INTRODUCTION

Corneal collagen crosslinking (CXL) treatment is based on a photochemical reaction between ultraviolet light A (UVA) and riboflavin (1-3). This minimally invasive procedure interferes with corneal biomechanics, stabilizing corneal ectatic disorders such as keratoconus and iatrogenic keratectasia (after corneal refractive surgery) (1-3). The introduction of CXL in routine clinical practice has changed the management of the above entities; furthermore, it provides a true treatment, by inhibiting progression of keratoconus and corneal ectasia. Prior to CXL, all interventions (glasses, contact lenses, and intracorneal ring segment implantation [4]) were used to improve visual function of patients, while they did not interfere or treat the underlying pathophysiology of the corneal tissue.

PURPOSE. To evaluate the time-dependent degradation rate of riboflavin after ultraviolet A (UVA) irradiation.

METHODS. Two solutions of commercially available riboflavin solution (0.1%) were used; one served as control, while the second was irradiated using UVA light at 370 nm wavelength. Four samples of riboflavin solution were retrieved prior to irradiance and at 1, 5, 15, 30, and 60 minutes after irradiation (group A); at the same time points samples of riboflavin were retrieved from the control solution in order to assess environmental time-induced degradation of riboflavin (group B). All samples were immediately analyzed using liquid chromatograph mass spectrometry to detect riboflavin and its 2 subproducts, lumiflavin (LF) and lumichrome (LC).

RESULTS. Mean percentage of riboflavin degradation was 0.0, 5.3, 9.1, 15.3, 20.6, and 33.3 at 0, 1, 5, 15, 30, and 60 minutes after UVA irradiation, respectively (group A). The time-dependent riboflavin degradation was statistically significant (p<0.05), while for group B there was no change in riboflavin concentration at all time intervals. In group A, mean LC concentration demonstrated a gradual concentration increase, reaching 2.386±1.526 ppm after 60 minutes of UVA exposure.

CONCLUSIONS. The time-dependent degradation of riboflavin solution is significant, reaching 20.6% after 30 minutes of UVA exposure. It seems that only a small fraction of the overall riboflavin molecules break down since more than 65% remain intact even after 1 hour of UVA irradiation. Control riboflavin solution seems to be stable, as no degradation is evident even after 60 minutes.

KEY WORDS. Corneal collagen crosslinking, CXL efficacy, CXL safety, Degradation rate, LC-MS, Riboflavin, UVA

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Riboflavin is an important component of this photochemical reaction; also known as vitamin B₂ (belongs to the flavin group), riboflavin acts as a photosensitizer which is excited while irradiated with UVA into its triplet state generating in this process reactive oxygen species (ROS) (3). The ROS can react further with various molecules inducing chemical covalent bonds bridging amino groups of collagen fibrils (type II photochemical reaction), inducing crosslinks, which increases corneal rigidity (3). Furthermore, riboflavin serves as a protective agent during CXL treatment, since it concentrates and absorbs UVA irradiation at the level of the corneal stroma, in order to prevent UVA-induced damage to other ocular layers and tissues (corneal endothelium, crystalline lens, and retina). The presence of riboflavin molecules in the corneal stroma during CXL is critical due to its dual action as mentioned earlier. Even though there have been many reports concerning the outcomes of CXL treatment, little is known about the behavior of riboflavin in respect to its degradation rate which is directly related to the efficacy and safety of the technique. The purpose of the current study is to evaluate in an experimental setup the time-dependent degradation rate of commercially available riboflavin solution (0.1% concentration) used for CXL treatment, after UVA irradiation at 370 nm wavelength and an irradiance of 3mW/cm², using a liquid chromatograph mass spectrometer.

MATERIALS AND METHODS

Materials

Commercially available riboflavin solution (riboflavin 0.1% isotonic eyedrops with 20% dextran 500, Medicross, Medio–Haus, Behrensbrook, Neudorf, Germany) was used to assess its time-dependent degradation rate after irradiation using the standard CXL treatment setup of UVA light source at 370 nm wavelength (UVX illumination system version 1000, UVXTM, IROC AG, Zurich, Switzerland). Before irradiation, an intended 3.0 mW/cm² (5.4 J/cm² total surface dose after 30 minutes UVA exposure) of surface irradiance was calibrated using a UV light meter at a working distance of 5 cm. Riboflavin solution 0.1% and UVA source used in this experiment are in accordance with the specifications described by the original CXL–Dresden protocol. The photochemical reaction between riboflavin and UVA results in 2 subproducts: the pathway of the reaction and the derived subproduct is mainly dependent on the pH of the environment in which the reaction takes place (5). Stock and working solutions of lumichrome (LC) and lumiflavin (LF) (Sigma-Aldrich Inc. P.O., St. Louis, MO, USA) were used; LC–MS grade water and formic acid (98%) (Sigma-Aldrich) and LC-MS grade acetonitrile (Fisher Chemical, Fisher Scientific, Leicestershire, UK) were used.

Solutions

Working solutions of riboflavin were prepared by dilution of the initial commercially available stock solution (1000 ppm) using nanopure water to a final concentration of 10 ppm. Stock and working solutions of LF and LC were prepared in methanol (for LC) or in methanol/10 mM ammonium acetate (1:1 v/v) (for LF) at concentrations from 0 to 5 ppm (6). The calibration curves were linear with r²=0.9992 and r²=0.9999 for LF and LC, respectively.

Experiment

A total of 2.5 mL of riboflavin working solution was placed in a customized round container (glass, clear) with inner diameter of 11.50 mm and height of 40.00 mm (container volume of about 4.16 cm³). Riboflavin within the container was illuminated using a UVA standard CXL lamp (group A), with an irradiation diameter of 11.50 mm. The plane of focus of the UVA lamp was set at the middle of the height of the riboflavin solution (12 mm from the bottom of the container), in order to achieve a homogeneous illumination profile of the solution, while every 5 minutes the solution was stirred to further achieve its homogeneity. Irradiation was conducted for a total of 60 minutes, while at 0, 1, 5, 15, 30, and 60 minutes, 100 µL of riboflavin solution was retrieved and placed in a LC-MS autosampler vial for LC-MS analysis, to assess the time-dependent degradation rate of riboflavin. Another container (group B) with 2.5 mL of riboflavin of the above working solution was used, served as control, and did not undergo any irradiation. Control working solution was kept at room temperature and in mesopic conditions (same conditions as experimental solution): room illuminance of 1 lux (mean level of ambient illuminance was evaluated after 5 measurements, using a digital lux meter, OM 210; Robin Electronics Ltd., UK), while at 0, 1, 5, 15, 30, and 60 minutes, 100 µL was retrieved to assess the environmental induced degradation rate of ri-
boflavin. The same procedure was repeated 4 times, and a total of 4 samples of riboflavin solution, at every time point, was analyzed both for the experimental and control groups to quantify riboflavin, LC, and LF.

**LC-MS analysis**

Analysis was performed on a liquid chromatograph mass spectrometer (Shimadzu LC-MS, 2010 EV) system equipped with an electrospray ionization (ESI) interface (positive mode), an autosampler, solvent degasser, binary pump, and a heated/cooled column compartment. The column was a Discovery C18 HPLC Column (25 cm × 4.6 mm, 5 µm, SupelCo, Bellefonte, PA, USA). Both mass spectrometer and HPLC inlet were controlled by Shimadzu LC solution software which also was used for data acquisition and processing. The instrument was tuned and calibrated using autotune procedures recommended by the manufacturer. CDL and heat block temperatures were 250°C and 200°C, respectively. The detector voltage was 1.5 kV and the nebulizing gas flow 1.5 L/min.

Ten microliters from each extracted sample was entered to the chromatograph column, at a temperature of 30°C. The mobile phase of the HPLC system consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) (7). The separation of the target compounds was achieved using the following elution program: 10% of solvent B (held for 1 minute) then increased to 90% (B) (16 minutes linear ramp), held for 5 minutes at 90% (B). The total mobile phase flow rate was 0.6 mL/min. The detection was done in SIM (selected ion monitoring) positive mode using target ion fragments with m/z 377.15 for B2, m/z 257.10 for LF, and m/z 243.10 for LC. The retention times were 10.1 min for B2, 11.4 min for LF, and 12.4 min for LC.

**Statistical analysis**

Statistical analysis was performed with SPSS 19.0 (Statistical Analysis Software, IBM Corporation, USA). Data were analyzed for statistical significance using one-way analysis of variance and a p value less than 0.05 was considered to be statistically significant.

**RESULTS**

The working solution of riboflavin, which served as control and was kept in mesopic conditions (1.20 lux) at room temperature during the experiment (same room conditions as experimental solution, group A), did not demonstrate a measurable change in riboflavin concentration at all time intervals and no increase of the LF concentration was observed (Tab. I). On the other hand, small amounts of LC were created and the mean measured concentrations were 0, 0.046, 0.047, 0.058, 0.066, and 0.082 ppm at 0, 5, 10, 15, 30, and 60 minutes, respectively (Tab. I). The concentration increase concerning LC production in group B was statistically significant (p<0.05).

Mean percentage of time-dependent riboflavin degradation irradiated with UVA was 0, 5.3, 9.1, 15.3, 20.6, and 33.3 at 0, 1, 5, 15, 30, and 60 minutes, respectively (Tab. I, Fig. 1). The time-dependent decrease of riboflavin concentration was statically significant (p<0.05). Mean concentration of the produced LF was 0, 0.003, 0.005, 0.006, 0.010, and 0.016 ppm and for LC 0, 0.090, 0.315, 0.570, 0.963, and 2.386 ppm at 0, 1, 5, 15, 30, and 60 minutes, respectively (Fig. 2). Mean concentration increase of LF production in group A was insignificant (p=0.69), while the concentration increase of LC was statistically significant.

**TABLE I - THE DEGRADATION RATE OF THE RIBOFLAVIN AND THE CONCENTRATION (PPM) OF THE PRODUCED LUMICROME (LC) AND LUMIFLAVIN (LF)**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Rivoflavin</th>
<th>LF</th>
<th>LC</th>
<th>LC-no UV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Degradation ± SD</td>
<td>C (ppm) ± SD</td>
<td>C (ppm) ± SD</td>
<td>C (ppm) ± SD</td>
</tr>
<tr>
<td>0</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>1</td>
<td>5.31 ± 5.34</td>
<td>0.002 ± 0.003</td>
<td>0.006 ± 0.006</td>
<td>0.090 ± 0.089</td>
</tr>
<tr>
<td>5</td>
<td>9.14 ± 2.11</td>
<td>0.005 ± 0.005</td>
<td>0.006 ± 0.006</td>
<td>0.315 ± 0.290</td>
</tr>
<tr>
<td>15</td>
<td>15.34 ± 0.75</td>
<td>0.006 ± 0.007</td>
<td>0.570 ± 0.50</td>
<td>0.401 ± 0.058</td>
</tr>
<tr>
<td>30</td>
<td>20.57 ± 1.73</td>
<td>0.010 ± 0.005</td>
<td>0.963 ± 0.963</td>
<td>0.460 ± 0.067</td>
</tr>
<tr>
<td>60</td>
<td>33.34 ± 7.28</td>
<td>0.016 ± 0.012</td>
<td>2.386 ± 2.386</td>
<td>1.526 ± 1.526</td>
</tr>
</tbody>
</table>
Riboflavin’s degradation rate induced by UVA

(p<0.05). Even though in groups A and B LC demonstrated a statistically significant concentration increase, LC concentration 60 minutes after UVA irradiation was almost 30 times higher when compared with the control solution (no irradiation) (Fig. 2).

DISCUSSION

Corneal CXL has changed dramatically the treatment approach of corneal ectatic disorders (keratoconus and post-laser-assisted in situ keratomileusis corneal ectasia). The current treatment protocol (Dresden protocol) is based on the saturation of the corneal stroma with riboflavin solution of 0.1% concentration (1-3). Riboflavin is instilled every 5 minutes on the cornea after epithelial removal for 30 minutes, and then the cornea undergoes irradiation using a UVA source which emits at 370 nm wavelength with an intensity of 3 mW/cm², while riboflavin continues to get instilled (every 3 minutes) (1-3).

Riboflavin is a unique molecule, which demonstrates several peaks in its absorption spectrum at 270, 366, and 445 nm. The UVA–tissue interactions differ at these frequencies; at 270 nm there is high absorption by cellular DNA, while this wavelength may induce tissue damage, such as photoconjunctivitis and photokeratitis (8). At 445 nm wavelength, there is potential for photochemical toxicity at the level of the retina (9). Finally, at 366 nm, there is absorption by pigmented tissues, but relative high transmission of DNA; this wavelength was chosen and is used during CXL treatment. The standard UVA source used in CXL has an output frequency of 370 nm and an intensity of 3 mW/cm², while the commercial riboflavin solution has an osmolarity of 400 mOsmol/L (similar to corneal stroma osmolarity) and a concentration of 0.1% (1-3).

The photochemical reaction between riboflavin and UVA results in 2 subproducts; the pathway of the reaction and the derived subproduct is mainly dependent on the pH level (of the environment in which the reaction takes place). In acidic and neutral pH values, LC is the main degradation product, while in basic values LF is produced (5). As human tear film (10) and corneal stroma (11) have a slightly alkaline pH, the working solution of riboflavin in this experiment was prepared to correlate with the pH conditions of the human ocular surface. This resulted in significant increase of LC concentrations, reaching 2.321 ppm 60 minutes after UVA irradiation, while LF revealed small concentration changes, reaching 0.011 ppm 1 hour after UVA exposure.

The presence of riboflavin in the corneal stroma is essential, since it acts both as a photosensitizer and as a protective shield. When irradiated riboflavin breaks down, providing ROS which induce chemical covalent bonds bridging this way amino groups of collagen fibrils; the mentioned riboflavin-UVA reaction results in strengthening of the corneal tissue (efficacy). Furthermore, riboflavin plays a significant role in the safety of the CXL procedure, since it absorbs and concentrates UV light in the corneal stroma, preventing UVA-induced toxicity at the level of the corneal endothelium, crystalline lens, and retina (safety). More than 90% of the UV...
light is absorbed in the cornea and in addition the anterior chamber riboflavin reduces the UV intensity to a level that is a factor of 1000 smaller than the recommended safety levels (10). It has been described by Wollensak et al that the cell damage threshold of UVA irradiation combined with riboflavin is 10 times lower than UVA alone (12).

Little is known about the amount of CXL needed (treatment quantification) to offer optimum outcomes; in addition, riboflavin molecules needed to break down for ROS production, resulting in crosslinking induction, are also unknown. Even though CXL treatment quantification is not yet determined, there are several studies in the literature in respect to the outcomes of CXL demonstrating its effectiveness (13-16). Moreover, several clinical studies have shown the increase of corneal strength and the stabilization of corneal ectatic disorders (keratoconus and iatrogenic keratectasia) (13-16). These findings suggest that the current widely used CXL protocol demonstrates an excellent efficacy profile. In this study, it seems that only 20% of riboflavin molecules decompose after 30 minutes of UVA irradiation, while after 1 hour riboflavin degradation did not exceed 35%. This experimental setup does not exactly simulate human CXL treatment where riboflavin is continuously instilled, constantly modifying the concentration of riboflavin molecules and probably altering the breakdown behavior of the agent. Furthermore, a significant amount of riboflavin solution, in human treatments, is inside the corneal stroma, which may further alter its degradation patterns. Nevertheless, it seems that a small fraction of riboflavin molecules break down (20%) after 30 minutes of UVA irradiation (human treatment), signifying that the induced CXL results after partial riboflavin breakdown.

Besides the need for riboflavin degradation during CXL, its presence is equally significant for the safety of the procedure. This study reveals that, even though the time-dependent degradation of riboflavin is statistically significant, over 65% (even after 1 hour of UVA exposure) remains intact and thereby absorbs the excess UVA irradiance, acting this way as a protective shield. The UVA absorbance at the level of the corneal stroma leads to decreased risks for induced toxicity at the level of the corneal endothelium, crystalline lens, and retina. Several studies have presented the high safety profile of CXL treatment, which is based on 2 factors: preoperative corneal thickness and corneal stromal penetration of riboflavin (3, 17, 18). The degradation patterns of riboflavin have been demonstrated by other studies (19, 20); based on these, the current CXL protocol states that riboflavin is instilled every 5 minutes during UVA irradiation, thus the solution on the surface of the cornea is constantly renewed and the effect of degradation (photobleaching) of riboflavin is compensated.

Another important finding of the current study is that the control working solution did not demonstrate a measurable change in riboflavin concentration even 1 hour after exposure in mesopic conditions. Standard CXL treatment is performed in low luminance conditions (in our institute illuminance in the room CXL is performed is 1.20 lux—mesopic conditions—this room was used for the experiment as well); it seems that riboflavin solution remains stable in this light condition, since no degradation is evident. Even though riboflavin solution was not affected in our experimental conditions (1.20 lux illuminance), riboflavin should be stored prior to surgery in scotopic conditions (dark bottles; protected from UV irradiation). This is important, as photopic conditions (excess light) would have an impact on the stability of the solution, which may undergo degradation, compromising the safety and efficacy of CXL.

The industry prepares new UV instrumentation for CXL on the way to decrease treatment duration. The intensity in these new devices will be increased from the known 3.0 mW/cm² and thereby the total energy needed as described by the standard CXL treatment (5.4 J/cm²) will be delivered on the corneal tissue in a shorter time interval (decreasing treatment time). An intensity increase will also affect positively the degradation rate of riboflavin; this raises issues concerning the concentration of the riboflavin solution used for CXL, which also has to increase, in order to avoid any possible negative effects on the method’s safety.

In conclusion, this experimental study demonstrates that the time-dependent degradation of standard riboflavin solution is significant, reaching 20.57% after 30 minutes of UVA exposure. It seems that only a small fraction of the overall riboflavin molecules break down since more than 65% remain intact even after 1 hour of UVA irradiation, while riboflavin solution which is not irradiated with UVA remains stable (no degradation) in mesopic conditions.
REFERENCES


