Comparative study on corneal cross-linking with isotonic and hypotonic riboflavin: can hypotonic riboflavin be applied in thinner corneas?

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Abstract

● AIM: To compare the results of corneal cross-linking (CXL) with isotonic (IR) and hypotonic riboflavin (HR) in patients with keratoconus and to verify the efficacy of keratoconus in thinner corneas.

● METHODS: Retrospective study of 29 eyes/keratoconus patients submitted to CXL, 15 eyes with application of IR (IR group) and 14 eyes with application of HR (HR group). The parameters analysed included (1-year follow-up): best corrected visual acuity (BCVA), sphere and cylinder, central and finer pachymetry, mean and maximum keratometry (Km and Kmax respectively), complications and progression.

● RESULTS: An increase on the BCVA scale (logMAR, logarithm of the minimal angle of resolution) was observed in the two groups: 0.26±0.57 (IR) and 0.47±0.72 (HR) before treatment, and 0.13±0.79 (IR) and 0.29±1.52 (HR) at the 1y. Only at 1y, the difference was statistically significant (P=0.018, group IR with higher BCVA). The central pachymetry (μm) decreased at 1mo in both groups, and increased in the following months: 497±28 μm (IR) and 432±14 μm (HR) before treatment, and 480±31 μm (IR) and 424±15 μm (HR) at 1y. The thinner pachymetry (μm) presented the same evolution: 487±29 μm (IR) and 410±20 μm (HR) before treatment, and 468±33 μm (IR) and 413±13μm (HR) at 1y. Km and Kmax decreased in both groups (P>0.05). Six eyes from each group presented transitory haze. No eye progressed to the 1y.

● CONCLUSION: The use of hypotonic riboflavin seems to be a valid alternative for performing the traditional corneal cross-linking technique in eyes with a central corneal thickness of <400 μm.

Keywords: keratoconus; cross-linking; riboflavin; thin cornea

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INTRODUCTION

Keratoconus is a disorder that is characterized by conical shape ectasia and progressive thinning of the cornea, which cause irregular astigmatism and myopia, resulting in a significant decrease in visual acuity[1-3]. Its pathophysiology is not completely known, but seems to consist mainly of a reduced number of connections between collagen fibres and increased activity of proteolytic stroma enzymes, compared to normal corneas. These changes result in greater biochemical and biomechanical fragility of the corneal stroma with keratoconus[1-3].

Currently, corneal cross-linking (CXL) is the only treatment option that allows for the stabilisation or even decrease of keratoconus progression, as it acts precisely on the biochemical and biomechanical characteristics of the stroma, attenuating or reverting the aforementioned changes and strengthening the corneas with keratoconus (action mainly evident in the previous 300 μm of the stroma)[4-8]. With the application of ultraviolet A (UV-A, 370 nm) and riboflavin (photosensitiser) in the presence of oxygen, an oxidative photopolymerization reaction occurs, resulting in the creation of new covalent bonds between collagen fibrils[5-8]. Riboflavin also has a “buffering” effect that protects the incidence of UV-A radiation in deeper ocular structures, such as the corneal endothelium, lens, and retina[8-9]. Therefore, the main limitation of the application of CXL in thinner corneas is that their reduced thickness increases the risk of absorption of UV-A by the endothelium, with subsequent injury of same, as demonstrated in the study by Kymionis et al[5-9]. In this context, Wollensak et al[10], pioneers in 2003 of CXL treatment to halt the progression of keratoconus, have demonstrated that the application of the...
parameters of the conventional protocol (Dresden protocol, epithelium-off, with isotonic riboflavin, IR-0.1% riboflavin in 20% dextran-and 3 mW/cm² of UV-A for 30min) in corneas with a central thickness of < 400 μm achieved the cytotoxicity limit of 0.35 mW/cm² for endothelial cell damage. This is why 400 μm was the central corneal thickness limit identified for reducing the referred risk. However, because, in practice, this aspect limits the application of the conventional CXL protocol. In this sense, Fernando Fonseca EPE (HFF, Lisbon, Portugal), submitted to the Ophthalmology Department at the Hospital Professor Doutor Fernando Fonseca EPE (HFF, Lisbon, Portugal), submitted to the following criteria during the period considered were included. The study followed the principles of the Helsinki Declaration and was approved by the hospital Ethical Committee. Data was collected for the study through consultation of patients’ clinical processes and topographic parameters obtained by Pentacam®.

The clinical parameters studied were: best corrected visual acuity (BCVA, in logarithm of the minimal angle of resolution scale-logMAR), manifest value of the sphere and cylinder (in dioptres -D), and complications. The topographic parameters of Pentacam® studied were: central and thinner pachymetry (in μm) and mean (Ks) and maximum (Kmax) keratometry parameters in D. Clinical (BCVA and cylinder value) and topographic parameters (Kmax) were used to identify the presence of progression, this being defined in previous studies as the loss of ≥2 lines of the BCVA or an increase of ≥1 D in the manifested cylinder or in the Kmax, in the 1y. The aforementioned parameters were analysed during a 1-year follow-up: prior to the CXL and, in the 1 and 6mo and in the 1y, after the CXL. The results of the study parameters were compared between the 2 groups. The division of eyes/patients between the IR and HR groups was based on the value of the central corneal thickness as measured by ultrasonic pachymetry following the de-epithelialization procedure: IR group if ≥400 μm and HR group if <400 μm. The CXL technique applied to corneas with a central thickness of ≥400 μm followed the Dresden protocol and the technique applied to corneas with a central thickness of <400 μm followed the modified technique described by Hafezi et al[13]. The procedure commenced with pachymetry corneal ultrasonography, followed by corneal de-epithelialization in the central 7 mm, using a scalpel and methylcellulose sponge, and subsequent repeated determination of pachymetry. One drop of the riboflavin solution was applied every 3min for 30min: isotonic solution of 0.1% riboflavin-5-phosphate with 20% dextran if pachymetry was ≥400 μm, and hypotonic solution of 0.1% riboflavin-5-phosphate without dextran if pachymetry was <400 μm. In the cases with pachymetry >400 μm, the irradiation of the cornea with the source of UV-A radiation (370 nm) was initiated, whereas in the cases with pachymetry <400 μm, distilled water was applied before a corneal thickness ≥400 μm. Irradiation of the cornea was carried out for 30min, with the source of UV-A radiation placed at 45 mm from same, with a power of 3 mW/cm², maintaining the application of riboflavin solution every 3min, interspersed with the application of distilled water. To ensure the safety of the technique in the HR group, the pachymetry was repeated intraoperatively after application of riboflavin. At the end of the procedure, a silicone-hydrogel contact lens was placed in each patient’s eye for better pain relief, to aid stable keratoconus in stage 4. All consecutive patients meeting the criteria during the period considered were included.

Ethical Approval
Retrospective study of 29 eyes of 29 patients with keratoconus from the Cornea Clinic of the Ophthalmology Department at the Hospital Professor Doutor Fernando Fonseca EPE (HFF, Lisbon, Portugal), submitted to CXL between 2012 and 2018, 15 eyes with IR application (IR group) and 14 eyes with HR application (HR group). Inclusion criteria consisted of: keratoconus in stages 1 to 3, according to Amsler-Krumeich classification; keratoconus with documented progression in the 12mo prior to the start of treatment, through refraction and/or corneal topography. Exclusion criteria consisted of: previous corneal surgery; history of herpetic keratitis; recurrent corneal infections; severe dry eye, and
in the epithelial healing and to accelerate recovery in the eyes with corneal haze. Patients were medicated with ofloxacin and dexamethasone/neomycin eye drops.

A statistical analysis was performed with the SPSS program (Statistical Package for Social Sciences, version 22.0), involving descriptive statistical measures; Chi-square and Fisher tests to analyse the differences between groups in qualitative variables and the Student’s t test to test the differences in quantitative variables; the assumptions of normal distribution and variance homogeneity were analysed with the Kolmogorov-Smirnov tests and Levene’s test, and in those cases where these assumptions were not satisfied, the aforementioned tests were replaced by the Mann-Whitney test. The results were considered statistically significant when p-value was less than 0.05 (power of the study: 95%).

RESULTS

The study sample included 29 eyes from 29 CXL-treated keratoconus patients (16 males and 13 females), with an average age of 21.4±6.0 (20.1±4.0 in the IR group and 21.3±5.0 in the HR group). CXL was performed with the application of IR on 15 of the 29 eyes and with HR on the remaining 14. The results are expressed as mean±standard deviation, in absolute values, and are shown in Tables 1 and 2 and in Figures 1-7. Those tables also include the statistical significance (P) of the differences between the two groups. For each group, the statistical significance difference (P) of all the parameters between the preoperative evaluation and each moment of the follow-up (Table 3).

1) BCVA: there was an improvement in BCVA in both groups, without statistical significance between the preoperative evaluation and each moment of the follow-up. In the first year alone, the difference between groups was statistically significant (P=0.019), with the IR group showing a better BCVA.

2) Manifest value of the sphere and the cylinder: the sphere increased slightly in the IR group, while in the HR group it decreased. The cylinder decreased in both groups. In each group, the results were not statistically significant between the preoperative evaluation and each moment of the follow-up. Both parameters did not present statistically significant differences between the groups during follow-up.

3) Central and thinner pachymetry: a reduction in central pachymetry was observed at the 1st month, with a progressive increase up to the 1st year of follow-up in both groups (IR group with higher values). In the IR group, both central and thinner pachymetry showed statistically significant differences at all the follow-up moments, while in the HR group only the thinner pachymetry showed a statistically significant difference between the preoperative evaluation and the evaluations of the 1st and the 6th month. Differences between the groups were statistically significant (P<0.05).

4) Central and thinner pachymetry: a reduction in central pachymetry showed a statistically significant difference at all the follow-up moments, while in the HR group only the thinner pachymetry showed a statistically significant difference between the preoperative evaluation and the evaluations of the 1st and the 6th month. Differences between the groups were statistically significant (P<0.05).

### Table 1 Comparison of BCVA and refraction between groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Preoperative</th>
<th>1mo</th>
<th>6mo</th>
<th>1y</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCVA (logMAR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR</td>
<td>0.26±0.57</td>
<td>0.30±0.0</td>
<td>0.15±0.58</td>
<td>0.13±0.79</td>
</tr>
<tr>
<td>HR</td>
<td>0.47±0.72</td>
<td>0.30±0.0</td>
<td>0.27±1.30</td>
<td>0.29±1.52</td>
</tr>
<tr>
<td>P</td>
<td>0.327</td>
<td>1.001</td>
<td>0.400</td>
<td>0.041</td>
</tr>
<tr>
<td>Sphere (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR</td>
<td>2.1±0.68</td>
<td>2.3±0.55</td>
<td>2.4±0.54</td>
<td>2.6±0.50</td>
</tr>
<tr>
<td>HR</td>
<td>3.4±2.5</td>
<td>2.6±1.9</td>
<td>1.5±1.6</td>
<td>2.5±1.6</td>
</tr>
<tr>
<td>P</td>
<td>0.305</td>
<td>0.340</td>
<td>0.082</td>
<td>0.569</td>
</tr>
<tr>
<td>Cylinder (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR</td>
<td>6.0±2.2</td>
<td>5.8±0.0</td>
<td>5.4±1.1</td>
<td>4.3±1.0</td>
</tr>
<tr>
<td>HR</td>
<td>6.2±2.1</td>
<td>6.5±1.8</td>
<td>6.6±1.7</td>
<td>5.2±0.2</td>
</tr>
<tr>
<td>P</td>
<td>0.100</td>
<td>0.400</td>
<td>0.401</td>
<td>0.251</td>
</tr>
</tbody>
</table>

*P<0.05.

### Table 2 Comparison of queratometry and pachymetry between groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Preoperative</th>
<th>1mo</th>
<th>6mo</th>
<th>1y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean keratometry (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR</td>
<td>50.2±2.9</td>
<td>49.6±0.1</td>
<td>48.9±1.9</td>
<td>48.5±2.5</td>
</tr>
<tr>
<td>HR</td>
<td>48.9±3.5</td>
<td>48.6±2.6</td>
<td>48.2±2.9</td>
<td>47.9±2.5</td>
</tr>
<tr>
<td>P</td>
<td>0.444</td>
<td>1.000</td>
<td>0.327</td>
<td>0.915</td>
</tr>
<tr>
<td>Maximum keratometry (D)</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>IR</td>
<td>60.6±5.2</td>
<td>59.6±0.1</td>
<td>58.7±4.3</td>
<td>58.2±5.0</td>
</tr>
<tr>
<td>HR</td>
<td>59.4±7.2</td>
<td>58.8±5.9</td>
<td>58.3±5.1</td>
<td>57.6±6.6</td>
</tr>
<tr>
<td>P</td>
<td>0.833</td>
<td>1.000</td>
<td>0.428</td>
<td>1.001</td>
</tr>
<tr>
<td>Central pachymetry (µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR</td>
<td>497±28</td>
<td>397±0.1</td>
<td>471±43</td>
<td>480±31</td>
</tr>
<tr>
<td>HR</td>
<td>432±14</td>
<td>427±8</td>
<td>413±18</td>
<td>424±15</td>
</tr>
<tr>
<td>P</td>
<td>0.002</td>
<td>0.501</td>
<td>0.030</td>
<td>0.011</td>
</tr>
<tr>
<td>Thinner pachymetry (µm)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>IR</td>
<td>487±29</td>
<td>375±0</td>
<td>454±42</td>
<td>468±33</td>
</tr>
<tr>
<td>HR</td>
<td>410±20</td>
<td>406±26</td>
<td>388±21</td>
<td>413±13</td>
</tr>
<tr>
<td>P</td>
<td>0.002</td>
<td>0.501</td>
<td>0.018</td>
<td>0.019</td>
</tr>
</tbody>
</table>

*P<0.05.

### Table 3 Statistical significance between the preoperative evaluation and each moment of the follow-up, for each group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Preoperative</th>
<th>1mo</th>
<th>6mo</th>
<th>1y</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCVA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR</td>
<td>0.77</td>
<td>0.75</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>0.25</td>
<td>0.194</td>
<td>0.194</td>
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</tr>
<tr>
<td>Sphere</td>
<td></td>
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</tr>
<tr>
<td>IR</td>
<td>0.40</td>
<td>0.317</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>0.266</td>
<td>0.29</td>
<td>0.285</td>
<td></td>
</tr>
<tr>
<td>Cylinder</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>IR</td>
<td>0.45</td>
<td>0.655</td>
<td>0.273</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>0.281</td>
<td>0.109</td>
<td>0.593</td>
<td></td>
</tr>
<tr>
<td>Mean keratometry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR</td>
<td>0.068</td>
<td>0.075</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>0.178</td>
<td>0.177</td>
<td>0.715</td>
<td></td>
</tr>
<tr>
<td>Maximum keratometry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR</td>
<td>0.275</td>
<td>0.251</td>
<td>0.045</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>0.412</td>
<td>0.344</td>
<td>0.715</td>
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<tr>
<td>Central pachymetry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR</td>
<td>0.045</td>
<td>0.046</td>
<td>0.026</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>0.061</td>
<td>0.077</td>
<td>0.46</td>
<td></td>
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<tr>
<td>Thinner pachymetry</td>
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<td></td>
</tr>
<tr>
<td>IR</td>
<td>0.026</td>
<td>0.028</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>0.038</td>
<td>0.042</td>
<td>0.47</td>
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</tr>
</tbody>
</table>

*P<0.05.
statistically significant in all follow-up moments except for the 1st month.

4) $K_m$ and $K_{max}$: a $K_m$ reduction was observed throughout the follow-up in both groups, with only the IR group showing a statistically significant result between the preoperative evaluation and the 1st year evaluation. There were no statistically significant differences between the groups.

5) Complications: 6 eyes of each group (total of 12 eyes) presented a transient haze, with spontaneous resolution until the 3rd week after treatment. All epithelial defects were resolved within 1 wk after treatment and no cases of corneal or permanent stromal scarring were observed.

6) Progression: no eye presented progression in the 1st year in either group, according to the criteria used to define progression.

**DISCUSSION**

The study showed an improvement in the studied parameters (BCVA, refraction, $K_m$ and $K_{max}$), without relevant complications and without progression, in both groups, which is in agreement with the literature and other studies[4,16]. These results in both groups corroborate the objectives of the CXL itself, culminating in the stability or even reduction of the progression of keratoconus by strengthening the covalent bonds between the collagen fibres of the cornea. Thus, the primary objective of CXL was achieved through the application of both IR and HR.

According to the results obtained, the application of HR seems to be an option to consider in thinner corneas. Its effect can be explained by its lower oncotic pressure, leading to an increase in corneal swelling until the corneal thickness threshold of 400 μm is reached for the procedure showing a lower risk of endothelial toxicity associated with UV-A radiation. The study by Nassaralla et al[16] confirmed the efficacy of HR application in corneas of <400 μm, with a statistically significant reduction of keratometry and BCVA in the first year of follow-up[16]. The study by Raiskup and Spoerl[15] demonstrated no statistically significant stabilization of keratometry and of BCVA with the same follow-up period[15]. Although our results are consistent with the literature available, there are still few comparative studies between CXL with IR and HR. Similar to our study, those conducted by Raiskup and Spoerl[15] and Nassaralla et al[16], mentioned
above, also revealed more discrete results with HR, contrary
to previous studies with corneas of ≥400 μm treated with
IR[4,10-12,15-16]. On the other hand, the study by Rosenblat and
Hersh[14], showed that there were no statistically significant
differences between IR and HR groups regarding improvement
of Kmax, uncorrected visual acuity and the thickness of the
cornea[14]. The study by Chen et al[19] also showed the absence
of a statistically significant difference between the IR and
HR groups in relation to keratometry, BCVA and a spherical
equivalent.

A theory discussed regarding the possible lower efficacy of
HR is the eventual lower biomechanical effect with greater
difficulty in establishing connections between collagen fibres,
due to the lower oncotic pressure associated with corneal
intumescenting, resulting in lower concentration and greater
distance from collagen fibres[14-18]. However, even if HR is not
as effective as IR, showing no statistically significant change
in the parameters under study, and/or showing stabilization
of parameters rather than improvement, its application is still
promising because by stabilizing the parameters, it is already
delaying progression without any apparent increased risk for
the endothelium.

Regarding the modifications in BCVA, cylinder and sphere,
some studies demonstrate results similar to those obtained in
our study, with varying significance; others show no relevant
changes in these parameters[4,10-12,15-16,18]. The study conducted
by Hashemi et al[4] indicates as a possible justification the
differences in sample size and base refractive error.

The changes in pachymetry in our study, with a decrease in
the comparison between the initial value and the first month and
with a later increase up to the first year, are in agreement with
other studies[11,17-18,20,21]. Gu et al[14] and Hassan et al[20]
suggest that the initial change is due to measurement errors due to
the initial swelling of the cornea caused by the procedure.
Also Wollensak et al[22] demonstrated that the swelling of the
cornea depended on the degree of CXL: the higher the CXL,
the smaller the swelling, and vice versa. On the other hand,
de-epithelialization was initially performed, which alone can
increase the rate of evaporation of stromal water and, as it is
not resistant to dehydration, makes the cornea vulnerable to a
increase the rate of evaporation of stromal water and, as it is

depended on the degree of CXL: the higher the CXL,
the smaller the swelling, and vice versa. On the other hand,
de-epithelialization was initially performed, which alone can
increase the rate of evaporation of stromal water and, as it is
not resistant to dehydration, makes the cornea vulnerable to a
increase the rate of evaporation of stromal water and, as it is

depend on the degree of CXL: the higher the CXL,
the smaller the swelling, and vice versa. On the other hand,
de-epithelialization was initially performed, which alone can
increase the rate of evaporation of stromal water and, as it is
not resistant to dehydration, makes the cornea vulnerable to a
increase the rate of evaporation of stromal water and, as it is

Regarding complications, the results are also in agreement
with the literature and other studies, including the presence of
corneal haze[21-22]. In our study, the absence of complications
affecting the final outcome of CXL supports the safety of
applying HR. This is also a controversial topic given that
some studies, as one study of Kymionis et al[23], documented
no complications after standard CXL with the standard IR
in patients with corneal thickness <400 μm. In that study,
the author showed that the corneal endothelium (evaluated
postoperatively by corneal confocal microscopy) did not
undergo any significant changes.

The limitations included the retrospective profile of the study,
as a small sample and a short follow-up. These limitations are
considered relative, since they are resolved with the continuity
of the study. It is a type of study that may have an impact on
clinical practice, as it allows more effective and safer treatment
of more advanced cases of keratoconus, associated with thinner
corneas, where current treatment options are still limited.

To conclude, the application of HR is promising in the
treatment of thinner corneas, stopping the progression of
keratoconus without seeming to compromise the safety of the
procedure. Further studies on this type of procedure, including
an increase in the number of cases and greater follow-up, will
contribute to a greater and better knowledge of its long-term
efficacy and safety in clinical practice.

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Henriques S, None; Pégo P, None; Vendrell C, None; Prieto
I, None.

Peer Review File: Available at:

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