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BASIC SCIENCE

Evaluation of antibacterial efficacy of photo-activated riboflavin using ultraviolet light (UVA)

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Abstract
Background To evaluate the antibacterial efficacy of photo-activated riboflavin using Ultraviolet A (UVA) on three bacterial strains commonly detected in keratitis.
Methods Three bacterial strains (Staphylococcus epidermidis, Staphylococcus aureus and Pseudomonas aeruginosa) were cultured on blood/hematin–agar plates and dispersed in PBS. Dispersion was done of 10 μl of bacterial stock-solutions in 90 μl of RPMI, where different riboflavin molarities had been added, to achieve a bacterial concentration of 1-4×10 ^{4}/ml. Riboflavin end molarities before illumination were 0, 100, 200, 300 and 400 μM. Each solution had a negative control. The solutions were illuminated with UVA (365 nm) for 30 minutes (5.4 J/cm^2) and then continued for a total time of 60 minutes (10.8 J/cm^2). A count of CFU was conducted after incubation and results compared.
Results In all tested strains, a slight decrease of bacteria was seen when exposed to UV for 30 minutes. A doubling of the UV dose showed a marked decrease of bacterial count in all bacteria tested. The combination of UV and riboflavin showed a more extensive reduction of CFU, confirming an interaction effect between UV and riboflavin.
Conclusion Riboflavin photo-activation using UVA (365 nm) can achieve an extensive eradication of bacteria, and the combination is more potent in reducing bacterial number than UV alone.

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Introduction

Pathogen inactivation by way of riboflavin photosensitization, using ultraviolet or visible light, was first recognised in the 1960s [1]. Extensive investigation of this phenomenon indicates that microbes are damaged by non-specific oxidative stress, mediated by reactive oxygen species as well as by intercalation of the riboflavin molecule into the RNA and DNA of micro-organisms [2–4]. A wide range of micro-organisms including viruses, bacteria, and parasites have been effectively inactivated by the photosensitization of riboflavin using UV light. Mirasol™, a commercially available device for the purpose of pathogen inactivation in transfusions, is based on the UV/riboflavin combination [5–12]. Corneal cross-linking is also a procedure that is mediated by ultraviolet light photo-activation of riboflavin, and promising clinical results have been reported in the treatment of keratoconus, pellucid marginal degeneration and corneal ectasia [13–21]. It has been proposed that photosensitization of riboflavin, using ultraviolet light A (UVA) at 365 nm (Collagen Cross-linking), perhaps could be applied as a treatment against infectious keratitis. This is supported by published case reports [22–24] and in vitro experiments [25]. At a time when antibiotic-resistant micro-organisms are becoming an increasing problem [26–36], introduction of a new method to treat corneal infections would be of great benefit. It is crucial to elucidate whether the photo-activation of riboflavin using UVA at 365 nm is a method which is possible to incorporate in the treatment of microbial keratitis. There is to date no published data either on the efficiency of standard treatment protocol for keratoconus

(Collagen Cross-linking) on bacterial growth inhibition, or whether the antibacterial effect is dependent on riboflavin concentration. We present a model to increase the understanding of the antibacterial efficacy of photo-activated riboflavin using UVA.

Materials and methods

Bacterial isolates were received from the microbiological department of Örebro University Hospital, and all bacterial strains were known reference bacteria. Isolates of *Staphylococcus epidermidis* RS 30 (s1) (ATCC 12228), *Staphylococcus aureus* RS 12 (s1) (CCUG 15915, ATCC 29213) and *Pseudomonas aeruginosa* RS 11 (s1) (CCUG 17619, ATCC 27853) were chosen for these experiments.

The bacteria were cultured on blood/hematin–agar plates, and the strains dispersed in PBS (GIBCO no: 14190, Invitrogen) to a concentration of approximately 10^8/ml. Counting of microbes was performed in a Burker chamber. Dilution of the above in PBS was made to reach concentrations of bacteria in stock solutions 2.0×10^5/ml (*S. epidermidis*), 3.5×10^5/ml (*S. aureus*) and 5.0×10^5/ml (*P. aeruginosa*). During the test period, the solutions were kept at room temperature (approximately 6 hours). Uncolored fluids were chosen in order to avoid disturbance or bias of pigment from broth in the photo-activation procedure. Dispersion was done of 10 μl of bacterial stock-solutions in 90 μl of riboflavin (No: R7649-25G, Sigma-Aldrich) in RPMI (GIBCO no: 11835, Invitrogen). This was done to achieve a bacterial concentration of $1\text{-}4\times10^4$/ml and riboflavin end molarities before illumination of 100, 200, 300 and 400 μM, in order to obtain a trend where eradication was most efficient. The addition of riboflavin was performed just before illumination with UV-light.

The total volume of fluid in the vessel was 100 μl, sufficient to maintain a thin layer of bacteria during 60 minutes incubation. The vessel chosen for the experiments were the inside of sterile corks from Sarstedt laboratory test tubes (No: 60.540.012, Sarstedt, Newton, NC, USA). These are white in color, and were selected to minimize light absorption during illumination. The inside diameter of the vessels was 8.5 mm and did not exceed the 9 mm spot, enabling illumination of the whole surface of solution.

For every preparation of riboflavin/bacteria solution, a non-illuminated control vessel was prepared. Each test series also had an illuminated control vessel with preparation of solution containing a similar bacterial end concentration without riboflavin. This solution without addition of riboflavin also had a negative control.

Prior to each illumination period, preparation for calculation of bacteria in solution and colony-forming units (CFU, 50 μl of diluted sample on each plate) determination was carried out.

The UV source used was UV-X (Peschke Meditrade™, Switzerland) with a wavelength of 365 nm. Calibration of the diode lamp was carried out before each experiment to determine that the value was between 2.7 and 3.3 mW/cm^2. The distance of light source to surface of solution was 5 cm and illumination was conducted in a dark room, to prevent photosensitization of riboflavin from background visible light. After UV illumination for 30 minutes, resulting in a total dose of 5.4 J/cm^2, 10 μl of the solution was extracted and determination of CFU/ml was performed. The remaining 90 μl of solution was illuminated directly after for another 30 minutes, resulting in a total UV dose of 10.8 J/cm^2.

Subsequently all the solutions were diluted in PBS for count of CFU and incubated on agar plates for 18-20 hours at 37 centigrades. The number of CFU (mostly around 50-150 colonies/plate) was counted for each solution as well as the corresponding control solution, and the concentration of bacteria was calculated.

Each experiment was performed completely three times, thus resulting in three measurements at each point. A total of three numbers of CFU were estimated, since values received were either improbable or neglected and this was done to balance statistical analysis. During analysis these values were varied without any major changes in statistical results.

Statistical analysis was executed using a two-way ANOVA with factors UV and riboflavin and their interaction term was calculated. The results from bacterial count at 30 and 60 minutes were analyzed separately. Differences in bacterial number between start and end point (30 and 60 minutes respectively) were calculated at each measurement and used as outcome variable in the ANOVA. Comparison between solutions with and without riboflavin was carried out and statistical significance calculated. An unpaired *t*-test was done at each measured molarity comparing the differences between UV-exposed/unexposed solutions at end-point. Significance level was set to 5%. Software used for analysis was SPSS® 15.0 (SPSS Inc. Chicago, IL, USA).

Results

In the first experiment (Figs. 1 and 2) preparations were made with solutions of *Staphylococcus epidermidis* RS 30 (s1) (ATCC 12228) into start concentrations ranging between 1.6 and 2.5×10^4/ml. After 30 minutes non-illuminated solutions showed no clear tendency towards reduction in bacterial number. When exposed to UV, a slightly larger reduction was observed in all solutions. No significant differences were seen between vessels containing riboflavin and the simply UV-exposed vessels.

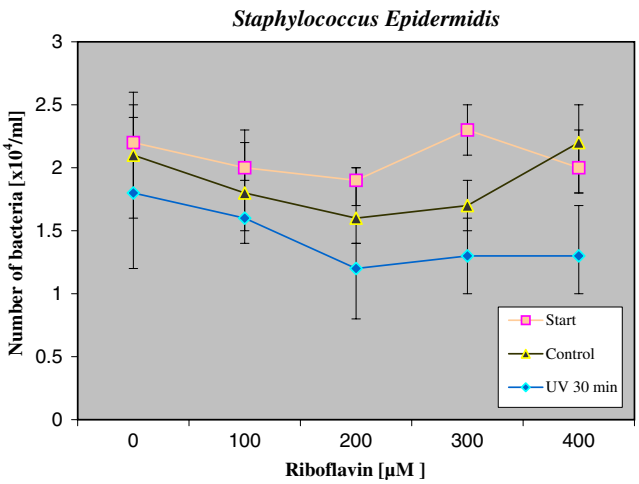

Fig. 1 Staphylococcus epidermidis

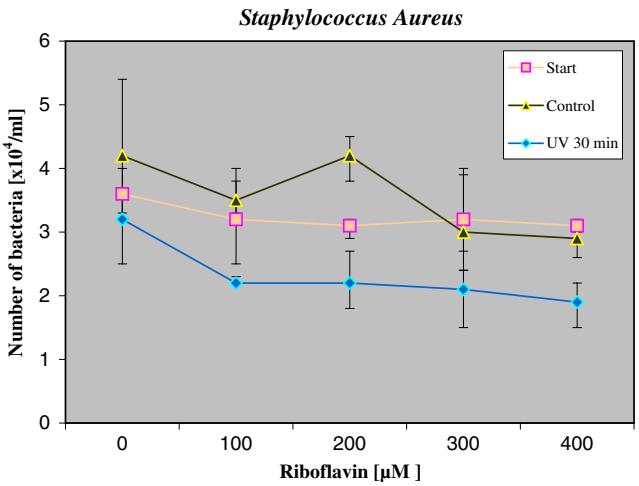

Fig. 3 Staphylococcus aureus

The decrease in bacterial count after 60 minutes of storage in RPMI was at all riboflavin concentrations similar to the one found after 30 minutes. UVA illumination without riboflavin was comparable to loss of bacteria from storage in RPMI (negative control), i.e. 27 and 23 percent respectively. The vessels containing riboflavin showed a clear reduction in bacterial count (mean reduction from start point ranging between 86 and 90%). No clear variation was seen at the diverse riboflavin levels. At all measured molarities, the differences between the riboflavin-absent solutions (simply UV-exposed) were statistically significant ($p<0.001$). *T*-tests at each riboflavin level between UV-irradiated and negative controls were also statistically significant ($p<0.05$). The *p* value for the interaction term of UV and riboflavin was 0.008.

In the next experiment, preparations with *Staphyloccus aureus* (Figs. 3 and 4) were dispersed to concentrations in final solutions varying between 2.5 and 4.0×10^4/ml. UV exposure for 30 minutes resulted in a decrease of bacteria in all vessels. Mean reduction varied between 11 and 39 percent. *T*-tests with negative controls revealed a statistical significance ($p<0.05$), with the exception of the measurements at the 300 µM riboflavin level ($p=0.056$). There was a tendency towards a higher reduction in the containers with riboflavin; however, this was in no case statistically significant. Again, no obvious pattern was observed regarding whether the extent of riboflavin in dispensers had significance in the eradication of micro-organisms.

As illumination time was doubled, the number of bacteria in solutions containing riboflavin was further reduced, with a tendency towards a slightly higher reduction as molarity of riboflavin increased. The mean eradication of bacteria in the containers without riboflavin compared to the number at the starting point equalled 28%, whereas the decrease in presence of riboflavin ranged between 61 and 71 percent. The reduction compared to negative controls was at all riboflavin levels statistically significant ($p<0.05$). When comparisons were made with

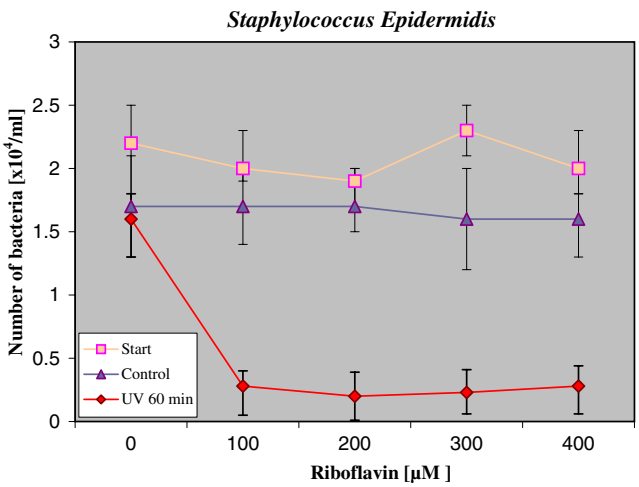

Fig. 2 Staphylococcus epidermidis

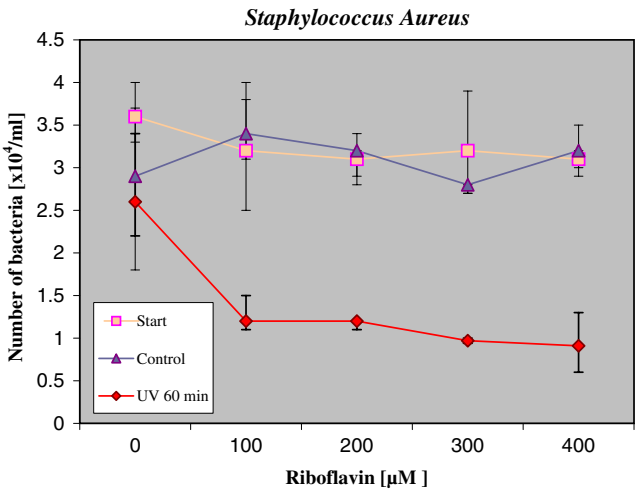

Fig. 4 Staphylococcus aureus

riboflavin-absent solutions, illuminated with UV, statistical significance was seen at 300 and 400 μM (p values 0.018 and 0.021 respectively) and the p value for the interaction term between UV and riboflavin was 0.038.

For the final experiment (Figs. 5 and 6), solutions were prepared with start concentrations of *Pseudomonas aeruginosa* between 9.4×10^3/ml and 3.1×10^4/ml. At evaluation after both 30 and 60 minutes, UV exposure alone showed no further reduction than control solutions. The mean CFU count even somewhat exceeded the one found in negative controls, yet the differences were not statistically significant at either of these points. When subjected to UV for 30 minutes, in presence of riboflavin, a decrease of bacteria was seen at all molarities, ranging between 39 and 53 percent; however, t-tests showed a statistical significance only at the 300 μM level ($p<0.05$).

All the solutions containing riboflavin showed after 60 minutes illumination a nearly complete eradication of micro-organisms, averaging between 98 and 100 percent. Storage of bacteria in presence of riboflavin presented a slightly variable pattern, but no additional reduction in bacterial number was observed when compared to the 30-minutes reading. The differences compared to non-illuminated controls were statistically significant at the 100 and 400 μM readings ($p<0.05$) and bordering statistical significance at the 200 and 300 levels ($p=0.051$ and $p=0.054$ respectively). The ANOVA showed statistical significance at all levels containing riboflavin when compared to the riboflavin-absent solutions ($p<0.05$). The interaction term was also statistically significant ($p=0.034$).

Discussion

The results of our in vitro experiments clearly show that photo-activation of riboflavin using UVA at 365 nm achieves eradication of all three tested bacteria. An augmentation of UV exposure time increased this effect in all tested strains. It is also evident that the combination of riboflavin and UV was considerably more efficient in reducing micro-organisms than UV single-handedly. A dose of 10.8 J/cm^2 (or UV exposure for 60 minutes) resulted in a high degree of eradication of each bacterium. Doubling the length of UV irradiation led to a response of greater magnitude than variations in riboflavin molarity. This might be explained by the fact that an increase in the UV dose should also amplify the amount of oxidative stress generated; however, an augmentation in riboflavin concentration does not necessarily do the same. Perhaps only a small amount of riboflavin is required to achieve the effect studied, and excess riboflavin may even block UV penetration into deeper layers of the fluid solution.

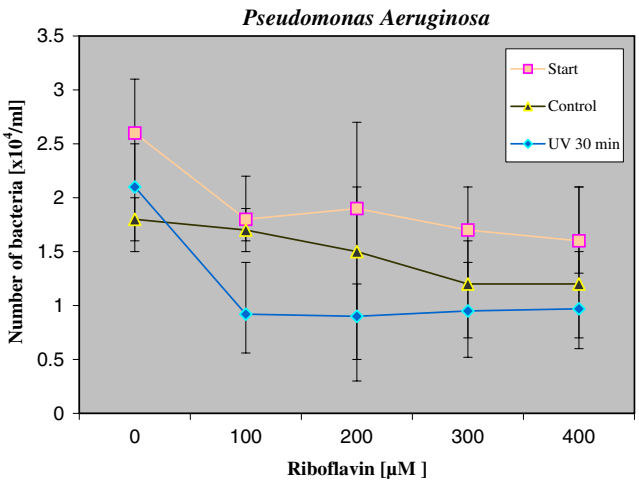

Fig. 5 Pseudomonas aeruginosa

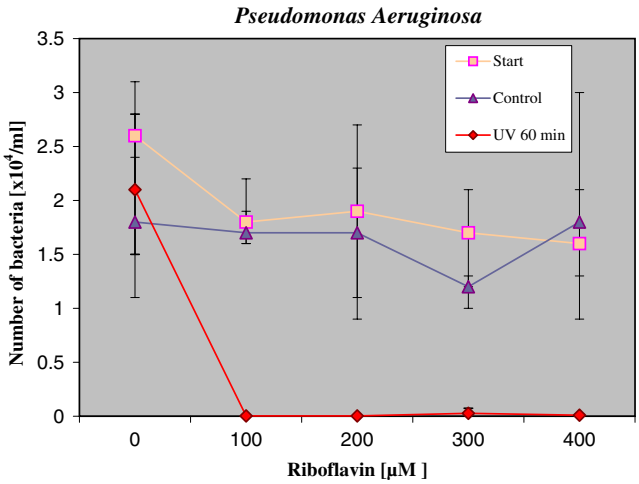

Fig. 6 Pseudomonas aeruginosa

Interestingly, the bacteria tested showed a rather heterogeneous response to the procedure, indicating a variation in sensibility between different micro-organisms. Perhaps diversities in metabolism and sensitivity to oxidative stress could explain the differences in sensitivity between the tested bacteria. Several factors could be of importance for these differences. The length of cell cycle could be of significance, since a shorter cell cycle would increase the possibility for exposure during cell division, leading to a higher risk of errors in genetic material of micro-organisms, followed by mutations and cell death. Cell wall structure, intracellular transport systems and metabolic pathways are other factors that may well influence sensitivity to oxidative stress.

These results support the observations of Martins et al. [25] that the photo-sensitization of riboflavin seems to have an efficient antimicrobial effect, especially if the dose of UV is increased.

It is therefore a future possibility that this procedure could be developed into a treatment option in handling infectious keratitis. This could conceivably be an alternative, but more likely a complement to the topical antibiotic therapy. A new mechanism of action against pathogens would be of great benefit and maybe a considerable improvement in the treatment of microbial keratitis. Antibiotic-resistant bacterial strains could also be of less significance when using photo-activation procedures.

Incubation time for 30 minutes resulted in only a limited bacterial eradication in this model, signifying that the settings used in treatment of keratoconus might not be sufficient for complete sterilization of an infectious ulcer during a keratitis. The layer of fluid in our model may also in part explain a retardation of the UV penetration in the solution, resulting in a reduced effect of the treatment. A decrease of the fluid layer thickness would increase the UV penetration in the whole fluid layer, possibly increasing the eradicating effect. Interfacial tension and fluid evaporation are two factors making it very difficult to use a small enough volume of bacterial solution in order to enable UV penetration in the whole fluid thickness.

The model described presents an opportunity to study the effect of the UV–riboflavin combination in multiple micro-organisms, as well as determining the importance of riboflavin concentration for each tested strain. The outcome observed in these experiments at different levels of riboflavin did not show clear differences in efficacy. However, it is important to realize that the different molarities translate only to minute variations in riboflavin concentration. It would naturally be of interest to investigate exactly at which riboflavin level the peak eradication is located; however, this is beyond the scope of this particular work.

The antimicrobial effect of riboflavin photosensitization using UVA should be further explored, as it might have the potential to be incorporated into the therapy of infectious conditions, such as keratitis.

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