for the control to $62.9 \pm 1.1 \times 10^{-4}$ mM/g tissue after 1 day of normal wound healing ($P > 0.001$). After 1 week and 4 weeks of normal wound healing, there were gradual improvement in TAC with values of $83.5 \pm 1.5 \times 10^{-4}$ mM/g tissue ($P < 0.001$) and $97.3 \pm 1.8 \times 10^{-4}$ mM/g tissue ($P < 0.01$) respectively, with percentage change of -34%, and -23%, with respect to the control group. Moreover, the groups treated with Q-switched Nd:YAG laser showed

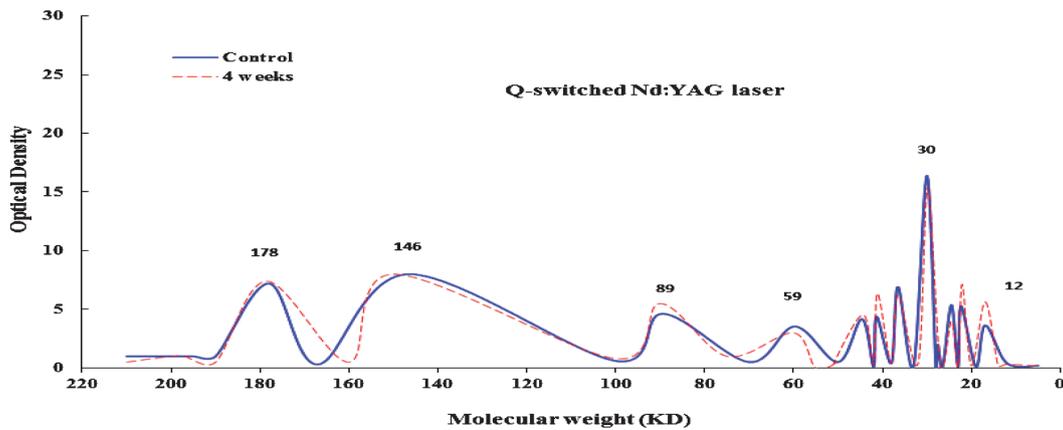


Figure 7. SDS- electrophoresis scanning patterns for control corneal protein and after 4 weeks of wound healing using Q-switched Nd:YAG laser.

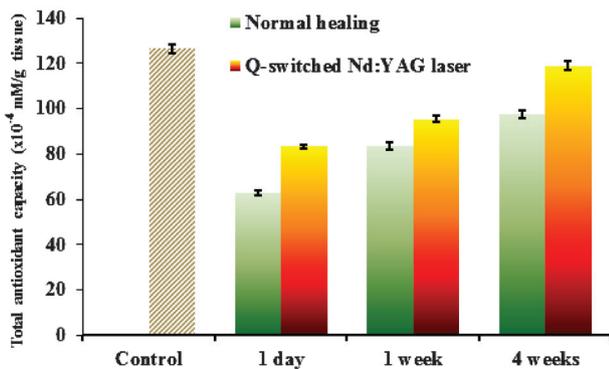


Figure 8. Total antioxidant capacity of the corneal tissues after different periods of normal healing and treatment with Q-switched Nd: YAG laser.

less decrement in TAC than in normal wound healing. The values of total antioxidant capacity were $83.4 \pm 2.0 \times 10^{-4}$ mM/g tissue ($P < 0.001$), $95.5 \pm 2.0 \times 10^{-4}$ mM/g tissue ($P < 0.001$) and $119.2 \pm 1.4 \times 10^{-4}$ mM/g tissue ($P < 0.01$) with percentage changes of -34%, -24% and -5% after 1 day, 1 week and 4 weeks respectively.

Total oxidative capacity (TOC)

Figure 9 showed a very high significant increase in the total oxidative capacity (TOC) after one day of normal healing (166%, $P > 0.001$) with values of 8.4 ± 0.3 mM/g tissue for the control to 22.4 ± 0.5 mM/g tissue. After 1 week and 4 weeks of normal wound healing, there were dramatic improvement in TOC with values of 14.0 ± 0.4 mM/g tissue and 11.6 ± 0.5 mM/g tissue respectively, with percentage change of 66%, $P < 0.001$ and 38%, $P < 0.001$ with respect to the control group. Moreover, the groups treated with Q-switched Nd:YAG laser showed better improvement than in normal wound healing. The values of total oxidant capacity were 13.1 ± 0.5 mM/g

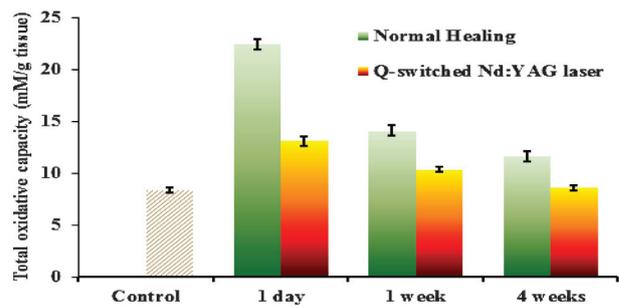


Figure 9. Total oxidative capacity of the corneal tissues after different periods of normal healing and treatment with Q-switched Nd: YAG laser.

tissue ($P < 0.001$), 10.4 ± 0.3 mM/g tissue ($P < 0.01$) and $8.6 \pm 1.4 \times 10^{-4}$ mM/g tissue ($P < 0.05$) with percentage changes of 56%, 24% and 2.3% after 1 day, 1 week and 4 weeks respectively.

Oxidative stress index (OSI)

Oxidative stress index (OSI) illustrated the ratio of TOC to TAC and determined the oxidative/ antioxidative balance (Figure 10). In the case of normal healing groups, the data indicated shifting towards oxidative

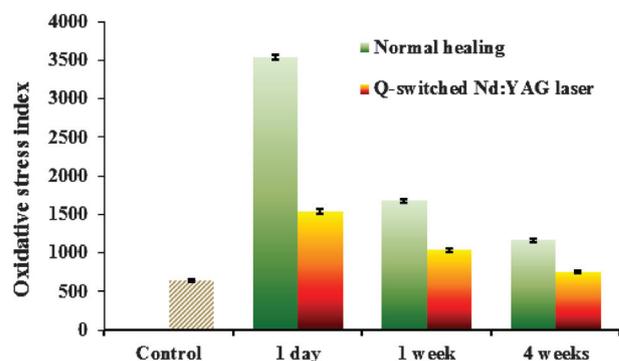


Figure 10. Oxidative stress index of the corneal tissues after different periods of normal healing and treatment with Q-switched Nd: YAG laser.

status (3536 ± 26) after one day which was very high compared with the control group (648 ± 14). After 1 week and 4 weeks of healing the oxidative stress was shifted towards antioxidative status with values of 1675 ± 25 and 1170 ± 16 respectively. By contrast, the laser treated groups showed more improvement in the oxidative/antioxidative balance than normal healing groups with values of 1589 ± 31 , 1049 ± 32 and 752 ± 16 after 1 day, 1 week and 4 weeks respectively.

Discussion

This work studied the biochemical analysis of proteins synthesized during epithelial migration. The change in corneal protein after injury was evaluated. The results indicated elevated levels of corneal protein percentage above the baseline of the control after one day in normal healing group (93%) and in laser treated group (58%). Furthermore, decreases in protein contents were observed after the next four weeks by values of 39% and 7.5% for normal healing group and laser treated group respectively. A comparison of total protein in the control group and protein synthesized during migration in both groups of normal healing and in laser treated groups indicated that the increased synthesis is the result of the enhanced synthesis of many of the proteins present in corneal epithelium. Moreover, the results indicated better improvement in laser treated group than in normal healing as indicated in Figure 1.

These elevations in total protein during wound healing are in agreement with previous investigation that devised an *in vitro* system that facilitated the analysis of biochemical events occurring during the migration of corneal epithelium¹⁸. Using this system, it was reported that: protein and glycoprotein synthesis as measured by [¹⁴C] leucine and [³H] glucosamine incorporation showed a large increase during epithelial sheet migration as compared to normal epithelium. Furthermore, another investigation indicated that, corneal epithelium migrating to cover a wound, synthesized protein and glycoprotein at a faster rate than did normal stratified epithelium¹⁹. The authors have found that the maximal rate of synthesis, as indicated by the incorporation of leucine and glucosamine, occurred 16 hours after injury and 6 hours before wound closure.

To explain the cause of the synthesis of new proteins involved in migration, an analysis was made in the present study using SDS-gel electrophoresis. Many differences were apparent between the migrating tissue and the controls: one protein band with a molecular weight of

110 KD was present to a much greater extent in migrating tissue than in normal epithelium. A time course analysis indicated that this band was apparent during migration in normal healing groups for four weeks and was not present after one and four weeks after injury by treatment with Nd: YAG laser. A comparison of SDS- PAGE in the control group, normal healing and laser treated groups indicated that protein in the 40- 70 KD range may form another part of newly synthesized molecules. This separation technique used did not allowed the assaying of such changes other than molecular weight.

Using these data together, the present study has attempted to ascertain the molecular nature of these changes in synthetic rates. Increased rates of protein synthesis could presumably result from the synthesis of specific molecules necessary for migration, or could be the result of a higher rate of synthesis of the proteins found in normal stratified tissue.

Only a few studies of the biosynthesis of proteins during epithelial migration have been reported. A 58 KD keratin-like protein was detected in the epithelium that was apparent 1 day after injury in rabbit corneas²⁰. In addition, it was noted that a keratin normally found in superficial cells was present in all cell layers following injury. The immunofluorescent study suggested that the basal cells may synthesize this keratin during migration²¹.

Indeed, protein and glycoprotein synthesis, increase dramatically during wound healing^{22,23}. The maximum increase in leucine and glucosamine incorporation occurred 16hrs after injury, and 6hrs before wound closure. It is possible that, the need for new proteins is high and the wound size has not decreased enough to increase the level of inhibition of synthesis that may occur with contact inhibition. It is interesting that the N-linked glycoprotein synthesis inhibitor, tunicamycin, blocks wound healing at about this same time point²⁴.

Again, the only major difference is that the 110-K band is present to a greater extent in migrating tissue. Because of this band's intensity, it is unlikely that it can account for the total increase in glucosamine incorporation. The increase may be the result of normal glycoproteins incorporating more glucosamine during migration.

However, the increase in incorporation could be the result of the increased synthesis of glycoproteins found in stratified tissue. An increase in protein and glycoprotein synthesis could reflect contributions by pre-existing migrating cells and/or synthesis by new cells moving into the wound area.

In the present study, the change in the keratin region (40-70 K) was detected which was more pronounced in

normal healing groups than laser treated groups. In normal healing groups, the change in keratin region were detected for all periods with slight improvement while laser treated groups showed decreasing in peaks intensities indicating recovery in the corneal protein after 4 weeks. This result is in agreement with the results of Kinoshita et al. who reported that, a 58KD keratin-like protein was present in migrating rabbit corneal epithelium which was not present in stationary epithelium¹⁸. Another possibility is that the conjunctival epithelium may migrate into the wound area when a large wound is created. It was known that, transparent corneal stroma contains a population of corneal fibroblasts termed keratocytes, which are interspersed between the collagen lamellae. Under normal conditions, the keratocytes are quiescent and transparent. However, after corneal injury the keratocytes become activated and transform into backscattering wound-healing fibroblasts resulting in corneal opacification. At present, the most popular hypothesis suggests that particular abundant water-soluble proteins called enzyme-crystallins are involved in maintaining corneal cellular transparency²⁵.

Another technique was applied to assess the mechanism of oxidant-antioxidant imbalance produced after injury and treatment of cornea with Nd:YAG laser. Based on that, the present study evaluated the total antioxidant capacity (TAC), total oxidative capacity (TOC), and oxidative stress index (OSI) of normal healed corneas and laser treated corneas. It was well known that various antioxidants had an additive effect, protecting the organism from free radicals²⁶. In this respect, evaluation of TAC provides information about the antioxidant capacity of the tissues²⁷.

Based on current knowledge, reactive oxygen species (ROS) are produced in metabolic and physiological processes, and harmful oxidative reactions may occur in organisms that remove them via enzymatic and non-enzymatic antioxidative mechanisms. Unstable free radical species attack cellular components causing damage to lipids, proteins, and DNA, which can initiate a chain of events resulting in the onset of a variety of diseases. Meanwhile, living organisms have developed complex antioxidant systems to counteract ROS and to reduce their damage²⁸⁻³⁰. These antioxidant systems include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase; macromolecules such as albumin, ceruloplasmin, and ferritin; and an array of small molecules, including ascorbic acid, α -tocopherol, β -carotene, reduced glutathione, uric acid, and bilirubin. The sum of endogenous and food-derived antioxidants

represents the total antioxidant activity of the system. The cooperation among different antioxidants provides greater protection against attack by reactive oxygen or nitrogen species, than any single compound alone. Thus, the overall antioxidant capacity may provide more relevant biological information compared to that obtained by the measurement of individual components, as it considers the cumulative effect of all antioxidants present in tissues¹⁴.

Oxidative stress results from increased number of lipid and protein oxidation products and decreased number of antioxidant enzymes and vitamins³¹. In addition, oxidative stress index (OSI), the ratio of the corneal TOC level to TAC, is an indicator of oxidative stress; reflecting the redox balance between oxidation and antioxidation^{16,32,33}.

In the present study, a decrease in the TAC (-49%) and increase in TOC (166%) of the normal healed cornea after on day of injury, induced change in oxidative stress index by 445 %. Moreover, after 1 week and 4 weeks the OSI decreased to 158% and 80% respectively. This was considered the cause of an imbalance between oxidative and antioxidative status.

Comparing these data with the groups treated with Nd:YAG laser, it seems that laser stimulates the antioxidant defense system by elevation of TAC than in normal healing because it induces more oxidative stress. Furthermore, these changes appeared to be time dependent. The results indicated gradual improvement in the OSI of the cornea after the 1 and 4 weeks of laser treatment that reached 62% and 16% respectively compared with the control. Interestingly, these results suggested that the oxidative/antioxidative balance shifted towards the antioxidative status and treatment with Nd:YAG laser helped to prevent further damage and give the cell time to repair the defect. In conclusion, anterior stromal puncture by Nd:YAG laser is an effective, simple, quick and easy procedure to treat recurrent corneal erosion.

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