·Basic Research·

Evaluation of the *in vitro* antimicrobial properties of ultraviolet A/riboflavin mediated crosslinking on *Candida albicans* and *Fusarium solani*

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Abstract

• **AIM: To evaluate the antimicrobial properties of ultraviolet A (UVA) (365 nm)/riboflavin against *Candida albicans* and *Fusarium solani*.**

• **METHODS: Two fungus isolates were cultured *in vitro* and prepared with 10-fold serial PBS dilutions of cell concentration. For each dilution of fungus suspension, the concentration (colony-forming units/mL, CFU/mL) and the inactivation ratio of fungal cells were evaluated under 4 conditions: no treatment (control), UVA (365 nm)/riboflavin, riboflavin, and UVA (365 nm).**

• **RESULTS: The cell concentration decreased in UVA (365 nm)/riboflavin group for *Candida albicans* at each dilution and *Fusarium solani* at dilutions of 10^4, 10^3, 10^2 CFU/mL, when compared with that in control, riboflavin, and UVA (365 nm) groups (P<0.01). No difference of cell concentration was detected amongst the culture of control, riboflavin, and UVA (365 nm) groups for the two fungus. There is a negative correlation between suspension concentration (log-transformed) and the inactivation ratio in UVA (365 nm)/riboflavin group for *Candida albicans* and *Fusarium solani* (P<0.01).**

• **CONCLUSION: According to the standard protocol of corneal collagen cross-linking, UVA (365 nm)/riboflavin combination treatment is found to moderately inactivate the viability of *Candida albicans* and *Fusarium solani in vitro*. The inactivation ratio was found to increase with the decrease of cell concentration under UVA (365 nm)/riboflavin condition.**

• **KEYWORDS:** antimicrobial; crosslinking; fungal; riboflavin; ultraviolet A
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INTRODUCTION

Fungal ulcerative keratitis is a vision-threatening condition. If not managed properly, it could have a profound and devastating impact on visual function. In some cases it could even lead to vision loss. Antifungal drugs are not always effective in severe keratomycosis [1,2]. In some cases, corneal transplantation is required as the only alternative after ineffective chemotherapy.

Ultraviolet A (UVA)/riboflavin induced cross-linking (CXL) is a new approach to increase the mechanical and biochemical stability of the corneal stromal tissue [3-5]. During the CXL procedure, drops of 0.1% riboflavin solution in 20% dextran are instilled onto the cornea every 5min for 30min. After allowing riboflavin to permeate through the cornea and appear in the anterior chamber, the cornea is exposed to ultraviolet (UV) light with a wavelength of 370 nm and an irradiance of 3 mW/cm^2 for a total time of 30min. To date, the CXL treatment was clinically applied on the keratoconus, pellucid marginal degeneration, and iatrogenic keratectasia after laser *in situ* keratomileusis (LASIK)[6-10], bacterial[11,12] and *Acanthamoeba* keratitis [13,14]. Laboratory studies have shown that the exposure of UVA light combined with riboflavin application inactivate a large variety of microorganisms [15-18]. But some studies showed that *in vitro* the standard CXL was no effect on *Candida albicans (C. albicans)*, *Fusarium solani (F. solani)*, and *Aspergillus fumigatus* by testing with Kirby-Bauer discs[19,20].

CXL, in which riboflavin is excited to a triplet state by UV exposure and releases reactive oxygen species (oxygen radicals and singlet oxygen) into the surrounding circumstance, is also a photodynamic therapy method.

Photodynamic therapy (PDT) is a process in which cells are treated with an agent that makes them susceptible to killing by exposure to light in a specific wavelength[9]. Antimicrobial therapy is one of the possible future applications of this technique. PDT is known to be effective against viruses, bacteria, and fungi [21,22]. Fungus is a type of eukaryotic organism having a greater cell size and thicker cell wall than those of bacterial, virus. This cell structure allows fungi to resist the inactivation of the photodynamic therapy (PDT)[23,24]. It has been widely noted that *C. albicans* is more difficult to be killed by PDT than bacterial cells [24,25]. Demidova and Hamblin [25] described that the effectiveness of antimicrobial PDT increased dramatically in phosphate-buffered saline (PBS) with the decrease of *C. albicans* cell concentration.

Based upon these researches and developments, we proposed that the efficacy of UVA (365 nm)/riboflavin combination to reduce pathogens may be manifested best in phosphate-buffered saline (PBS) and in lower concentration fungal pathogens. Our study has been designed to evaluate the standard protocol of UVA (365 nm)/riboflavin CXL treatment for 10-fold serial PBS dilutions of *C. albicans* and *F. solani in vitro*.

MATERIALS AND METHODS

Fungi Cultivation *C. albicans* isolate (Biochemical Laboratory of Shandong Success Pharmacectic Co. Ltd, China) was cultured on Sabouraud glucose agar (Beijing Sanyao Technique Development Co. China) at 25℃ for 48h. The cultures were washed with sterile PBS. Using Remel McFarland equivalence standards, a 0.5 McFarland turbidity solution of *C. albicans* (approximate cell concentration of 1 to 2×10^8 colony-forming units/mL, CFU/mL) was produced and diluted 1:10 with PBS to 10^7 CFU/mL as the stock suspensions.

An isolate of *F. solani*(No.3.4489) were obtained from China General Microbiological Culture Collection Center (CGMCC, Beijing, China). The frozen isolate was sub-cultured twice and then plated onto Sabouraud glucose agar (Beijing Sanyao Technique Development Co. China) at 25℃ for 7d. Cultures gently were dissolved in sterile PBS to obtain the mixture suspensions (conidia and hyphal particles). The suspension was permitted to sit for 5min at room temperature to allow large particles to settle out, and then the upper part of the homogeneous suspension (3-4 mL) was adjusted to a 1.0 McFarland turbidity standard using a turbidimeter, providing an cell concentration of 0.4 to 5×10^8 CFU/mL (conidia/mL) (in this paper, CFU/mL was applied for consistent). A 10^7 CFU/mL stock suspension was produced as above.

These stock suspensions were diluted 10-fold serially to the final cell concentrations at 10^6, 10^5, 10^4, 10^3 and 10^2 CFU/mL with PBS in no treatment (control, C) groups and UVA (365 nm) (UVA) groups, and with PBS and riboflavin sodium phosphate injection (riboflavin-5-phosphate, 0.3%. Jiangxi Pharmacectic Co. Ltd. China) in riboflavin (R) groups and UVA (365 nm)/riboflavin (UVAR) groups. The final concentration of riboflavin in R and UVAR groups was 0.1%.

Ultraviolet Irradiaton The UV irradiation was performed with UV-X1000 system (IROC Innocross AG, Zurich, Switzerland) with a wavelength of 365±10 nm. Calibration of the light energy was carried out before each experiment to ensure that the energy density was in range of 2.7 and 3.3 mW/cm^2.

Suspensions with different cell concentration in four groups were incubated in culture tubes and shaken in an orbital shaker in the dark at room temperature. After 30min, 200 micro liter aliquots of the suspensions were subjected to a 48 well cell culture cluster (Costar Corning, New York, USA). The diameter of the well on the cluster is about 11 mm, 200 μL liquid in the well is sufficient to maintain a thin layer of liquid during 30min irradiation. The suspension whose volume less than 200 μL will not form liquid layer in the well because of surface tension. For UVAR group and UVA group, the suspensions in the wells were irradiated as the standard CXL procedure. The distance of light source to surface of solution was 5 cm, the illumination diameter was 11 mm and illumination was conducted in dark room to prevent photosensitization of riboflavin from background visible light. The energy density was in range of 2.7 and 3.3 mW/cm^2. The inside diameter of the well was 11 mm and equal to the diameter of light spot which enables satisfying irradiation of suspension surface with UV light. For R group and C group, the suspensions in the 48 well plate were kept in the dark at room temperature for 30min.

Cell Concentration Determination After the experimental treatments, the suspentions in each wells were diluted 10 fold serially with saline individually and 100 μL liter aliquots of the dilutions were seeded in duplicate onto sabouraud glucose agar (Beijing Sanyao Technique Development Co. China). For the 10^2 cell concentration array, duplicate 50 μL aliquots of the suspensions in the wells were directly seeded on sabouraud glucose agar. After incubation for 48h at 25℃, colonies were counted. Cell concentration was calculated.

Statistical Analysis Each experiment was repeated five times for the above serial dilutions in four groups. Descriptive statistics were expressed as mean±SD.

One-way analysis of variance (ANOVA) combined with Turkey test was used for the analysis of cell concentration. Because no treatment was taken on the dilutions in C group, the cell concentration of C group was theoretically equal to that before treatment, and also equal to the cell concentration of other group before treatment at a certain dilution. The inactivated cell number per ml suspension of any group was calculated by subtracting the cell concentration of C group by

that group. The inactivation ratio in UVAR group was calculated as follow: inactivation ratio (per cent)=[inactivated cell number (CFU/mL) of R+UVA]/ [cell concentration (CFU/mL) of C]×100%. ANOVA and multiple comparisons (Turkey test) were analyzed for the inactivated ratio among all dilutions. The regression analysis was carried out between the log-transformed cell concentration (before treatment) and its inactivated ratio under UVA+R condition.

P <0.05 was determined as significance. Data were analyzed with the SPSS software (16.0 Version for Windows).

RESULTS

Two Fungus Cell Concentration After Treatment For *C. albicans*, the cell concentration of UVAR group decreased at all dilutions comparing with that of C, R and UVA group (10^6: F=8.803, P =0.001; 10^5: F=11.56, P =0.000; 10^4: F=22.28, P=0.000; 10^3: F=40.934, P=0.000; 10^2: F=30.612, P= 0.000), while no difference of cell concentration was found among C, R and UVA groups (P >0.05) (Figure 1). For *F. solani*, at dilutions of 10^4, 10^3, 10^2 CFU/mL, the cell concentration of UVAR group decreased comparing with that of C, R and UVA group (10^4: F=5.505, P =0.009; 10^3: F= 9.518, P =0.001; 10^2: F=18.027, P =0.000), while no difference of cell concentration was found among C, R and UVA groups (P >0.05) (Figure 2). There was no difference of cell concentration of *F. solani* among four groups after treatment at dilutions of 10^6, 10^5CFU/mL(P >0.05)(Figure 2).

The Inactivation Ratios at All Dilutions for Two Fungus After Treatment The inactivation ratios of *C. albicans* in UVAR group were 32.2%, 33.1%, 49.0%, 57.8%, and 62.3% at dilutions of 10^6, 10^5, 10^4, 10^3 and 10^2 CFU/mL respectively (Figure 3). The inactivation ratios of *F. solani* in UVAR group were 25.7%, 28.2%, and 45.1% at dilutions of 10^4, 10^3 and 10^2 CFU/mL (Figure 4).

Relation Between the Fungal Cell Concentration (Before Treatment) and the Inactivation Ratio Under Ultraviolet A/riboflavin Condition for Two Fungus The regression analysis demonstrated a negative relation between the fungal cell concentration (before treatment) and the inactivation ratio under ultraviolet A/riboflavin condition with a formula: *C. albicans* [Inactivation ratio (per cent)]=82.043-8.452 [log-transformed cell concentration (Lg CFU/mL)] (P< 0.0001) (Figure 3), *F. solani* [Inactivation ratio (per cent)]= 66.060-9.567 [log-transformed cell concentration (Lg CFU/mL)] (P <0.01) (Figures 5, 6.)

DISCUSSION

The therapy for fungal keratitis is relative difficult due to its latent initiation and resistance to lots of existing anti-fungal drugs [26-28]. The corneal transplantation is nowadays a preferred therapeutic modality for keratitis in late stage or unresponsive to anti-fungal drugs. UVA (365 nm)/riboflavin mediated CXL, which was developed in the last decades, was

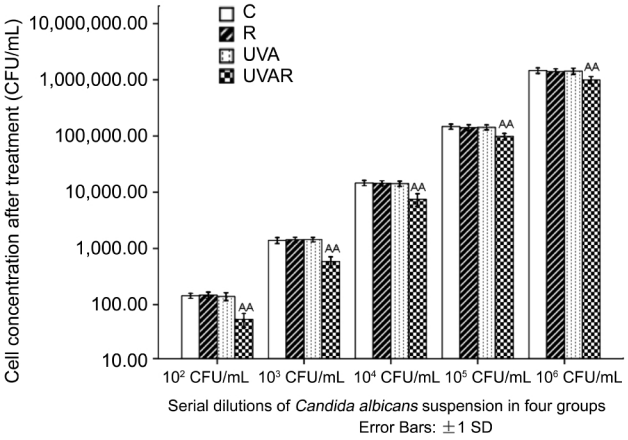

Figure 1 Comparing the cell concentration of *Candida albicans* suspensions under 4 conditions at 10-fold serial dilutions: no treatment, riboflavin, UVA (365 nm), and UVA (365 nm)/ riboflavin C for no treatment, control group; R for riboflavin; UVA for UVA (365 nm); and UVAR for UVA (365 nm) /riboflavin. Bars are calculated means from five samples, and error bars represent standard deviation. AA on the bars indicate a statistically significant decrease (P <0.01) in cell concentration of *Candida albicans* comparing with other groups at a certain concertration.

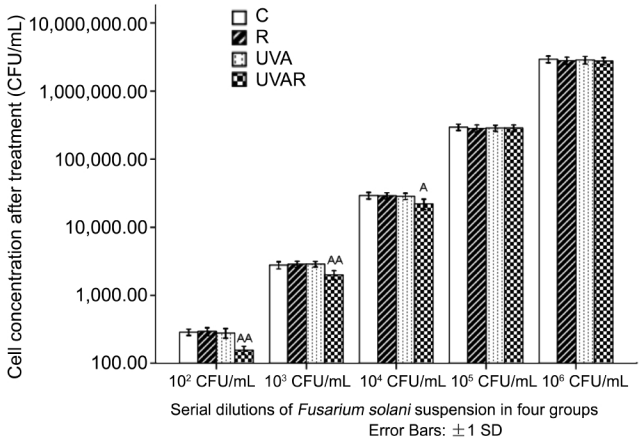

Figure 2 Comparing the cell concentration of *Fusarium solani* suspensions under 4 conditions at 10-fold serial dilutions: No treatment, riboflavin, UVA (365 nm), and UVA (365 nm)/ riboflavin C for no treatment, control group; R for riboflavin; UVA for UVA (365 nm); and UVAR for UVA (365 nm) /riboflavin. Bars are calculated means from five samples, and error bars represent standard deviation. A on the bars indicate a statistically significant decrease (P <0.05) and AA on the bars indicate a statistically significant decrease (P <0.01) in cell concentration of *Fusarium solani* comparing with other groups at a certain concertration.

initially described for the treatment of keratoconus, and then applied in cases with bacterial and Acanthamoeba keratitis clinically [11-14]. Recent studies have demonstrated the therapeutic effect of CXL on fungal keratitis on animals [29]. The possible mechanism of the bioactivity of CXL may include the suppression of replication of the pathogens through a chemical alteration to the nucleic acids [30,31], inactive effect of PDT [11-14,29] and an improvement in the resistance of corneal collagen fibers against destructive

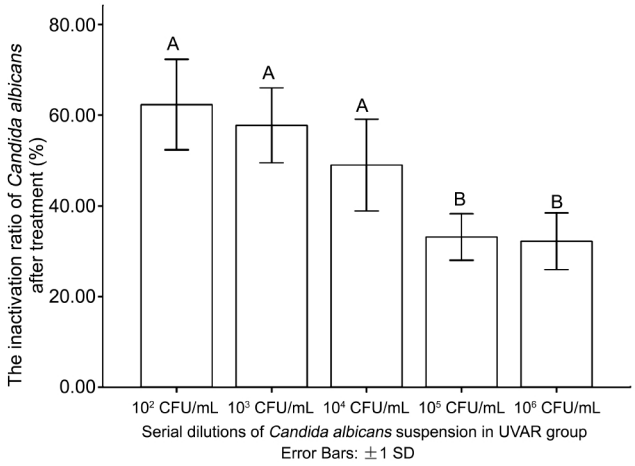

Figure 3 Comparing the inactivation ratio (per cent) of *Candida albicans* after treatment in UVA (365 nm)/riboflavin (UVAR) groups at 10−fold serial dilutions. Bars are calculated means from five samples, and error bars represent standard deviation. Different capital letters (A with B) indicate there is a significant difference between the two bars (P<0.05). Same capital letters (A with A, or, B with B) indicate no difference between the two bars.

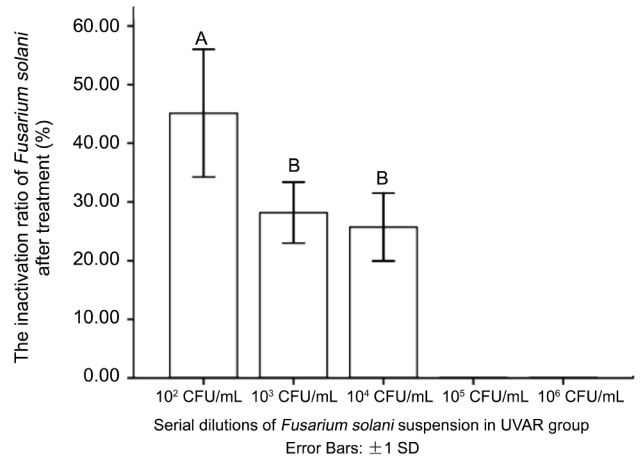

Figure 4 Comparing the inactivation ratio (per cent) of *Fusarium solani* after treatment in UVA (365 nm)/riboflavin (UVAR) groups at 10−fold serial dilutions. Bars are calculated means from five samples, and error bars represent standard deviation. Different capital letters (A with B) indicate there is a significant difference between the two bars (P<0.05). Same capital letters (B with B) indicate no difference between the two bars.

enzymes that are released during corneal inflammation[8].

We used *C. albicans* and *F. solani* as targeted fungus based on the consideration of the high prevalence of *Candida* and *Fusarium* keratitis worldwide [28,32-35]. Presenting data in this study highlights the impedance of CXL on *C. albicans* and *F. solani* proliferation *in vitro*. No change of fungal cell number was found after the treatment with the sole UVA or riboflavin. To our knowledge, this is the first *in vitro* experiment to access susceptibility of standard CXL treatment for fungal isolates in PBS. Riboflavin is water soluble and is more effective in PBS solution than dropping

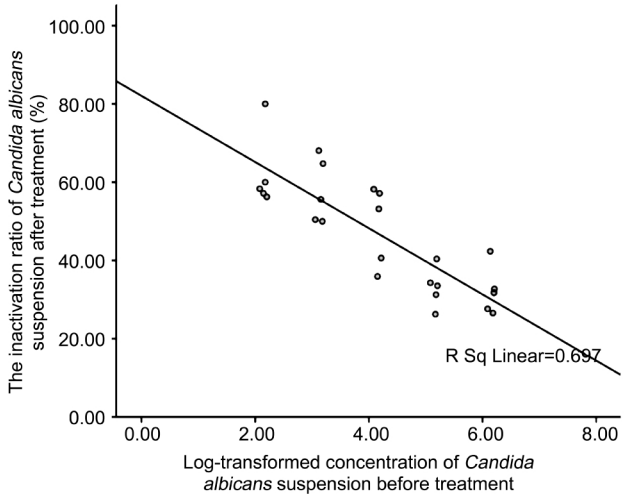

Figure 5 The regression analysis of the inactivation ratio (per cent) of *Candida albicans* after UVA (365 nm)/riboflavin treatment and log −transformed cell concentration before treatment (Lg CFU/mL).

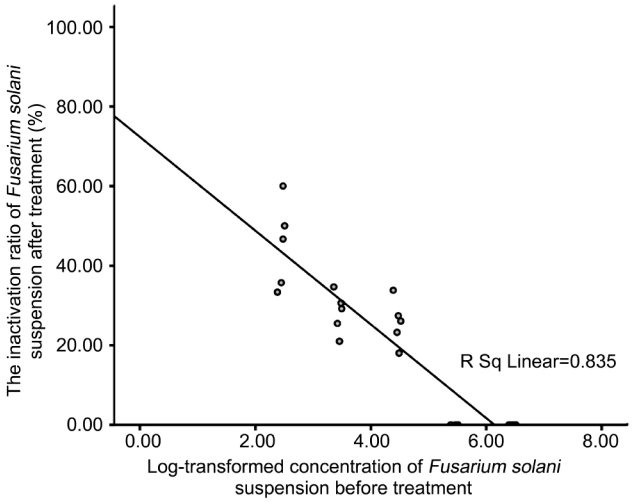

Figure 6 The regression analysis of the inactivation ratio (per cent) of *Fusarium solani* after UVA (365 nm)/riboflavin treatment and log −transformed cell concentration before treatment (Lg CFU/mL).

on agar plate. This may explain that growth inhibition zone was not shown after the standard CXL-riboflavin and UVA combination treatment when fungus were tested by using Kirby-Bauer discs in previous studies[19,20].

In our experiments, the inside diameter of the well was 11 mm and equal to the diameter of light spot which enables the completely contact of suspension surface with UV light, but the depth of liquid in the well is about 2 mm. It is well known that the penetration of UVA light is poor. That mean only a portion of suspensions in the well was actually treated with UVA irradiance because UVA light was blocked by the riboflavin and the fungal cells in the suspension. So there were only partly antifungal effect in UVAR groups for *C. albicans* at each dilution and for *F. solani* at dilutions of 10^4, 10^3, 10^2 CFU/mL in our experiments. This paper was to verify the antifungal ability of CXL, so the riboflavin

concentration in suspension at each dilution was prepared as 0.1%. Thus the interference of UVA irradiation depended on the cell concentration and cell characteristic of fungal suspension. Our experiments showed that the inactivation ratios at high concentrations were smaller than those at low concentrations for two fungus. The result was in agreement with that of Demidova and Hamblin's study [25], in which 3 to 7 line $\log_{10}$ of killing were achieved towards *C. albicans* at 10^7 and 10^6 cells/mL while 10^8 cells/mL were resistant to PDT's inactivation. The regression analysis demonstrated that the inactivation ratio under UVA+R condition was negatively related to the log-transformed cell concentration (LgCFU/mL) (Figures 3, 4). Although the resistance to CXL bioactivity in fungus suspensions with high concentrations may be induced by the insufficient irradiation of UVA light, the exact underlying mechanism are needed to be explored in the further study.

Because there was no difference on cell concentration among C, R, UVA groups at all dilutions for two funguses after treatment and the cell concentration of C group was theoretically equal to that before treatment, cell inactivation did not take place theoretically in C, R, UVA group at all dilutions for two fungus. Traditional applications of UVC light (200-275 nm wavelength) are used for drinking water and air/surface disinfection. UVA is light in the range of 320-420 nm wave length which has no antimicrobial activity as UVC. Riboflavin, or vitamin B2, is a naturally occurring compound and an essential human nutrient. As such we concluded that there was no antifungal effect under riboflavin or UVA condition alone.

Because of low power irradiance and poor transmittance of UVA applied in this study, fungal cells in suspensions were partly inactivated. This partial pathogen inactivation may be clinically important in our view. Infectious keratitis with the edema, opacity corneal stroma will not benefit from the CXL treatment unless UVA penetration strengthened with scraping the necrosis tissues, increasing the UVA irradiance, decreasing the riboflavin shielding. Continued studies are required to evaluate these methods in the clinical setting.

In conclusion, our study showed that the UVA (365 nm) /riboflavin mediated CXL has anti-fungal effect *in vitro* and the inactivation ratio of CXL increases along with the decrease of the cell concentration for *C. albicans* and *F. solani*. To improve the understanding of the anti-fungal spectrum of CXL, further investigations are needed to evaluate the bioactivity of CXL on others fungal strain commonly encountered in clinical keratitis and anti-fungal viability of CXL in trials *in vivo*.

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