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**Corneal cross-linking: Intrastromal riboflavin concentration in iontophoresis-assisted imbibition versus traditional and transepithelial techniques**

**Short Title: Corneal cross-linking: Intrastromal riboflavin concentration**

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## Introduction

1  
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3 Keratoconus is a progressive corneal degenerative disorder in which a  
4 biomechanical weakness modifies the normal gradual curve into a more  
5 conical shape thus thinning. Substantial visual disturbance, ghost imaging,  
6 and decreased visual acuity ensue.

7  
8 Corneal collagen cross-linking (CXL) is the only conservative treatment  
9 currently available to halt or reduce progression by improving the  
10 biomechanical rigidity of the corneal stroma.<sup>1</sup> This technique consists in  
11 exposure to ultraviolet-A (UVA) irradiation at 370 nm, in the presence of  
12 stromal riboflavin (vitamin B2: a chromophore) and a UVA blocking agent. It  
13 combines the principles of chemical non-enzymatic CXL with the photo-  
14 oxidative CXL where riboflavin is the photosensitizer.<sup>2</sup>

15  
16 The basic principle behind CXL is that UVA excites the riboflavin in the  
17 corneal stroma to its triplet state, thus generating singlet oxygen species.  
18 These species can react with various other molecules, inducing the formation  
19 of covalent bonds that bridge the amino and carbonyl groups of collagen fibrils  
20<sup>3</sup> and proteoglycan core proteins.<sup>4</sup> This process requires the presence of an  
21 adequate concentration of exogenously-applied riboflavin to guarantee a  
22 sufficient amount of cross-linking while avoiding the absorption of UVA  
23 irradiation of deeper ocular tissue, such as the lens and retina, which could  
24 result in cellular damage.<sup>5</sup>

25  
26 Different techniques have been proposed to improve intrastromal  
27 penetration of riboflavin molecules. In the standard procedure<sup>6-7</sup> (Epi-off  
28 technique), the corneal epithelium is first removed and then drops of 0.1%  
29 riboflavin are administered at 1-5 minute intervals for 15-30 minutes before  
30 irradiation (30 min exposure to 370 nm UVA radiation at 3 mW/cm<sup>2</sup>). Removal  
31 of epithelium allows riboflavin penetration, which is otherwise impeded by the  
32 epithelial cell tight junctions due to its molecular weight.<sup>8</sup>

33  
34 Recently, several methods of trans-epithelial cross-linking were  
35 proposed to allow the penetration of riboflavin across the epithelium.<sup>9-11</sup> In  
36 these procedures (Epi-on techniques),<sup>6</sup> removal of the epithelial layer is not  
37 necessary because chemical agents or enhancers, such as benzalkonium  
38 chloride, sodium EDTA and tromethamine are added individually or  
39 concurrently to the riboflavin solution to facilitate passage of riboflavin into the  
40 stroma.

41  
42 A novel approach to promote riboflavin saturation into the corneal  
43 stroma is based on iontophoresis, a noninvasive delivery system designed to  
44 enhance the penetration of molecules into tissue using a small electric  
45 current. This technique was used to deliver drugs in various fields of  
46 medicine, including intraocular compartments.<sup>12-14</sup> It generates a higher  
47 concentration compared to agent-free techniques<sup>6</sup> and has been used in  
48 ophthalmology mainly for the treatment of posterior segment diseases.<sup>14,15</sup>  
49 Recently the use of iontophoretic systems was proposed for riboflavin  
50 intracorneal delivery in CXL.<sup>16,17</sup>

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52 The present study aimed at determining differences in riboflavin  
53 concentration in the anterior, intermediate and posterior stroma after three  
54 types of imbibition procedures (standard Epi-off, Epi-on and Iontophoresis-  
55 assisted administration) of 0.1% riboflavin in human donor corneas.  
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## Material and Methods

### *Riboflavin imbibitions*

After approval by the Review Board of the Department of Medicine and Ageing Sciences (University of Chieti, Italy), ten human eye bank corneal buttons with scleral rims were included in the study. The mean donor age was 66.8 years (range 56 to 72 years). The average death-to-enucleation time was 6 hours (range 4 hours and 30 minutes to 11 hours and 15 minutes). The mean storage time (between eye bank procedures and cross-linking imbibitions) was 29.3 hours (range 20 to 48 hours).

For CXL imbibition, the corneoscleral buttons were mounted on an anterior chamber maintainer (Barron artificial anterior chamber; Katena Products Inc, Denville, NJ), filled with Eusol-C storage media (Alchimia s.r.l., Ponte S. Nicolò, Italy) to obtain adequate pressure and stability of the corneal tissue.

Three corneas were treated according to a standard protocol, which consisted in removal of the central 10 mm of corneal epithelium and soaking the stroma with 0.1% riboflavin (Ricrolin; SOOFT Italia S.p.A., Montegiorgio, Italy) for 20 minutes within a dedicated silicon ring (Epi-off group).

Three corneas received riboflavin imbibition via a transepithelial approach. Ricrolin TE (0.1% riboflavin plus enhancers [including destrane T500, edetate sodium, tromethamine, bihydrate sodium phosphate monobasic, and bihydrate sodium phosphate bibasic]; SOOFT Italia S.p.A., Montegiorgio, Italy) was applied with the same procedure described for the Epi-off group, for 30 minutes, using the same silicon ring imbibition system (Epi-on group). The enhancers in Ricrolin TE facilitate riboflavin penetration into the corneal stroma in the presence of an intact epithelium. In addition, penetration was increased by a longer duration of pretreatment with Ricrolin TE, as per reported protocols.<sup>8,9</sup>

Three corneas were soaked with riboflavin using a iontophoresis system, composed of a power supply (I-ON CXL; SOOFT Italia S.p.A., Montegiorgio, Italy), two electrodes (the return electrode in the artificial system was a stainless steel wire inserted into the saline circuit and the negative electrode was a steel grid contained in a corneal applicator; Iontofor CXL, SOOFT Italia S.p.A., Montegiorgio, Italy) and a connection cable. Adherence of the corneal applicator to the eye was maintained with a vacuum system.

After connecteing the vacuum syringe to the luer-lock connector and verifying that the stop clamp was in the "open" position, the corneal applicator was put onto the cornea to be treated. Light suction was applied with the syringe (at least 1 mL) and the stop clamp was closed. The operator verified the applicator was secured to the cornea.

The steel grid (negative electrode) was covered with Ricrolin+ (0.1% riboflavin solution specifically formulated to allow quick passage into the corneal stroma, through intact epithelium with corneal iontophoresis; SOOFT Italia S.P.A., Montegiorgio, Italy).

The electrical generator was in the "OFF" position up to this point. After connecting the two electrodes to the power generator (the female red plug to

1 the connector on the Iontofor CXL and the white male plug to the return  
2 electrode), the power generator was turned on (acoustic confirmation). The  
3 operator selected 1 mA and pressed START again. The procedure took 5  
4 minutes (total current intensity 5 mA). The power supply software indicated  
5 the continuity of the procedure (the generator sounds an alarm if the current is  
6 interrupted).

7 The operator controlled that the steel grid was covered with riboflavin  
8 solution for the entire duration of the procedure, thus maintaining a regular  
9 flow of the electrical current.

10 After the iontophoretic procedure, the remaining riboflavin was removed  
11 through the specific tube and the stop clamp was opened to allow the air to  
12 enter the vacuum ring. The applicator was removed from the cornea and the  
13 power generator disconnected from the applicator and from the return  
14 electrode. The cornea was rinsed with saline solution.

15 One cornea was used as a control and was washed with a saline  
16 solution.

### 17 *Femtosecond Laser (FSL) stromal dissections*

18 Three stromal slices of the corneal samples were prepared with a 500-  
19 kHz VisuMax femtosecond laser (Carl Zeiss Meditec, Jena, Germany)  
20 immediately after the imbibition or control, maintaining the corneoscleral rims  
21 mounted in the artificial anterior chamber with controlled pressure. Before FSL  
22 stromal dissections of the corneas that underwent transepithelial riboflavin  
23 imbibition (belonging to Epi-on and Iontophoresis group) and in the control  
24 cornea, the epithelium was removed using a blunt spatula for a 10-mm  
25 diameter. Therefore, all FSL cuts were performed on denuded stroma in order  
26 to standardize the thickness of the two superficial slices and to ensure that  
27 High-Performance Liquid Chromatography (HPLC) was conducted only on  
28 stroma.

29 The central corneal thickness was measured using an ultrasound  
30 pachymeter (Optikon 2000 S.p.A, Altair, Rome, Italy).

31 Each cornea was divided into three 8.00 mm circular slices by  
32 performing two sequential lamellar dissections first at 300 and then at 150  $\mu\text{m}$   
33 from the corneal surface. A 90° (perpendicular to the corneal surface) side cut  
34 was performed. The FSL parameters were set as follows: spot-line separation  
35 between 2.0 and 2.5  $\mu\text{m}$  for the lamellar cuts and 3.0  $\mu\text{m}$  for the side cuts.  
36 The spot energy was between 135 nJ and 150 nJ.

37 The two anterior stromal slices were removed using corneal graft  
38 toothed forceps and blunt spatula. The residual deep corneal slice was freed  
39 using an 8.00 mm cornea punch. Stromal slices were placed in histological  
40 screw-capped containers, shielded from the light with aluminum foil, and  
41 stored at -20°C for subsequent HPLC analysis.

### 42 *High-Performance Liquid Chromatography (HPLC) analysis*

43 HPLC analysis was subsequently performed to quantify the  
44 concentration of riboflavin in each volume of tissue.

1 Corneal samples were vortexed 1 min in 500 $\mu$ L of methanol and  
2 centrifuged at 13200 rpm at room temperature. The supernatants were  
3 evaporated under a nitrogen stream at 40°C. The dry residue was dissolved  
4 in 50 $\mu$ L of ultrapure water/methanol 70/30 (v/v) and vortexed for 1min. Tubes  
5 were centrifuged for 2 min at 13200 rpm and 5  $\mu$ L of the supernatant was  
6 injected into a HPLC/MS 1100 series system equipped with a simple  
7 Quadrupole LC/MSD 1100 (both Agilent Technologies, Santa Clara, CA). The  
8 HPLC analysis of riboflavin was performed with a Zorbax SB C18 column (2.1  
9 mm, 50 mm, 3.5  $\mu$ m, Agilent Technologies, Santa Clara, CA). The mobile  
10 phase (90%, 0.1% formic acid and 10% acetonitrile) was used under isocratic  
11 conditions at a flow rate of 0.2 mL/min. Mass spectrometer parameters were:  
12 ionization mode: API-ES positive, drying gas flow: 11 l/min, nebulizer  
13 pressure: 20 Psig, drying gas temperature: 350°C, capillary voltage: 4000V  
14 and mass to charge ratio (M/Z): 377.20. The lower limit for riboflavin  
15 quantification was 200 ng/mL. Calibration curves were obtained by plotting  
16 riboflavin peak area as a function of concentration.  
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### 21 *Statistical analysis*

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23 Statistical analysis was performed using SPSS 20.0 (IBM, Armonk, NY).  
24 General Linear Model for repeated measures was used to compare the mean  
25 difference of riboflavin concentration over slices in the three treatment groups.  
26 *A priori*-specified contrast analysis was used for within treatment group  
27 comparisons of riboflavin concentration.  
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29 Data were expressed as median and interquartile range (IQR) and  
30 graphed as a box-whisker plot. Statistical significance was assigned at  
31  $p \leq 0.05$ .  
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### 35 **Results**

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37 The three groups did not present statistically significant differences in terms of  
38 donor age, death-to-enucleation time and storage time (Table 1). Central  
39 corneal thickness values ranged between 566 and 630  $\mu$ m (mean 592  $\mu$ m) in  
40 the Epi-off group, between 545 and 614  $\mu$ m (mean 575  $\mu$ m) in the Epi-on  
41 group, and between 542 and 619  $\mu$ m (mean 580  $\mu$ m) in the Iontophoresis  
42 group. Differences in corneal thickness were not statistically significant.  
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45 Riboflavin content in human de-epithelialized cornea slices after  
46 imbibition and in the untreated control cornea is reported in Table 1. The  
47 overall stromal concentration of riboflavin was  $34.1 \pm 7.1$   $\mu$ g/g in the Epi-off  
48 group,  $7.2 \pm 3.7$   $\mu$ g/g in the Epi-on group and  $15.0 \pm 5.1$   $\mu$ g/g in the  
49 Iontophoresis group.  
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51 In all groups riboflavin concentration was significantly higher in the  
52 anterior stromal slice with respect to the intermediate slice, while the posterior  
53 slice showed the lowest concentrations (Figure 2). As expected, no traces of  
54 riboflavin were measured in the control cornea.  
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56 Overall, the mean riboflavin content in the anterior 150  $\mu$ m of stroma  
57 (superficial slice) in the Epi-off group was approximately two-fold greater than  
58 that found in the Iontophoresis group ( $50.5 \pm 5.3$   $\mu$ g/g and  $23.6 \pm 2.5$   $\mu$ g/g,  
59 respectively) and four-fold greater than that observed for the Epi-on group  
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1 (11.7±3.3 µg/g). Similar differences among the three groups were observed  
2 for the intermediate (150-300 µm) and posterior stromal slices, presenting an  
3 evident reduction of riboflavin concentration with increasing depth in all  
4 groups (Table 1 and Figure 2).

5 The slice depth-dependent decrease in riboflavin concentration was  
6 statistically significant ( $F_{1,6}=62.265$ ,  $p<0.001$ ), as was the group-dependent  
7 variation ( $F_{2,6}=20.268$ ,  $p=0.002$ ). The slice depth-group interaction also  
8 showed a statistically significant difference in terms of riboflavin decrease with  
9 increasing slice depth ( $F_{2,6}=18.004$ ,  $p=0.002$ ). *A priori*-specified contrast  
10 analysis showed significant within-treatment differences for superficial and  
11 intermediate slices versus the deepest slice for the Epi-off and Iontophoresis  
12 groups.  
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## 15 Discussion

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19 The standard corneal collagen cross-linking protocol proposed by Wollensak  
20 at al. recommended epithelium removal before corneal exposure to the  
21 riboflavin solution (Epi-off technique) in order to allow a proper penetration of  
22 the molecule into the stroma.<sup>1</sup> Experimental studies reported that the intact  
23 epithelium did not limit the UVA transmittance<sup>18</sup> but did reduce the  
24 effectiveness of CXL by preventing stromal penetration.<sup>19-22</sup> The corneal  
25 epithelium has multiple cell layers with intercellular tight junctions that  
26 represent a barrier to ion diffusion and to the passage of foreign molecules  
27 across the cornea.<sup>23</sup>

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30 However, de-epithelization of the cornea is associated with  
31 postoperative symptoms such as ocular discomfort, pain and photophobia. It  
32 can also lead to complications such as wound infection, infiltrates and  
33 transient or persistent sub-epithelial haze related to the activation of the  
34 stromal wound healing response.<sup>6,24,25</sup> Consequently, a modification to the  
35 original technique without epithelial removal was suggested.<sup>26</sup> This technique,  
36 based on the adjunction of epithelial penetration enhancers such as  
37 benzalkonium chloride,<sup>27</sup> sodium EDTA, and trometamol<sup>9,28</sup> to the riboflavin  
38 solution are termed transepithelial or Epi-on CXL. The results obtained with  
39 the currently available Epi-on techniques in treating keratoconus varied.<sup>7,9,27,28</sup>  
40 It is likely that the effect of transepithelial CXL is less pronounced than that  
41 obtained with Epi-off although comparative clinical trials are needed to  
42 establish the efficacy of this technique. One possible explanation for this  
43 reduced efficacy of Epi-on CXL is the lower stromal riboflavin saturation that  
44 may occur with the epithelium in place despite the presence of the above-  
45 mentioned enhancer molecules.  
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50 A previous HPLC study in human cadaver corneas reported that the  
51 riboflavin stromal concentration obtained with the transepithelial imbibition  
52 approach, in the absence of any substances that enhance epithelial  
53 penetration, was lower by a factor of 100 than that obtained with epithelial  
54 removal using the same riboflavin solution.<sup>8</sup> Increasing the exposure time to  
55 riboflavin did not improve penetration when the epithelium was in place.<sup>8</sup> A  
56 recent study on rabbit eyes showed that transepithelial riboflavin solution,  
57 containing 0.01% benzalkonium chloride and 0.44% NaCl but without dextran,  
58 promoted the permeability of riboflavin through the epithelium.<sup>10</sup> Thus it is  
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likely that the biomechanical effect produced by epi-on CXL may be limited in comparison to that obtainable with standard CXL, probably because of restricted and inhomogeneous stromal distribution of riboflavin.<sup>26</sup>

A novel modification to the standard protocol is the iontophoretic delivery of riboflavin. It is a noninvasive technique, in which a mild electrical current is used to enhance the penetration of electrically charged molecules into tissue.<sup>2,16,17</sup> Riboflavin is a negatively charged, highly water-soluble molecule with a relatively low molecular weight and, therefore, suitable for iontophoresis corneal delivery.<sup>6</sup> The theoretical advantages of this technique are circumventing corneal epithelial removal while significantly shortening to 5 minutes the pre-irradiation time; i.e., the time required for administering riboflavin to the cornea.<sup>2,6</sup> A sufficient riboflavin concentration in the stroma for efficient collagen cross-linking is nonetheless achieved. Another advocated beneficial effect of iontophoretic riboflavin corneal administration is that riboflavin penetration is more homogeneous and deeper than that achieved with Epi-on techniques. However, the riboflavin stromal concentration obtained using currently available transepithelial CXL imbibition techniques, including iontophoresis, as compared with the standard protocol, are not yet known.

In the present study we evaluated the differences in the riboflavin concentration in the anterior, intermediate and posterior stroma in human donor corneas obtained with the three imbibition techniques currently available for clinical use in Europe: standard Epi-off protocol, Epi-on and iontophoresis-assisted techniques available. Riboflavin concentration was determined with HPLC, a highly sensitive method for determining corneal riboflavin concentration after topical administration of 0.1% riboflavin in whole human corneal samples.<sup>8</sup>

The corneas were sliced in three sections using a highly precise femtosecond laser system capable of performing extremely regular lamellar dissections in the corneal stroma.<sup>29</sup> The femtosecond laser was programmed to execute two consecutive lamellar cuts in each donor corneas at 300  $\mu\text{m}$  first, and subsequently at 150  $\mu\text{m}$  of depth within the same contact-applanating procedure, thus yielding three slices with an 8.00 mm diameter. A similar double FSL cut technique was already validated in a previous study with the purpose of optimizing interfaces for endothelial keratoplasty buttons.<sup>30</sup> Although 8.00 mm wide corneal lenticules for the analysis of the riboflavin stromal concentration was an arbitrary choice, it should have represented a sufficiently large area to reflect the clinical features of the cross-linked corneas observed in vivo, in keratoconic patients, where the peripheral and peri-limbal corneal zones are generally not affected by the procedure.<sup>31</sup> Removal of the epithelium after transepithelial imbibitions (Epi-on and iontophoresis) was performed in order to standardize the thickness of the two most anterior slices (150  $\mu\text{m}$ ; anterior and intermediate stroma). Thus HPLC measurements were only performed on stromal tissue, avoiding any variability related to the riboflavin content within the epithelial cells that is not of clinical interest since it does not contribute to stromal collagen cross-linking in patient treatment. Due to the standardization of the thickness of the two anterior stromal slices and the slight variation of the total corneal thickness among the corneal tissues in the groups, the resulting posterior stromal slice had a variable thickness (250-340  $\mu\text{m}$ ). However, it is commonly thought that the



1 greater riboflavin penetration and, therefore, the major cross-linking effect  
2 take place in the anterior half of the cornea.<sup>2,32-35</sup> In addition, the stromal  
3 thicknesses observed in our samples differ from those generally encountered  
4 in the clinical practice for two reasons: the corneas were not affected by  
5 keratoconic thinning and stromal hydration was presumably higher than in-  
6 vivo due to the preservation procedure. This may represent a limitation of the  
7 study.

8 The three imbibition techniques yielded different overall concentrations  
9 of riboflavin, ranging from the lowest concentration in the Epi-on group,  
10 doubling in the Iontophoresis group and again doubling in the Epi-off group.  
11 These differences were also observed when comparing the stromal slices at  
12 different depths among the three procedures maintaining a ratio of  
13 approximately 4:2:1 in Epi-off, Iontophoresis and Epi-on, respectively. The  
14 concentration in the control cornea was, as expected, below measurable  
15 levels. Our results regarding the comparison of Epi-off and Epi-on technique  
16 differed from those reported in a previous investigation comparing HPLC-  
17 determined riboflavin corneal concentration after CXL 0.1% riboflavin  
18 exposure with epithelial debridement compared with epithelium *in situ*.<sup>8</sup>  
19 Baiocchi et al.<sup>8</sup> found that the overall corneal stromal concentration of  
20 riboflavin was 24 µg/g after 30 minutes exposure. This was comparable to  
21 that found in our Epi-off group (34 µg/g), but highly different from the riboflavin  
22 content in the stroma measured in the transepithelial method with a gap of  
23 two orders of magnitude on the log scale indicating a 1:100 ratio in favor of  
24 debrided corneas and a high amount of riboflavin was observed within the  
25 epithelium. Conversely, in our study the overall stromal concentration of  
26 riboflavin was higher in the samples treated without epithelium *in situ* by a  
27 factor of 4 (1:4 ratio) with respect of the Epi-on group. It is possible to  
28 hypothesize that this difference was related to the fact that in the previous  
29 study<sup>8</sup> the 0.1% riboflavin used for transepithelial procedure was exactly the  
30 same<sup>8</sup> as for the standard Epi-off procedure and did not contain any epithelial  
31 penetration enhancer substance, as opposed to the commercially available  
32 riboflavin solution containing enhancers specifically formulated for  
33 transepithelial CXL used in this study. In fact, in a recent study testing various  
34 solutions containing different NaCl and benzalkonium chloride concentrations  
35 for transepithelial cross-linking it was observed that compared to the standard  
36 protocol, these solutions resulted in a riboflavin absorption coefficient ranging  
37 from 33 to 37% of the standard epithelium-off procedure.<sup>10</sup> These findings  
38 were comparable to our results for the Epi-on riboflavin content (25% of the  
39 standard Epi-off).

40 In addition, our findings indicated that the riboflavin concentration  
41 decreased as a function of depth and the difference in the rate of decrease for  
42 the three imbibition techniques was statistically significant. While the rate of  
43 decrease was highest in the Epi-off group, the deepest stromal slices of this  
44 group had a higher riboflavin concentration compared to the most superficial  
45 layers of Epi-on group. The Iontophoresis group presented an intermediate  
46 rate of decrease in riboflavin concentration for slice depth, with the deepest  
47 slice presenting a similar concentration as the most superficial slice of the Epi-  
48 on group.

49 In conclusion, our results indicated that transepithelial iontophoresis  
50 imbibition allowed greater and deeper riboflavin saturation in the corneal  
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1 stroma with respect to the conventional Epi-on technique, while retaining the  
2 advantages of avoidance of epithelial removal and shortened procedure time.  
3 Although with iontophoretic-assisted delivery the riboflavin content did not  
4 reach the levels of the standard Epi-off protocol in this study, it has to be  
5 remarked that it is not completely known what level of riboflavin concentration  
6 is needed in the stromal tissue to achieve a full cross-linking effect in treating  
7 keratoconus patients. Comparative prospective clinical trials investigating the  
8 clinical efficacy of CXL via iontophoresis are needed to confirm whether this  
9 approach could represent a valid alternative to the Epi-off CXL (gold-  
10 standard) procedure.  
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## Figure Legends

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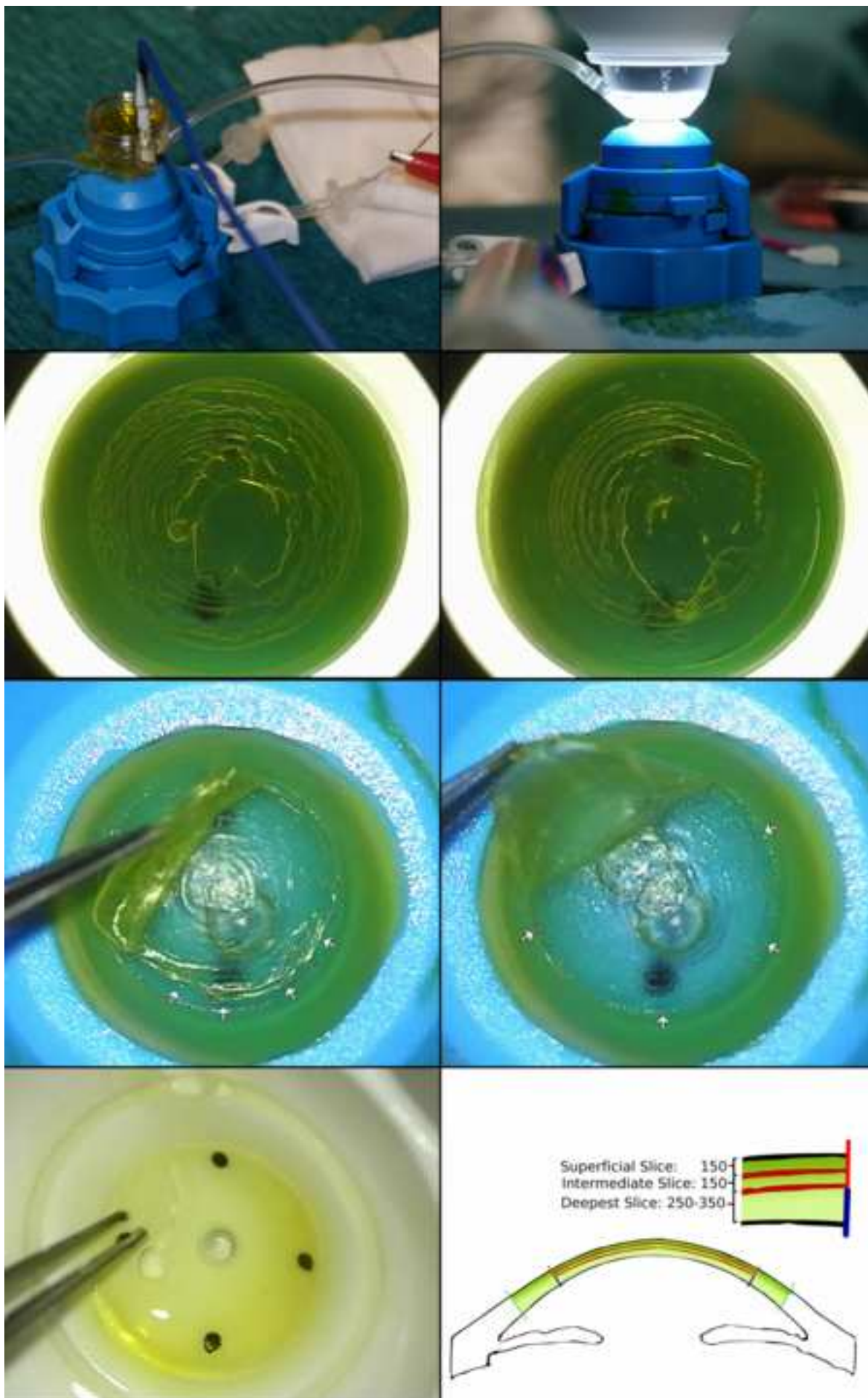
Figure 1. Experimental set up for the iontophoretic imbibition phase in corneal cross-linking of the corneal samples and preparation of the stromal slices using the femtosecond laser system. *First row left:* the cornea is mounted onto the artificial anterior chamber and the corneal applicator with a vacuum suction system, is placed on the cornea. The positive electrode is a stainless steel wire inserted into the saline circuit while the negative one is the steel grid contained in the corneal applicator. *First row right:* After the imbibition and epithelial removal the cornea is docked with the femtosecond laser plastic curved interface. *Second row left:* Frame of the videorecording system internal to the laser that illustrates the first lamellar deep stromal cut performed at 300  $\mu\text{m}$  of depth. Note the green fluorescent color of the corneal tissue indicating the presence of riboflavin. *Second row right:* Image illustrating the mid phase of second lamellar dissection, performed by the laser on top of the first, at a depth of 150  $\mu\text{m}$ . *Third row left:* the anterior 8.0 mm stromal slice is removed by the aid of surgical forceps. Note the edge of the intermediate slice that has been intentionally wrinkled in order to make it visible (arrows). *Third row right:* the intermediate 8.0 mm slice is gently removed by the aid of forceps. The circular borders of the trephination (arrows) and the stromal bed of the lamellar dissection appear regular and smooth. *Last row left:* after 8.0 mm mechanical punching the posterior stromal slices is isolated. *Last row right:* schematic diagram illustrating characteristics and thicknesses of the three stromal slices prepared using the described double-cut femtosecond laser dissections.

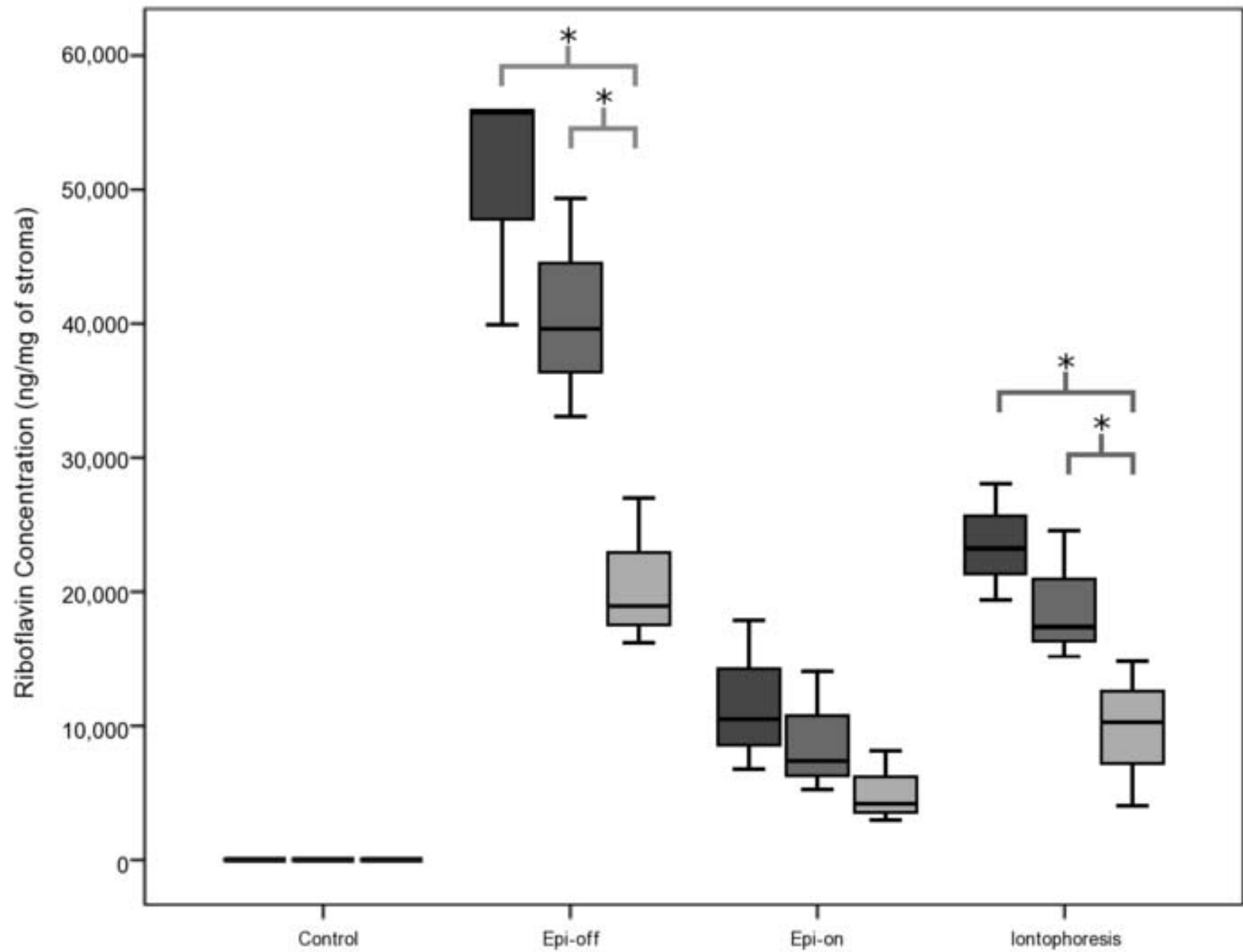
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Figure 2. Box-plot of the riboflavin concentration for the control cornea (n=1) and the three treatment groups (Epi-on, Epi-off and Iontophoresis imbibition phase of corneal cross-linking; n=3). Darker to lighter shades of grey indicate superficial to deepest slices, respectively. The mean riboflavin content in the anterior 150  $\mu\text{m}$  of stroma (superficial slice) in the Epi-off group was approximately two-fold greater than that found in the Iontophoresis group and four-fold greater than that observed for the Epi-on group. Similar differences among the three groups were observed for the intermediate (150-300  $\mu\text{m}$ ) and posterior stromal slices, presenting an evident reduction of riboflavin concentration with increasing depth in all groups. Differences in riboflavin concentration were statistically significant between the most superficial and intermediate slices compared to the deepest slice for the Epi-off and Iontophoresis groups.

**TABLE 1.** Donor age, time from death to enucleation, storage time, and riboflavin content in human disepithelialized cornea after 20 min corneal cross-linking imbibition with Epi-off, after 30 min imbibition with Epi-on, after 5 min of iontophoresis and control without imbibition.

Patient	Age of Donor	Death to Enucleation Time (Hours)	Storage Time (Hours)	Treatment Protocol	Riboflavin Concentration (ng/g of stroma)		
					Superficial	Intermediate	Deep
1	64	5.5	36	Epi-off	55756	39630	27014
2	71	7.0	20	Epi-off	55830	49274	18891
3	69	11.25	24	Epi-off	39898	33066	16274
4	64	6.5	32	Epi-on	17938	14116	8153
5	71	4.5	46	Epi-on	10500	7320	2938
6	56	8.0	32	Epi-on	6745	5215	4251
7	69	9.25	28	Iontophoresis	19430	15215	4083
8	72	8.5	48	Iontophoresis	23277	17333	10277
9	66	5.25	32	Iontophoresis	28009	24540	14816
10	66	4.5	22	Control	0	0	0
				Mean $\pm$ SD			
				Group			
				Concentration			
				Epi-off	50494 $\pm$ 5298	40656 $\pm$ 4707	20726 $\pm$ 3233
				Epi-on	11728 $\pm$ 3289	8884 $\pm$ 2686	5114 $\pm$ 1566
				Iontophoresis	23572 $\pm$ 2481	19029 $\pm$ 2822	9725 $\pm$ 3111
				Control	0	0	0







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