Original Article

Evaluation of effects of riboflavin and/or ultraviolet-A on survival of rat limbal epithelial stem cells in *ex-vivo*

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Purpose: To investigate the effects of riboflavin and/or ultraviolet-A (UV-A) irradiation on the cell viability of *ex-vivo*-cultured rat limbal stem cells (LSCs). **Methods:** LSCs of male Wistar rats ($N = 12$ eyes) were cultured, and immunofluorescence staining was performed to evaluate them. After characterization, these cells were assigned to four groups of control (C), a group that was exposed to UV-A radiation (UV), a group that was treated with riboflavin (R), and a group that cotreated with both UV-A and riboflavin (UV+R). To determine the cell viability of LSCs, these cells were subjected to MTT assay on days 1, 3, and 7 after exposure to UV-A and/or riboflavin. The duration of exposure to UV-A and riboflavin was similar to levels used during the conventional corneal collagen cross-linking procedure. **Results:** Compared with the viable cells in the control group, there was a significant decrease ($P < 0.0001$) in the number of LSCs in the UV group during all study days. In the R group, the level of viable LSCs was as same as the level of viable LSCs in the C group. Combined treatment with UV-A plus riboflavin significantly decreased the survival of LSCs on days 1 and 3 ($P < 0.0001$, $P < 0.001$, respectively) compared with the control group. Interestingly, in the UV+R group, the photosensitizing effect of riboflavin significantly decreased the cytotoxic effect of UV irradiation 7 days after exposure. **Conclusion:** These results suggest that the administered UV energy in the presence or absence of riboflavin can damage LSCs. Likewise, riboflavin could decrease the toxic effect of UVA on LSCs.

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Limbal stem cells (LSCs) are crucial for maintaining tissue integrity throughout the eye surface. These cells have specific properties of stem cells, including error-free proliferation, self-renewal, poor differentiation, and long lifespan.[1] Due to the lack of markers for LSCs, their localization is not completely understood. Corneal epithelium accompanied by peripheral limbal epithelium and conjunctival epithelium covers the ocular surface. Based on laboratory evidence, the basal layer of limbal epithelium (1.5–2.0 mm wide area) is the main stem cell niche in the cornea harboring a small population of stem cells. Therefore, protecting these cells is necessary to maintain a healthy cornea surface.[2]

Corneal cross-linking (CXL) is a promising treatment for arresting progressive keratoconus and other corneal ecstatic diseases. This powerful technique was introduced by Wollensak *et al*.[3] in 2003 causes a biomechanical stiffening effect on the cornea. During this procedure, intentional radiation of ultraviolet-A (UV-A) in the presence of riboflavin, a photosensitizer, mediates the cross-linking of collagen and proteoglycans and subsequently leads to stability of the cornea and halts disease progression.[4]

Riboflavin is a water-soluble member of the B-vitamin family with a protective effect on various tissues and organs due to its antioxidant, anti-inflammatory, anti-aging, anti-cancer, and anti-nociceptive properties. Nowadays, the combination of riboflavin and other treatments has received much attention for its protective properties and the diminishing toxicity of drugs. As a photosensitizer, riboflavin, due to its triplet-excitation state, can trigger oxidative damage to light-exposed cells by the degradation of a wide range of bio compounds.[5]

Despite extensive studies that confirm the effectiveness of CXL, there are some concerns about the safety of this treatment due to the danger of accidental UV-A irradiation and its collateral effect on limbal stem cells (LSCs). Safety measurement in CXL includes prevention of direct irradiation to the limbal region and using a ring-shaped sponge or the so-called Vidaurri suction ring to protect LSCs.[6]

The human limbus is in close vicinity of the cornea, which puts the limbal population at risk of exposure to UV-A during the CXL procedure. Therefore, partial irradiation of the limbal region is unavoidable. Previous studies provide evidence that UV-A radiation, used during the CXL procedure, induces apoptosis in LECs by regulating the expression of B-cell lymphoma (Bcl-2) protein family members.[7]

The main limitation of previous studies investigating the adverse effects of CXL on LSCs was their short-term duration. As it is difficult to evaluate the long-lasting effects of CXL on

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the LSCs in patients, in this study, we evaluate the safety of CXL on LSCs in *ex-vivo*.

Methods

Materials

All primary cultures and serial passaging were carried out in growth media consisting of Dulbecco's modified Eagle's medium/F12 culture medium (DMEM-F12, Sigma, Deisenhofen, Germany) supplemented with 10% fetal bovine serum (FBS; Moregate BioTech, Queensland, Australia), L-glutamine, and antibiotics (streptomycin-penicillin [Gibco 15140-122; Thermo Fisher Scientific, Waltham, MA, USA]). Trypsin (Invitrogen, Carlsbad) and collagenase I (Sigma, NY, USA) were used for digestion. Paraformaldehyde (Merck, USA) was used for cell fixation and 3-(4, 5-dimethyl) thiazol-2-yl-2, 5-dimethyl tetrazolium bromide (MTT) (Sigma) was used for the viability assay. Monoclonal antibodies used for immunocytochemical staining included rabbit ABCG2 primary antibody, P63 rabbit monoclonal primary antibody, goat anti-rabbit FITC-conjugated secondary antibody purchased from (Biorbyt, USA), and dimethyl sulfoxide (DMSO) (Sigma, Saint Louis, MO).

Animals

Six male Wistar rats (250–280 g) were obtained from the laboratory animal unit. Animals were housed in standard rat cages in a well-ventilated room with constant temperature (21 ± 2°C) and under a 12-h light/dark cycle with free food and tap water access. All experimental procedures were performed according to the National Research Council Committee for the care and use of laboratory animals. The Ethical Committee approved the present study.

Limbal stem cells isolation and culture

Primary LSCs were isolated from the corneoscleral rims of 8-weeks-old male Albino Wistar rats and propagated by a protocol described elsewhere[8] with some modifications. Briefly, limbal tissues were obtained and first washed three times with phosphate-buffered saline (PBS) solution containing 1× streptomycin/penicillin and the remained parts of the conjunctiva and Tenon's capsules were mechanically removed under a stereomicroscope. Next, excised tissues were treated with Dulbeccoæs modified Eagleæs medium (DMEM) containing collagenase I (1 mg/mL) for 50–60 min at 37°C. Subsequently, digested tissues were incubated in 0.25% trypsin for 15 min at 37°C. The remaining tissue was mechanically dissociated and centrifuged for 10 min at 1200 rpm. After the dissociation period, the cells were plated in a flask previously coated with collagen I, supplemented with DMEM-F12 (3:1) and 10% Fetal Bovine Serum (FBS). The medium was changed every 72 h until it reached confluence. Cells were cultured in 25-cm tissue culture flasks in a 37°C, 5% CO_2 tissue-culture incubator. The cultured cells were harvested for passage after reaching 70–80% confluency at 3 days.

Limbal stem cell characterization

Immunocytochemistry was done for monoclonal antibodies against ABCG2 and P63 markers. For immunocytochemical staining, the cells that were cultured in a 24-well plate and first washed with PBS, fixed with 4% paraformaldehyde for 10 min, washed again with PBS, washed with 0.2% Triton X-100 for 10 min, and incubated in blocking buffer (3% BSA, 0.02% azide in PBS) containing primary antibodies for overnight. Then, cells were incubated with secondary antibodies (goat anti-rabbit FITC-conjugated secondary antibody). Finally, the slides were visualized with fluorescent microscopy (Olympus IX71).

Study design

Isolated LSCs were seeded in a four-well plate, and each well was specified to one of the bellows mentioned cell groups. C group, control group without any modification; UV group exposed to UV-A irradiation 365 nm 3 mW/cm^2 for 30 min; R group treated with an isotonic 0.1% riboflavin solution (a photosensitizer) for 30 min; and UV+R group that was co-treated with both UV-A and riboflavin.

One UVA double diode (365 nm wavelength) was fixed at a 5 cm distance from the respective well using a stand. The actual irradiation lasted for 30 min, which is regularly used in the clinical setting. After the irradiation, the riboflavin solution was discarded and replaced by the cell medium. One, 3, and 7 days after the treatment, the cell viability of all groups were evaluated by the MTT assay [Fig. 1].

Cytotoxicity assay

To assess the cell viability of LSCs using the MTT assay, 100 µL of MTT was added to each well and incubated for 4 h at 37°C under dark conditions. After this, 200 µL of DMSO was added to each well and incubated for 5 min at 37°C. The absorbance of each well was then read at 570 nm using a BioRad ELISA reader (Model 680). This procedure was performed on other days of the study.

Statistical analysis

Statistical significance was assessed by the GraphPad software (San Diego, CA, USA) for Windows. First, Shapiro–Wilk normality test was performed to check the sample normality. Additionally, the two-way analysis of variance (ANOVA) followed by a *post hoc* Tukey's test was applied to evaluate data. All results are expressed as the mean ± standard deviation (SD). P <0.05 was assumed as statistically significant.

Results

Microscopic assessment of LSCs

In vitro culture of limbal stem cells in flasks coated with collagen feeder was used for evaluation of the proliferation of cultivated LSCs [Fig. 2].

Characterization of LSCs

Immunofluorescence expression of LSC markers, namely, ABCG2 and p63 was examined. ABCG2 and p63 were expressed in LSCs. Extremely clear blue staining of LSCs nuclei was observed when the growing cells were stained with the DAPI staining method [Fig. 3].

Effect of UV and riboflavin on cell viability

The viability of LSCs was assessed using the MTT assay on days 1, 3, and 7 after treatment with UV, R, and UV+R. As depicted in Fig. 4, the results from the two-way ANOVA of cell survival in the MTT test using group and day as factors demonstrated the main effect of group ($F(3, 8) = 252.4$; $P < 0.0001$), day ($F(2, 16) = 0.4571$; $P > 0.05$), and group × day interaction ($F(6, 16) = 12.72$, $P < 0.0001$).

Intergroup analysis indicated that the number of viable LSCs was significantly decreased in the UV group on days 1, 3, and 7 ($P < 0.0001$ for all days) compared with the control

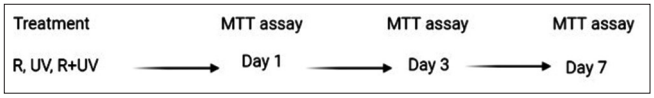

Figure 1: Scheme of the study design. R: Riboflavin, R + UV: Riboflavin + UV

group. Moreover, there was no significant difference between the riboflavin-treated and control groups during all study days [Fig. 4].

However, combined treatment with UV-A plus riboflavin significantly decreased the survival of LSCs on days 1 and 3 ($P < 0.0001$, $P < 0.001$, respectively) compared with the control group. Interestingly, we did not observe a significant difference between UV+R and C groups on day 7.

Therefore, it might be inferred that riboflavin could decrease the toxic effect of UVA on the LSCs [Fig. 4].

Comparison between different days in all treatment groups depicted that the difference between days 1 with 3 and 7 was not significant ($P > 0.05$) in the R and C groups. Nevertheless, there was a significant ($P < 0.001$) difference between day 1 with days 3 and 7 in UV and UV+R groups [Table 1].

Discussion

The present study investigates the results of exposure to UV-A and riboflavin both alone and after combined treatment on LSCs to evaluate the safety of CXL protocols.

Collagen is the main extracellular matrix protein of the cornea, and it is naturally biocompatible and relatively inexpensive to isolate. LSCs can be successfully cultivated on collagen carriers while maintaining normal phenotypes and achieving multilayered stratification.[9] We cultured LSCs in a flask coated with a collagen feeder in this study.

Our data demonstrated that UV-A induced the loss of LSCs *in vitro*. This cytotoxic effect of UV on corneal cells, including LSCs, is already reported by several studies.[9–11] The reason for this is that both UV-A and UVA-induced reactive oxygen species (ROS) can induce DNA damage and consequently cell death.[12,13]

To our knowledge, this is the first study investigating the effect of UV/riboflavin on LSCs viability during 7 days. Our results have shown that LSCs that were only exposed to UV were not able to renew their populations during 1, 3, and 7 days after exposure. These findings agree with previous case reports that showed delayed corneal epithelial healing in patients after exposure to UV/riboflavin.[14,15]

Consistent with other studies, our data showed that riboflavin alone could not induce cell damage,[16] which was

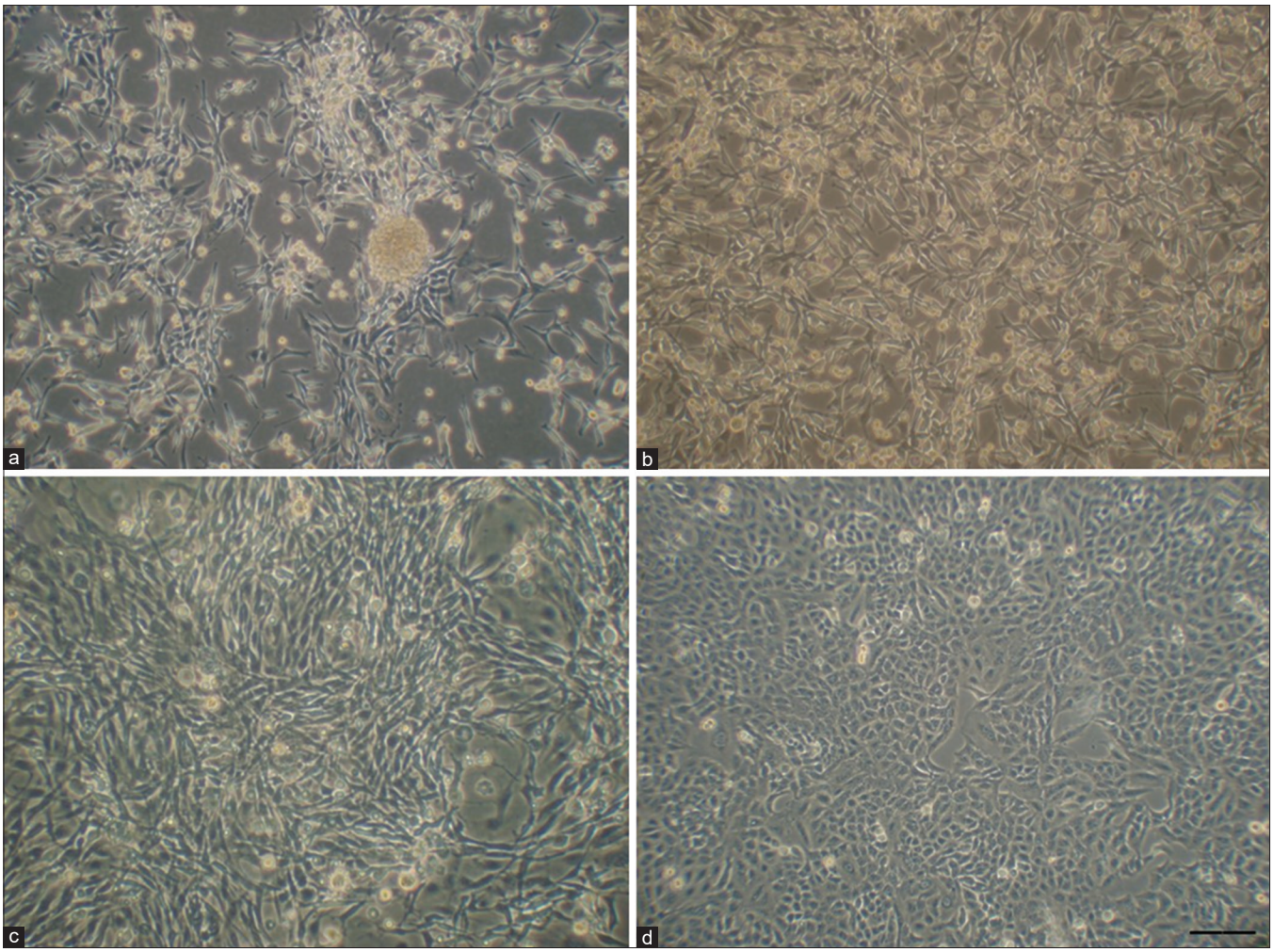

Figure 2: Microscopy images show a primary culture of rat limbal stem cells (LSCs) on the collagen-coated plate for 3 days. (Passage 0, day 1 (a), and day 3 (b)). Microscopy images show a mixed population of cells cultured on Passage 1, after 6 days (c). The characteristic cobblestone morphology of an LSC cluster is visible after Passage 3, day 14 (d). The scale bar is 200 μm

predictable because riboflavin or vitamin B12 is an essential compound in nutrition that presents several tissues, including several tissues in the retina.[17]

Table 1: Effects of riboflavin, ultraviolet-A (UV-A) irradiation, and their combinations on the viability of *ex vivo*-cultured rat limbal stem cells 1, 3, and 7 days after exposure

Groups	1	3	7
UV-A (UV)	0.15 ± 0.07	0.16 ± 0.07*	0.16 ± 0.09**
Riboflavin (R)	0.48 ± 0.03	0.49 ± 0.009	0.45 ± 0.02
UV+Riboflavin (UV+R)	0.37 ± 0.11	0.39 ± 0.06**	0.36 ± 0.1**
Control (C)	0.54 ± 0.07	0.56 ± 0.03	0.52 ± 0.05

Data are expressed as mean ± SD. Values are statistically significant at *P<0.001,**P<0.0001 compared with day one in each group

In our study, riboflavin could not significantly reduce the cytotoxic effects of UV on LSCs 1 day after exposure. However, it exhibits a meaningfully protective effect against the toxic effect of UV irradiation on days 3 and 7 after exposure. In line with these, a study reported that the threshold for UVA-induced damage in keratocytes was shown to be lowered when UV treatment was combined with riboflavin (range of 0.5–0.7 mW/cm^2) compared to when UV was just used (5 mW/cm^2).[18] Moreover, the UVA damage threshold in corneal endothelial cells 10 times declined when exposed to both riboflavin and UVA, compared to when only treated with UVA.[19] Another interesting finding of our study was that the combined group showed faster recovery results on day 3 than the UV group.

Using a rabbit model, Armstrong *et al*. compared the corneal injury/wound healing effect and biomechanical changes of traditional/standard epithelium-off riboflavin-UVA CXL with three transepithelial procedures, including benzalkonium chloride ethylenediaminetetraacetic acid (BKC-EDTA)

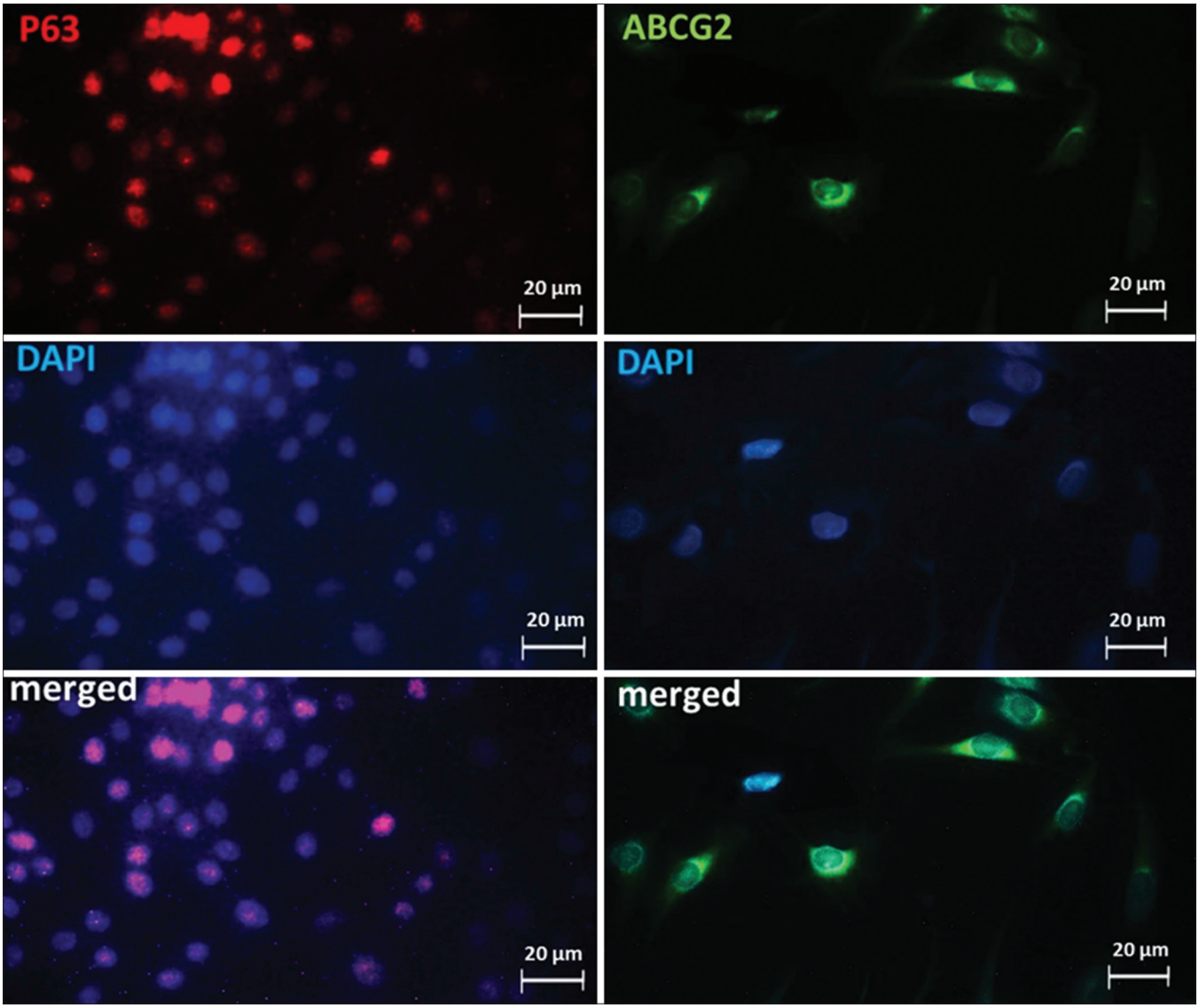

Figure 3: Immunohistochemistry for ABCG2 and p63 in rat limbus. The distribution of cells expressing ABCG2 and p63 was examined using immunostaining with anti-ABCG2 (green) and anti-p63 (red) antibodies, and DAPI (blue). Scale bars indicate 20 µm

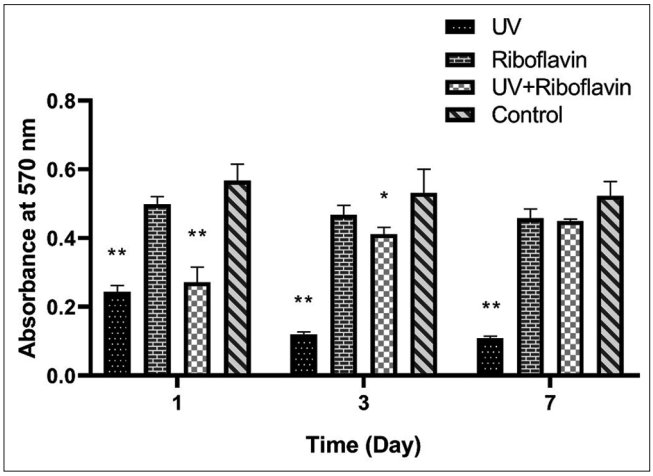

Figure 4: Quantification of viable limbal stem cells in the MTT assay 1, 3, and 7 days after exposure to UV and/or riboflavin. Each bar represents the mean ± SD; **P* < 0.001 and ***P*` 0.0001 vs. the control group

transepithelial, tetracaine transepithelial, or femtosecond laser-assisted transepithelial riboflavin-UVA CXL for 2 months. Their findings revealed that stromal cell death extended the full corneal thickness in femtosecond laser-assisted CXL and standard epithelium-off CXL groups. Importantly, in transepithelial CXL methods, the presence of the epithelium can block the penetration of UV light (not all) into the cornea.[20] In addition, another study conducted by Torricelli *et al*.[21] by comparing the effects of standard epithelium-off riboflavin-UVA crosslinking to transepithelial benzalkonium chloride-EDTA (BAC-EDTA) riboflavin-UVA crosslinking showed that the biomechanical stiffening effect created by standard epithelium-off CXL was greater than standard ones in the cornea of a rabbit model.

We agree that our study has limitations. First, we used rat LSCs due to some problems accessing human LSCs. However it is insightful, it may be challenging to translate these findings observed in rat LSCs to humans because of the differences in the behavior of LSCs *in vitro* and *in vivo* conditions.

Conclusion

CXL is becoming crucial for treating keratoconus and other corneal ecstatic disorders. Some advantages of this method include ease of administration, minimally invasive nature, and excellent efficacy claims. Nevertheless, there are sparse data on the adverse side effects of the CXL procedure on LSCs. We designed this study to identify the adverse effects of the CXL procedure on the survival of LSCs for 1 week after exposure to UV and riboflavin. Our data demonstrated that riboflavin and/or UVA induced the loss of LSCs in the *ex vivo* condition. However, these cells can restore their population 7 days after exposure to combined treatment. Because LSCs are vital for maintaining and repairing corneal epithelium under normal conditions and after wound healing, protection of these cells is essential to avoid CXL complications.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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