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## Comparison of Aqueous Humor Nitric Oxide Levels After Different Corneal Collagen Cross-Linking Methods

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### ABSTRACT

**Purpose:** Nitric oxide production can cause either apoptotic or necrotic cell death through oxidative stress. We aimed to investigate the nitrite oxide metabolites (NO<sub>x</sub>) and nitrite levels in the aqueous humor of rabbit eyes after different methods of corneal collagen cross-linking (CXL).

**Materials and methods:** Twenty-four eyes of 12 adult New Zealand rabbits were used. They were assigned into four groups, each including six eyes. Group 1 (control) consisted of eyes with no treatment. Group 2 received UV-A power setting at 3 mW/cm<sup>2</sup> for 30 minutes of continuous exposure and named as standard CXL group. Group 3 received UV-A power setting at 30 mW/cm<sup>2</sup> for 3 minutes of continuous exposure and named as accelerated CXL (A-CXL) group. Group 4 received UV-A power setting at 30 mW/cm<sup>2</sup> for 6 minutes of pulsed exposure (1 sec on, 1 sec off) and named as pulse-light accelerated CXL (PLA-CXL). Aqueous humors were aspirated from anterior chamber with a 27G needle after 1 hour UV-A exposure. NO<sub>x</sub> and nitrite levels were measured

**Results:** The nitrite levels in aqueous humor were significantly increased in Group 2 and Group 3 when compared with Group 1 ( $p = 0.000$ ,  $p = 0.036$ , respectively). When treatment modalities were compared with each other, high nitrite level in Group 2 was statistically significant when compared with Group 4 ( $p = 0.019$ ). NO<sub>x</sub> levels were higher in Group 2 when compared with Group 1 ( $p = 0.006$ ).

**Conclusions:** Numerous studies investigated the physiological and pathophysiological roles of NO. NO is considered one of the most important molecule for ocular health. According to NO<sub>x</sub> level in aqueous humor, it seems that PLA-CXL is the safest method due to the similar results with control group.

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Aqueous humor; corneal collagen cross-linking; nitric oxide

## Introduction

Corneal collagen cross-linking (CXL) changed our point of view to free radicals by demonstrating positive sides in the treatment of many corneal diseases. These free radicals induce new chemical bonds between and within the collagen fibers, which strengthens the cornea.<sup>1</sup> While allowing the formation of free radicals deliberately with our own hands, today it is well-known that they are playing a crucial role in damaging the cell by causing oxidative damage. Also, UV-A irradiation itself can induce oxidative stress and can potentially damage other ocular structures.<sup>2–4</sup>

Although the standard method for CXL was well-described in 2003,<sup>5</sup> investigators were not satisfied with the standard method, and began pursuing new methods. To shorten the overall treatment time, an accelerated CXL was developed by increasing the UV-A illumination intensity. Afterwards, considering the photo-dynamic types I and II reactions as the basis of CXL,<sup>6</sup> pulse-light accelerated CXL (PLA-CXL) was introduced. No data are currently available regarding comparing oxidative stress levels at a cellular basis among these different methods.

NO is a short-lived free radical that plays important roles in vasodilation, immunity, inflammation, and neurotoxicity.<sup>7,8</sup> In many physiological and pathological conditions, NO is produced by nitric oxide synthase (NOS) through oxidative deamination of

L-arginine, and it shows its effect after being synthesized. Because of NO's high reactivity and short half-life, it is converted to the relatively stable end-products of nitrite and nitrate. Therefore, measurement of nitrite and nitrate levels in biological fluids are used as an indicator of NO synthesis.<sup>9</sup>

While many attempts are made to define the safest method for CXL, it would be more accurate to evaluate the effect of CXL at the cellular level. Therefore, in this current study we aimed to investigate the nitrite oxide metabolites (NO<sub>x</sub>) and nitrite levels in the aqueous humor of rabbit eyes after using different methods of CXL.

## Material and methods

Animal study was approved by Gazi University Ethical Committee for Animal Experiments and all protocols were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

## Experiments

Twelve adult New Zealand rabbits weighing between 2.5 and 3 kg were used and both eyes of each rabbit were included in the study.

Animals were anesthetized with an intramuscular injection with ketamine and xylazine. The eyes of rabbits were divided into four groups, each group had six eyes. Before epithelial debridement, proparacaine HCl 0.5% (Alcaine; Alcon Laboratories, Fort Worth, TX, USA) was instilled. After specimens were obtained, the rabbits were euthanized.

Group 1 (control) consisted of eyes with no treatment. In Group 2 (standard CXL), riboflavin 0.1% within 20% dekstranT500 solution was applied every 3 min for 30 minutes following epithelial debridement on a 9 mm field. Then, all corneas were irradiated with UV-A light (at an irradiance of 3 mW/cm<sup>2</sup>) for 30 minutes of continuous exposure. During irradiation, riboflavin 0.1% within 20% dekstranT500 solution was applied at 3 min intervals.

In Group 3 (accelerated CXL, A-CXL), riboflavin 0.1% within 20% dekstranT500 solution was applied every 3 min for 30 minutes following epithelial debridement on a 9 mm field. Then, all corneas were irradiated with UV-A light (at an irradiance of 30 mW/cm<sup>2</sup>) for 3 minutes of continuous exposure.

In Group 4 (PLA-CXL), riboflavin 0.1% within 20% dekstranT500 solution was applied every 3 min for 30 minutes following epithelial debridement on a 9 mm field. Then, all corneas were irradiated with UV-A light (at an irradiance of 30 mW/cm<sup>2</sup>) for 6 minutes of pulsed exposure (1 sec on, 1 sec off).

In all groups, aqueous humors were aspirated from anterior chamber with a 27G needle after 1 hour of UV-A exposure. All specimens were frozen with liquid nitrogen and stored in a dark place at -80°C until biochemical measurements.

### Biochemical analysis

Nitric oxide metabolites (NO<sub>x</sub>) and nitrite levels in aqueous humor were measured by spectrophotometric method described by Miranda et al.<sup>10</sup> Aqueous samples were deproteinized with 96% ethanol at 1:2 (v/v) and vortexed for 5 minutes and then mixed samples were centrifuged at 10,000g for 5 min at 4°C. Supernatants were used for the analysis of NO<sub>x</sub> levels and nitrite. For NO<sub>x</sub> analysis, 100 µL of supernatant, 100 µL of vanadium (III) chloride and following Griess reagents, 50 µL of sulphanilamide and 50 µL of N-(1-naphthyl) ethylenediamide dihydrochloride (NEED) were pipette into each microplate well and then incubated for 30 min at 37°C. For determination of nitrite levels, 100 µL of supernatant, 50 µL of sulphanilamide, and 50 µL of NEED were pipette into each microplate well and then incubated for 15 min at 37°C. The absorbance of NO<sub>x</sub> levels and nitrite were read at 540 nm. NO<sub>x</sub> and nitrite concentrations were calculated from the linear standard curves prepared by sodium nitrate (10–100 µM range) and sodium nitrite (0–100 µM range), respectively. NO<sub>x</sub> and nitrite levels were expressed as micromoles per liter.

### Statistical analysis

Statistical analysis was made using SPSS software for Windows version 20.0 (Chicago, IL, USA). Data were evaluated for normal distribution using the Shapiro–Wilk test. Results were given as a mean ± standard error or median (minimum–maximum). One-way variance analysis (ANOVA)

followed Tukey's *post hoc* test was used to determine the statistical significance of the comparisons between groups ( $p < 0.05$ ). For the abnormally distributed data, Kruskal–Wallis variance analysis was used to the statistical significance of the differences. The significance of the observations was determined using a Bonferroni multiple comparison test (adjusted  $p < 0.05/n$  with  $n$  number of comparisons).

## Results

The comparisons of aqueous humor nitrite levels among the experimental groups are given in Figure 1. Aqueous humor nitrite levels were higher in Group 2 (8.42 ± 1.37 µmol/L), Group 3 (6.40 ± 1.96 µmol/L), and Group 4 (5.79 ± 0.94 µmol/L) than in Group 1 (4.0 ± 1.11 µmol/L). While high levels in Group 2 and Group 3 were statistically significant when compared with Group 1 ( $p = 0.000$ ,  $p = 0.036$ , respectively), high levels in Group 4 were not statistically significant ( $p = 0.155$ ). When treatment modalities were compared with each other, high levels in Group 2 were statistically significant when compared with Group 4 ( $p = 0.019$ ).

Figure 2 shows the NO<sub>x</sub> levels in aqueous humor of all experimental groups. NO<sub>x</sub> levels were higher in Group 2 (87.5 ± 19.6 µmol/L), Group 3 (58.8 ± 15.3 µmol/L), and Group 4 (62.2 ± 4.3 µmol/L) when compared with Group 1 (48.5 ± 12.6 µmol/L). It was only statistically significant in Group 2 ( $p = 0.006$ ).

## Discussion

The hallmark of the CXL treatment is the free radicals that were generated by photosensitization of riboflavin by UV-A light. In addition to photochemically induced free radicals, UV-A irradiation itself can potentially damage intraocular structures.<sup>2,3,11,12</sup> Although today free radicals are closely associated with pathogenesis of many diseases, they function to generate cross-linking within and between collagen fibers.<sup>1</sup>

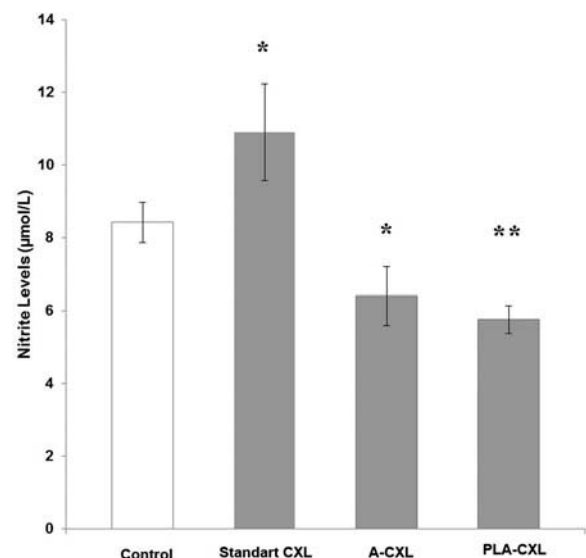
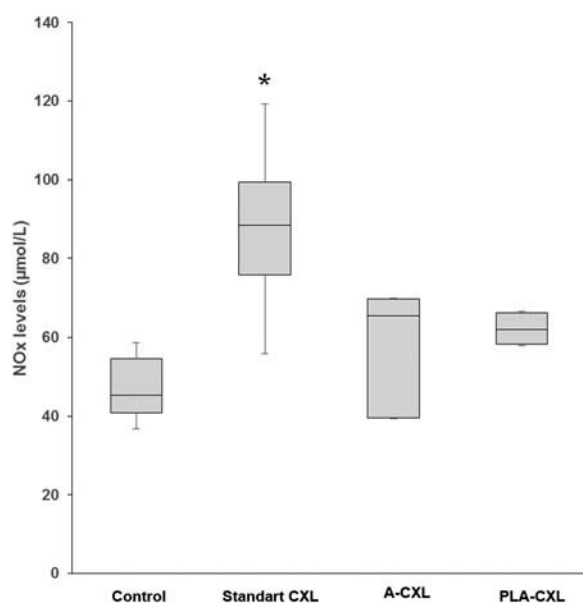


Figure 1. The comparisons of aqueous humor nitrite among the experimental groups. \* $p < 0.05$  compared with control, \*\* $p < 0.05$  compared with standard CXL group.



**Figure 2.** The comparisons of aqueous humor  $\text{NO}_x$  levels among the experimental groups. \* $p < 0.008$  compared with control.

NO is a free radical gas that was introduced as the molecule of the year in 1992.<sup>13</sup> It has vital physiological roles in many processes, such as vasodilation, inflammation, immunity, thrombosis, and neurotransmission.<sup>7</sup> After the discovery of nitric oxide, it has been a source of inspiration for understanding the pathophysiology of many diseases. Determining NO levels is difficult due to its short half-life and its rapid reaction with other molecules.<sup>14</sup> For this reason, measuring nitrite and nitrate levels as the stable end-products of the NO is commonly used to detect the production of NO in human body fluids.<sup>15</sup>

Aqueous humor helps the regulation and stabilization of the homeostasis of the anterior segment structures by being the main source of nutrition and removing excretory products from the metabolism. Particularly, aqueous humor behaves as a blood surrogate for vascular structures such as the cornea and lens.<sup>16</sup> Inflammatory cells and mediators circulate in the eye within the aqueous humor.<sup>17</sup> The aqueous humor is the main source of antioxidants in the anterior segment of the eye, particularly for the lens epithelium and corneal endothelium.<sup>18</sup> There are several reports which investigated the effects of aqueous humor NO in many eye disorders such as glaucoma, retinal disease, uveitis, and cataract.<sup>19–23</sup>

In this current study, we suggest that evaluating  $\text{NO}_x$  levels in the aqueous humor may help to predict the possible damage to the ocular tissues, such as the corneal endothelium, lens epithelium, and retina during CXL treatment. As mentioned before, CXL is mediated by free radicals. Superoxide anion is one of the main free radicals that is generated during CXL by photosensitization of riboflavin by UV-A light. In the presence of superoxide anion, it rapidly reacts with NO to generate peroxynitrite,  $\text{ONOO}^-$ , which is a highly biologic toxic molecule.<sup>24</sup> Yanagiya et al.<sup>25</sup> demonstrated a mild cytotoxic effect of peroxynitrite generated in the aqueous humor on corneal endothelial cells.

Kryczka et al.<sup>26</sup> investigated the glutathione levels as an important intracellular corneal antioxidant after a standard CXL procedure in porcine corneas. They detected partially depleted glutathione levels after the standard CXL, but they suggested that this result was not threatening and that CXL is a safe procedure. However, the standard procedure is time-consuming.<sup>27</sup> Although the efficacy and safety of the standard CXL procedure was established, researchers focused on shortening the treatment time based on the Bunsen–Roscoe law of reciprocity. This law expresses that the same photochemical effect can be achieved by reducing the duration and increasing the light intensity, and vice versa.<sup>28</sup> Subsequently, “accelerated” CXL was improved, which has the same treatment dose as a standard CXL, however a shorter exposure time than a standard CXL. With a better understanding of photochemical kinetics of CXL, a new method of PLA-CXL has been developed. This method involves pulsing the UV-A light during CXL treatment to replenish the oxygen concentration of the environment. The photochemical reaction that occurs under aerobic conditions is called a type II photochemical mechanism. Type I photochemical mechanism occurs in which riboflavin and substrate react after total depletion of oxygen.<sup>6</sup> It is accepted that pulsing the UV light during CXL treatment theoretically restarts the type II photochemical reaction by allowing oxygen replenishment, which means PLA-CXL causes more singlet oxygen releasing for cross-linking formation. Several studies compared the safety and efficacy of different CXL methods with devices on patients.<sup>29–32</sup> In this study, we aimed to evaluate the safety of different CXL modalities in a molecular base. We found that the standard CXL method causes more production of  $\text{NO}_x$  and nitrite when compared with the control group. This may be related to long-time exposure to riboflavin and UV-A. PLA-CXL caused similar  $\text{NO}_x$  and nitrite levels as with the control group. We do not have an exact explanation for this result. But, in our previous study, we compared advanced oxidative protein products (AOPP) in cornea tissues after different CXL methods as a marker of free radical production potential.<sup>33</sup> We found higher AOPP levels in cornea tissues treated with PLA-CXL. This information might indicate that in PLA-CXL, free radicals accumulate in corneal tissue and do not leak out to aqueous humor.

There are some limitations of this study. Besides  $\text{NO}_x$ , we should evaluate anti-oxidant parameters in the aqueous humor. Also, we measured  $\text{NO}_x$  levels 1 hour after CXL methods. Levels of oxidant and anti-oxidant parameters might change in subsequent experiments.

In conclusion, numerous studies reveal the physiological and pathophysiological roles of NO in the eye. It is well-known that unregulated NO production can cause either apoptotic or necrotic cell death through oxidative stress.<sup>34</sup> By evaluating  $\text{NO}_x$  level in aqueous humor, we suggested that PLA-CXL is the safest method due to the similar results with the control group. Because CXL is accepted as the standard treatment procedure for progressive keratoconus, it is important to ascertain the safest CXL method to minimize the possible cytotoxic effects on the anterior segment of the eye. Further studies will determine the most effective and least harmful CXL method.

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## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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