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193 nm ArF Excimer Laser and the Potential Risk for Cataract Formation

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Abstract: The present work is designed to evaluate the effect of two different intensities of excimer laser on soluble lens protein of rabbit's eyes. Samples of lens protein from twenty albino rabbits were examined for the effect of argon fluoride (ArF) excimer laser (193 nm). In the 1st group the right eyes were submitted to 300 mJ/cm^2 ArF excimer laser and left for 1 week, the 2nd group was submitted to 300 mJ/cm^2 and left for 4 weeks, the 3rd group was submitted to 500 mJ/cm^2 and left for 1 week and finally the 4th group was submitted to 500 mJ/cm^2 and left for 4 weeks. The left eyes from each group were used as control. Measurements of protein concentration, refractive index (RI), column chromatography, sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS- PAGE) and ultraviolet absorption spectrum of soluble lens protein were investigated. The results indicate significant alteration in lens protein concentration and the refractive index measurements. The electrophoresis mobility and column chromatography separation indicates significant changes in molecular weights of lens proteins. The ultraviolet absorption spectrum revealed apparent depletion in lens protein concentration with pronounced changes outside the range of protein absorbance. It was concluded that, 193 ArF excimer laser treatment has a direct deleterious effect on lens protein which may be a possible risk for cataractogenesis.

Key words: Excimer laser, lens protein, gel chromatography, refractive index, electrophoresis, UV spectroscopy.

INTRODUCTION

The use of excimer laser was first introduced by Trockel *et al.*,[1] for the correction of optical errors through a removal of the superficial stroma in the central part of the cornea (Photorefractive Keratectomy, PRK), which induces a flattening of the anterior cornea with a consequent correction of myopia[2]. The argon fluoride (ArF) excimer laser causes high- powered pulses of ultraviolet light (193 nm). Part of the energy delivered is reflected, part is consumed by the corneal surgery itself and part is transmitted into the eye as secondary radiation in the cataractogenic range of 290-320 nm[3]. In situ measurements via a quartz fiber introduced into the eye and advanced to the lens surface showed that secondary radiation in the cataractogenic range of 295-320 nm is transmitted by the cornea and reaches the lens. It was known that these wavelengths can induce cataract formation[4,5].

Aqueous humor has about 78% transmittance of wave length in the 300-320 nm range[6]; thus most of the secondary radiation due to fluorescence, and generated during PRK treatment, easily reaches the anterior surface of the lens. This radiation starts at 200 nm and extends into the visible spectrum. It contains the considerably more hazardous portion of the spectrum between 250 nm to 350 nm, which penetrate deeper into the eye, where they can generate phototoxic and cataractogenic effects[5,7]. There are insufficient data on the possible cataractogenic side effects of excimer laser. The present work aimed to investigate the effect of excimer laser corneal surgery on the lens.

MATERIALS AND METHODS

20 newzealand male rabbits weighing 2-2.5 kg were used in this study. The animals were selected from the animal house of Research Institute of Ophthalmology and were fed on balanced diet.

Clinical Examination: Slit lamp biomicroscopic examinations of the lens were performed before corneal ablation, at 24 hour, one week and four weeks after laser ablation. All observations were made following papillary dilation with tropocamide 1.0%, the results showed no signs of intraocular inflammation and no edema in all eyes.

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Laser Treatment: Animal were fully anesthetized by using intramuscular ketamine (katalar 2.5mg/ kg). Additionally they received 0.4% Benoxinate eye drops for local anesthesia. Four groups of rabbits underwent corneal ablation using argon fluoride excimer laser (ArF, 193 nm). The photoablation treatments were performed using Lampda Physics ArF excimer laser. The laser pulse rate was 0- 200 Hz the optical zone was 1cm^2, the energy/ pulse was 2.0 mJ, the wavelength was 193 nm and the duration of pulse was 6 ns. These parameters resulted in an estimated photoablation depth of 96.9µm.

The rabbits were classified into four groups of five rabbits each and the left eyes from each group were used as control and the right eyes were treated with laser as following:

Group I: received 193 nm ArF excimer laser intensity of 300 mJ/cm^2 and the animals were left for one week;
Group II: received 193 nm ArF excimer laser intensity of 300 mJ/ cm^2 and the animals were left for four weeks;
Group III: received 193 nm excimer laser intensity of 500 mJ/cm^2 and the animals were left for one week;
Group IV: received 193 nm excimer laser intensity of 500 mJ/cm^2 and the animals were left for four weeks.

After the demonstrated periods rabbits were decapitated and the eyes were enucleated. The lenses were removed, homogenized in bidistilled water and centrifuged at 8000 rpm for 30 minutes. The supernatant of the samples were removed for the analysis of soluble lens protein. The following measurements were carried out on soluble lens protein: Estimation of total protein concentration for lens protein by using the method of *Lowry et al,*.[8] ; measurements of the refractive index (RI) using Abbe´s refractometer attached with temperature control unit at 37°C ± 0.02; sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS- PAGE) using the method of *Laemmli*[9] ; gel filtration chromatography using sephacryl S-300 and ultraviolet absorption spectroscopy for lens protein using a spectrophotometer type UV-visible recording 240 graphical, Shimatzu, Japan.

Statistical Evaluation: The results were statistically evaluated according to the "Students" t- test[10] . "t" is the test of significance and was used in comparison between results.

RESULTS AND DISCUSSION

Soluble Lens Protein Content: The total soluble lens protein for all studied groups was illustrated in table (1). The soluble lens protein for control group is 288 ± 5.2 mg/g tissue wet weight. The data shows that there is a non significant change in the lens protein content after 1 week of treatment with 300 mJ/cm^2 of ArF excimer laser (193 nm). However, after 4 weeks there was a pronounced decrease in lens protein content. Moreover, for the groups treated with 500 mJ/cm^2 of ArF excimer laser (193 nm) there was a progressive decrease in protein content after 1 week and 4 weeks of treatment.

The Refractive Index of Lens Protein: Table (2) illustrates the refractive indices (RI) for lens protein of rabbits treated with ArF excimer laser (193 nm) with different intensities. The data indicates that, there is no change in RI of lens protein for the group treated with 300 mJ/cm^2 after 1 week. However, there is a pronounced increase in the RI after 4 weeks of treatment with 300 mJ/cm^2. In addition, the data indicates that, the RI for groups treated with 500 mJ/cm^2 of ArF excimer laser is increased after 1 and 4 weeks.

SDS-Polyacrylamide Gel Electrophoresis for Lens Protein: The scanning pattern for control group is characterized by the presence of 10 fractions represents different crystallin of lens protein (α, β_H, β_L, and γ crystallin).

Figure (1) illustrates the change in the electrophoresis pattern after 1week of treatment with 300 mJ/cm^2 of ArF excimer laser (193 nm) compared with control.

The second peak in the treated group was shifted towards the lower molecular weight which accompanied with appearance of a new fraction at 118.75 K.D. Moreover, the pattern indicates reduction in the intensities of fractions in the area between 28 - 14 K.D and formation of low molecular weight fractions at 29.17 and 18.72 K.D.

After 4 weeks of treatment with 300 mJ/cm^2 of ArF excimer laser (Figure 2), the SDS electrophoresis pattern of rabbits lens protein indicates the formation of new fractions at 118.75 K.D and 24.53 K.D. In addition, there was a significant decrease in the intensities of some peaks in the high and low mobile group.

Figure (3) illustrates the SDS-PAGE of rabbits soluble lens protein for control and after 1 week of treatment with 500 mJ/cm^2 of ArF excimer laser (193 nm). The pattern reveals a pronounced change in the low mobile group (high molecular weight region) which indicates the presence of two new fractions at 116 K.D, and 102.74 K.D. Moreover, there was a remarkable decrease in some peaks intensity in the high and low mobile group.

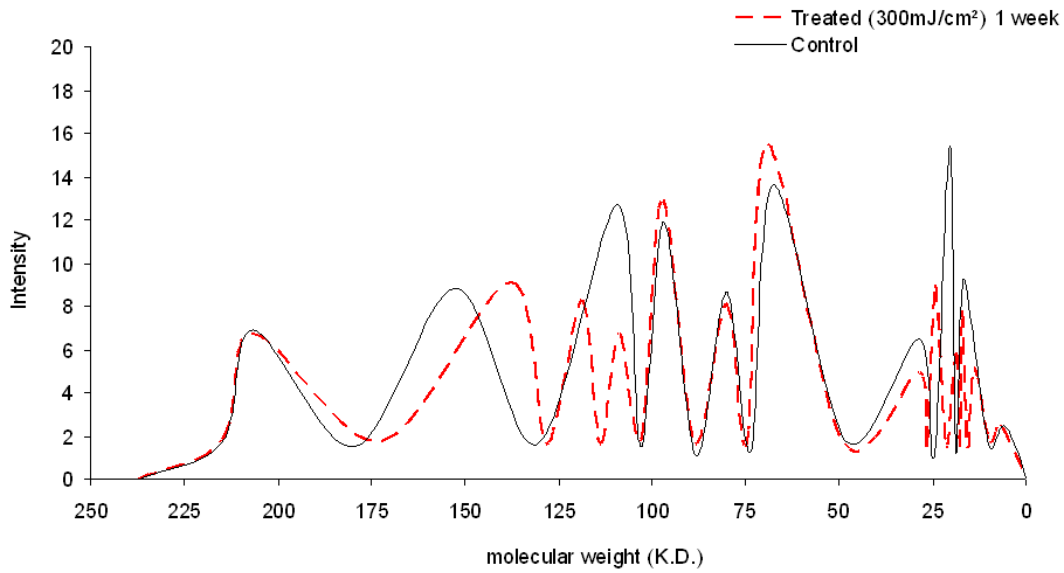

Fig. 1: Electrophoretic pattern for rabbit's lens protein after 1 week of treatment with 300 mJ/cm^2 of 193 nm ArF excimer laser.

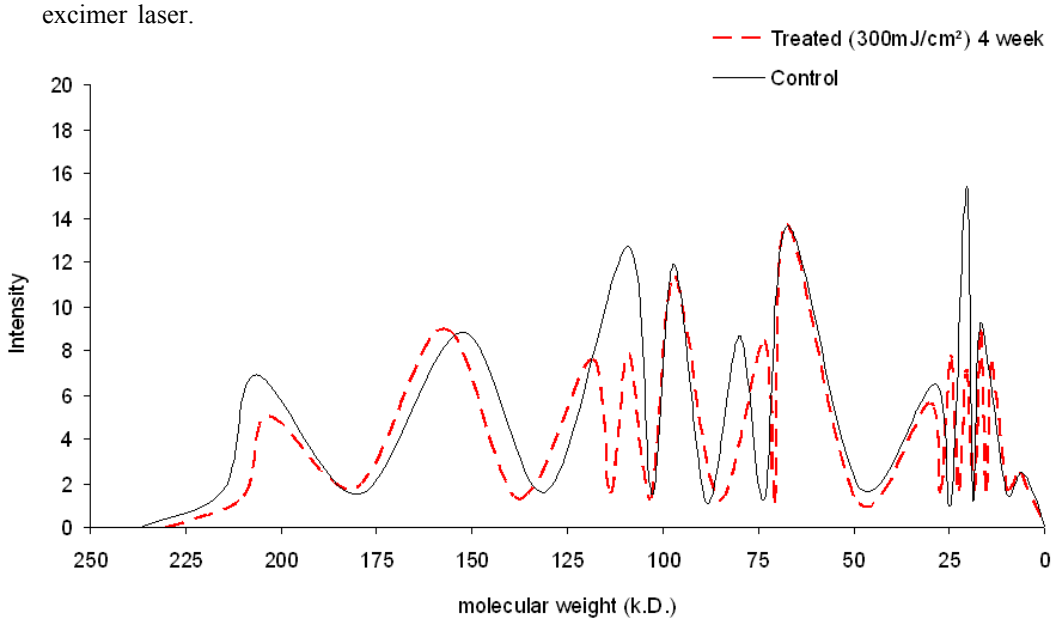

Fig. 2: Electrophoretic pattern for rabbit's lens protein after 4 weeks of treatment with 300 mJ/cm^2 of 193 nm ArF excimer laser.

Figure (4) illustrates the SDS-PAGE of rabbits lens protein after 4 weeks of treatment with 500 mJ/cm^2 of ArF excimer laser (193 nm) compared with control. The scanning pattern indicates the same phenomena as in the previous group that treated with 500 mJ/cm^2 for 1 week. Both height and width of the peaks were remarkably changed. In addition, some protein fractions were appeared at 113.44, 100.63 K.D instead of 108.99 K.D.

Gel Chromatography for Lens Protein: Figure (5) represents the chromatographic elution pattern of soluble lens protein for normal and treated rabbits submitted to 300 mJ/cm^2 of ArF excimer laser. The normal soluble lens crystallin was eluted into four main peaks corresponding to the different crystallin fractions which represented by α-crystallin, β_H-crystallin (high molecular weight), β_L-crystallin (low molecular weight) and γ-crystallin.

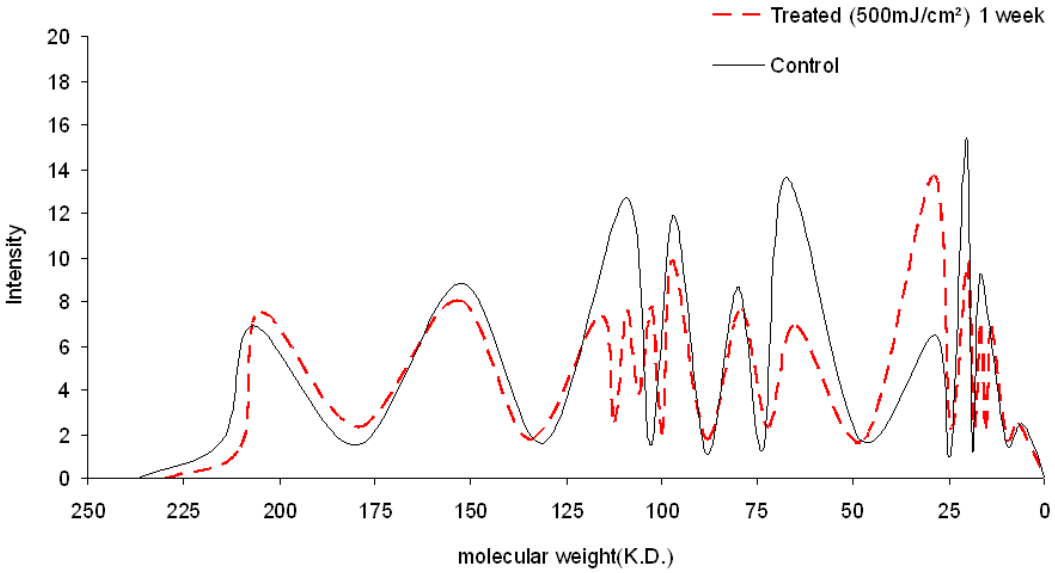

Fig. 3: Electrophoretic pattern for rabbit's lens protein after 1 week of treatment with 500 mJ/cm^{2}of 193 nm ArF excimer laser.

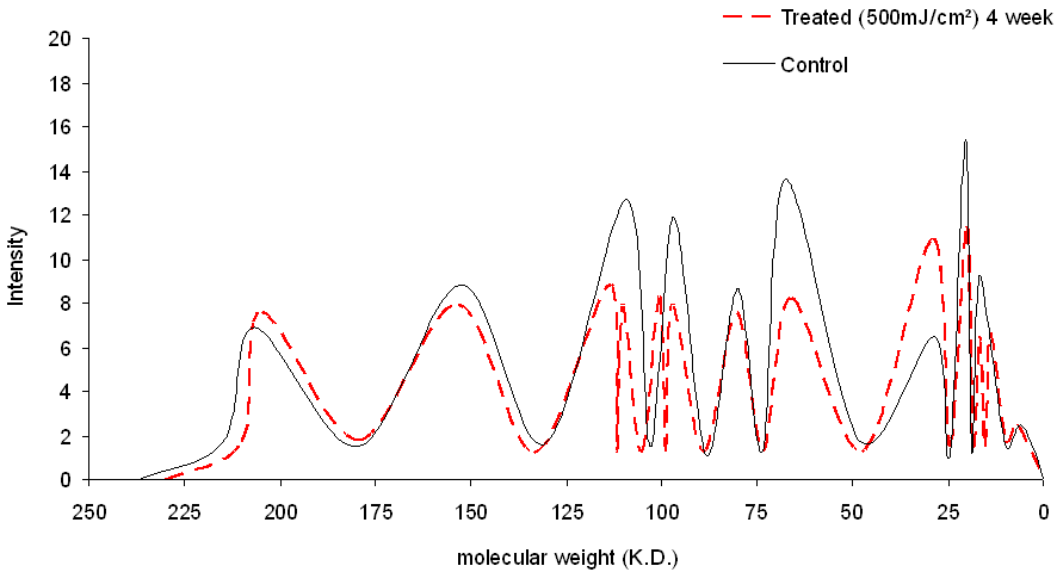

Fig. 4: Electrophoretic pattern for rabbit's lens protein after 4 weeks of treatment with 500 mJ/cm^2 of 193 nm ArF excimer laser.

After 1 week of excimer laser treatment there was a significant shift of all crystallin fractions towards higher molecular weight relative to the control. Also, α-crystallin and β_H-crystallin were diffused together. In comparison β_L & γ-crystallin were decreased in their intensities. After 4 weeks of treatment with 300 mJ/cm^2 of ArF excimer laser (figure 6), the molecular weights of all lens crystallin were changed and two peaks were appeared in α-crystallin region. Also γ-crystallin was changed accompanied with increasing in peak intensity.

Figure (7) illustrates the chromatographic elution pattern for normal and treated rabbit's lens protein after 1 week of treatment with 500 mJ/cm^2 of ArF excimer laser (193nm). The figure indicates a remarkable shift towards higher molecular weights. In addition, α-crystallin and β_H crystallin peaks were diffused together. Similarly, β_L and γ-crystallin peaks were diffused together and decreased in their intensities. After 4 - weeks (figure 8), the pattern showed a significant shift towards higher molecular weight region

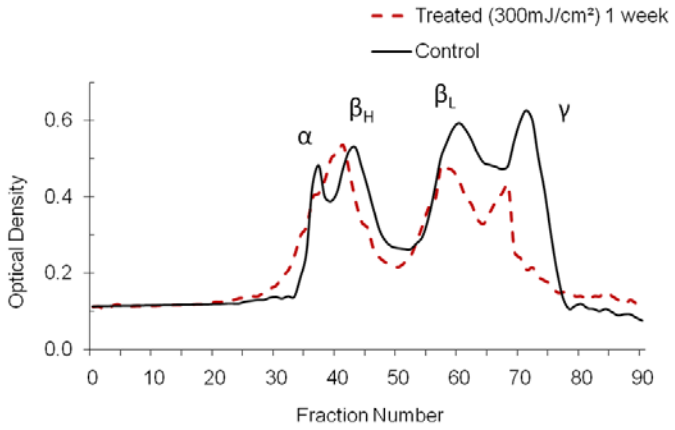

Fig. 5: Chromatographic elution pattern for rabbit's lens protein after 1 week of treatment with 300 mJ /cm^{2}of 193 nm ArF excimer laser.

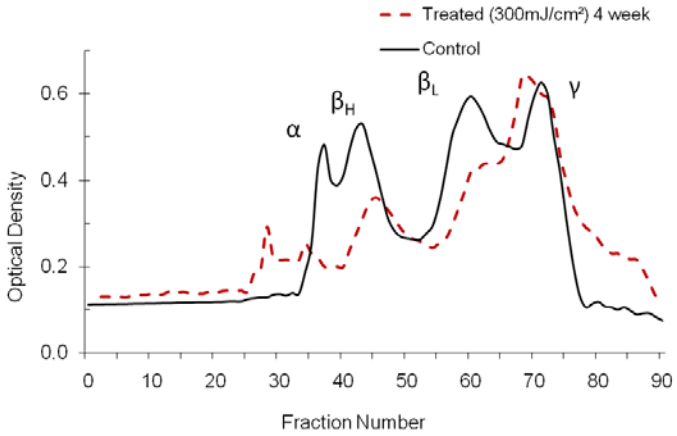

Fig. 6: Chromatographic elution pattern for rabbit's lens protein after 4 weeks of treatment with 300 mJ /cm^{2}of 193 nm ArF excimer laser.

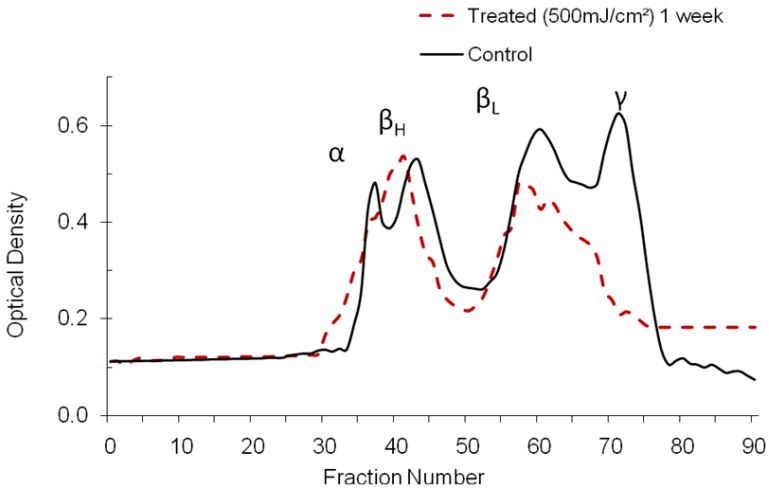

Fig. 7: Chromatographic elution pattern for rabbit's lens protein after 1 week of treatment with 500 mJ /cm^{2}of 193 nm ArF excimer laser.

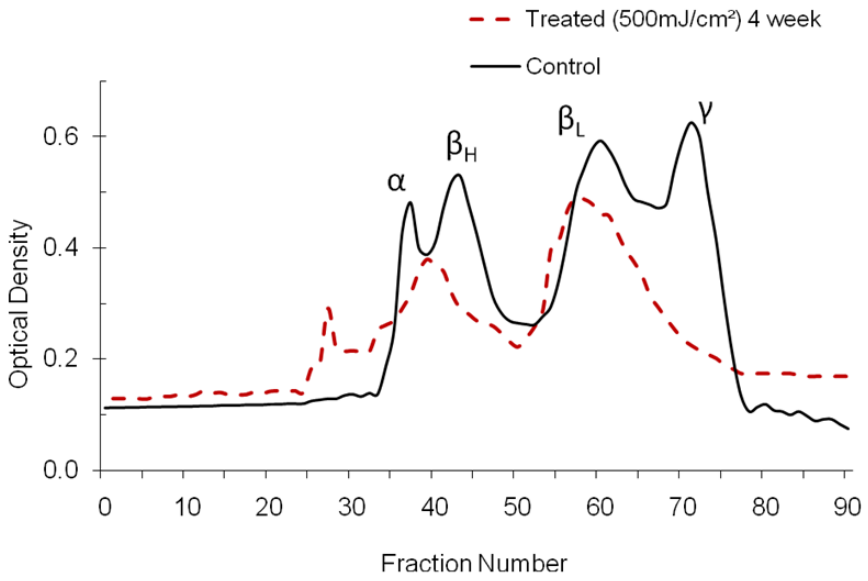

Fig. 8: Chromatographic elution pattern for rabbit's lens protein after 4 weeks of treatment with 500 mJ /cm^{2}of 193 nm ArF excimer laser.

accompanied with decreasing in peaks intensities and appearance of new fraction in the high molecular weight region.

UV- Absorption Spectra for Lens Protein: Figure (9) illustrates the absorption spectra for rabbit's lens protein for control and after 1 and 4 weeks of treatment with 300 mJ/cm^2 of ArF excimer laser (193 nm). The spectra for the control sample indicates the appearance of the characteristic absorption spectra at λ_{max} = 280 nm. The spectra shows a decrease in the intensities of the peaks after 1 and 4 weeks of treatment accompanied with remarkable absorption changes outside the range of protein absorption at λ_{max} = 355 nm. After 1 and 4 weeks of treatment with 500 mJ/cm^2 of ArF excimer laser (figure10), the spectra indicates disappearance of the peaks at λ_{max} = 280 nm with significant appearance of new broad peaks with high intensities at longer wavelength (λ_{max} = 355 nm).

Discussion: Cataract is defined as opacity in the normally transparent crystallin lens that impairs light transmittance through the lens and produces an impairment of vision [11]. In general, there are a number of components that absorbs ultraviolet radiation (UVR) and may induce photochemical damage to the tissue. The most common are chromophores, nucleic acids and proteins including various types of enzymes[12,13]. Epidemiological evidence indicates that long term exposure to ultraviolet radiation in the UVB range is associated with an increased risk of cortical and posterior subcapsular cataracts [14]. Additionally, experimental evidence suggests that the lens is susceptible to damage from ultraviolet radiation in UVB range of 290-320 nm.

Mammalian lens contain several classes of chromophores that are absorber of energy. Moreover these compounds, acting as photosensitizers, once excited by UV radiation in the presence of oxygen- can generate free radicals [15]. *Oliveira and charman*[16], concluded that, excimer laser- corneal tissue interaction librates highly reactive free radical species capable of attack tissue component leading to more serious damage.

In the present study, the total protein concentration of control rabbit lens is 288.0 ± 5.2 mg/g tissue wet weight. This value is in agreement with *Durchschlag et al.,*[17] and is significantly decreased after 4 weeks of treatment with 300 mJ/cm^2 excimer laser. The decrease is more pronounced after treatment of the rabbit ´s eye to 500 mJ/cm^2 excimer laser (table 1).

The drop in total soluble lens proteins was increased by increasing the time after UV exposure. This may be due to that exposure of rabbit lenses to UV lead to inhibition of protein synthesis and aggregation of soluble proteins. These findings agree with *Zigman et al.,*[18].

The decrease in the total protein content of rabbit's lenses after treatment with 300 mJ/ cm^{2}and 500 mJ/cm^2 excimer laser is confirmed by the chromatographic elution pattern obtained after 1 week and 4 weeks of treatment.

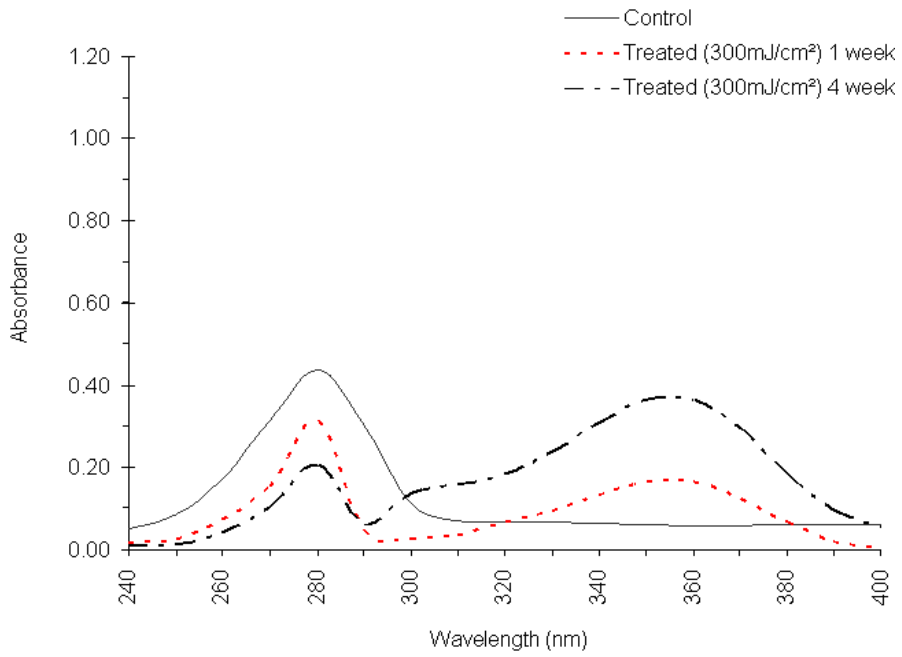

Fig. 9: UV - absorption spectra for rabbit's lens protein after 1 and 4 weeks of treatment with 300 mJ/cm^2 of 193 nm ArF excimer laser.

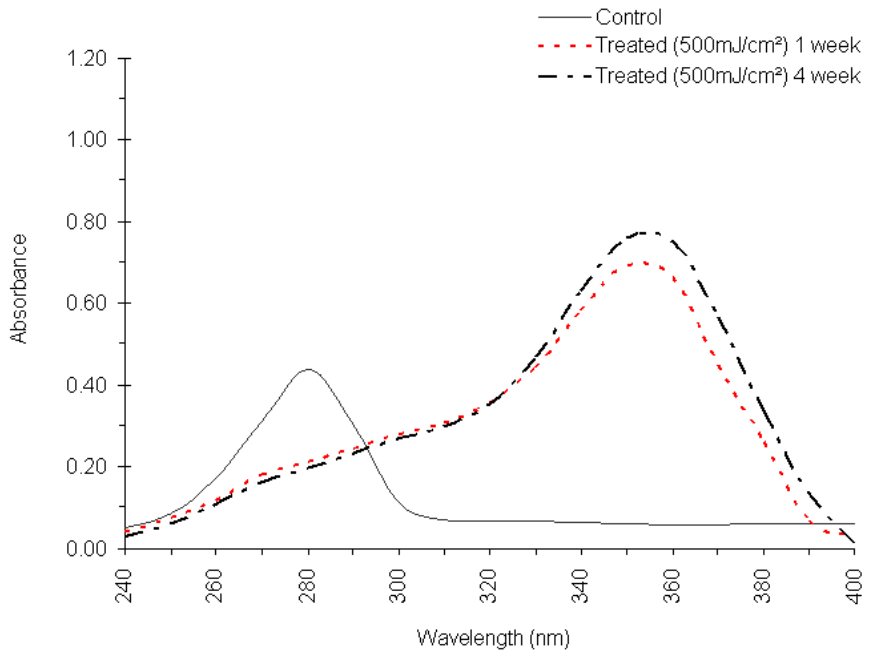

Fig. 10: UV - absorption spectra for rabbit's lens protein after 1 and 4 weeks of treatment with 500 mJ/cm^2 of 193 nm ArF excimer laser.

The chromatographic elution pattern obtained after 1 week of treatment with 300 mJ/ cm^2 indicate the aggregation of β and γ crystallin and their shift towards higher molecular weight. This phenomenon is propagated after 4 weeks of treatment with excimer laser in addition to the appearance of high molecular weight proteins in the void volume of the column. This means that protein aggregation and crosslinking is increased by increasing time after exposure.

Table 1: Effect of 193 nm ArF excimer laser with different intensities on lens protein after different periods.

Group	Soluble Lens Protein (mg/g tissue wet wt.)		
	mean ± SD	P value	Change % *
Control	288 ± 5.2	-	-
1 week (300 mJ/cm²)	283 ± 3.7	N.S	-0.02%
4 weeks (300 mJ/cm²)	231± 6.3	H.S (P<0.01)	-20%
1 week (500 mJ/cm²)	236 ± 8.13	H.S (P<0.01)	-18%
4 weeks (500 mJ/cm²)	193 ± 5.0	V.H.S (P<0.001)	-33%

** % Change with respect to the control group.*
N.S : Non significant
H.S : Highly significant
V.H.S : Very highly significant

Table 2: Refractive index for lens protein of rabbits after 1 and 4 weeks of treatment with 300 mJ/cm^2 and 500 mJ/cm^2 ArF excimer laser.

Group	Refractive index of lens protein	
	(300 mJ/cm^2)	(500 mJ/cm^2)
Control	1.3324	1.3324
1 week	1.3324	1.3344
4 week	1.3354	1.3384

The SD of these values is in the same sensitivity range of the instrument.

The mechanism of damage that occurred to the lens after exposure to UVR was explained previously[19] . It was reported that, absorbed UVR- photon excite lens molecules and create free radicals, which increase the oxidative stress on the lens that induced damage to DNA, proteins and lipids. This damage affected lens cell differentiation and protein synthesis.

In the present study, it is recognized that, by increasing the intensity of excimer laser to 500 mJ/ cm^2, there is increase in aggregation of $\beta_{H,}$ β_l and γ crystallin more than those associated with lens proteins exposed to 300 mJ/ cm^2. After 4 weeks of exposure, high molecular weight protein also appeared in the void volume of the column.

The changes in the molecular weight distribution of different lens crystallins and aggregation of soluble lens protein was increased by increasing the intensity and time post exposure. These changes in lens protein may lead to cataract formation. It is known that during cataract formation the decrease of biosynthesis of lens crystallin is followed by their aggregation which then leads to opacification of the lens. Similar changes in protein pattern were described by *Takemoto and coworkers* [20] who concluded that, the formation of aggregated covalently linked high molecular weight protein would play an important role in opacification of the lens.

From the forementioned obtained data (soluble lens protein content and chromatographic separation) it is clear that, the main effect of UVR exposure is the decrease of the solubility of lens proteins which leading to formation of new insoluble species. This new insoluble species may have new molecular weights leading to changes in the electrophoresis mobility.

The SDS- PAGE profiles of lenses submitted to 300 mJ/ cm^{2}and 500 mJ/ cm^{2}excimer laser and rabbits were decapitated after 1 and 4 weeks of treatment showed that, the lens proteins underwent changes including decrease and/ or increase in electrophoresis mobility, disappearance and appearance of some protein fractions, broadening and changes of the lens protein intensity. Such changes have been previously reported [21,22] in the UV irradiated lenses. Their data showed that the lens proteins underwent changes including polypeptide photocrosslinking to form dimers and higher molecular weight materials after exposure to excimer laser radiation.

The lens of the eye maintains a gradient of refractive index, which allows it to produce an image without significant spherical and chromatic aberration [23]. Since the transparency of the crystallin lens depends on the regular or ordered spacing of its cells and proteins. Disturbance of this order- such as protein aggregation, changes in tissue hydration, can results in local changes of refractive index which cause light scattering [24].

In the present study, the relative refractive index is elevated after exposure to excimer laser (193nm) (Table 2). The elevation in the refractive index is directly proportional to the decrease in the concentration of

soluble lens protein and also the increase in the aggregation of soluble lens protein which detected from gel chromatography and electrophoresis. These results are in accordance with the previous work [23].

UV-absorption spectroscopy experiments on soluble lens protein of rabbit's lenses submitted to300 mJ/ $cm^2$and 500 mJ/ cm^2excimer laser and decapitated after 1 and 4 weeks revealed that lens crystallin suffer from two major effects, firstly, a change in the aromatic amino acid residues (Tryptophan and Tyrosine), as may be concluded from the changes at λ= 280 nm caused by the changes in the aromatic chromophores. Secondary, there is pronounced aggregation, as indicated from the remarkable absorbance changes outside the range of protein absorption at λ= 355nm. At high intensity (500 mJ/cm^2) the aromatic residues (Tryptophan and Tyrosine) of the crystallin are completely depleted at λ= 280 nm verified the destruction of the aromatic residues and indicated a change of the secondary structure (decrease of the helix content).These changes were similar to those detected by previous works [17-25].

Li and Borkman [26] showed that the first photooxidation product of Tryptophan, N-formylkynurenine (NFK), could serve as a photosesitizer for further Tryptophan oxidation by UV light. Tryptophan was then found to be photolysed by UV light exposure to yield many pigmented and fluorescent compounds. Some of these may bind to human and animal lens proteins, thereby altering their physical and chemical properties. Such changes in the tryptophan residues in lens proteins would result in increased pigmentation of the lens and photosensitivity to excimer laser.

Conclusion: Excimer laser treatment of the cornea with UV beam of ArF (193 nm) generates secondary radiation in the cataractogenic range. These rays are transmitted by the cornea and reach the lens. Although the obtained data may not be similar to the situation in the human eye, they may provide a model with which to compare the relative effect of different treatment parameters to help to establish the optimum protocol.

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