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Comparative irradiation experiments on porcine corneal endothelial cells at 222 nm and 254 nm

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Abstract: Corneal diseases or injuries often result in vision loss, necessitating corneal transplantation. Donor corneas must meet strict quality standards, including sterility. To reduce annual corneal losses due to unintended contaminations, disinfection attempts using 222 nm irradiation were conducted and compared to 254 nm exposure. 254 nm is known for its antimicrobial impact but also for its danger to human cells, including corneal endothelial cells, while 222 nm is considered safe for human tissue, though it also exhibits high effectiveness against most microorganisms. In a laboratory study, porcine corneas were irradiated on the endothelial side with 150 mJ/cm² at 222 nm and 254 nm. Endothelial cells were counted after incubation in culture medium, salt solution, or demineralized water. The results were statistically analyzed and compared. For incubation periods up to 24 hours in culture medium, no significant differences ($p = 0.715$) between the applied wavelengths were observed. However, when demineralized water was used, both wavelengths induced cellular damage and a decline in cell density. Control samples revealed no detectable changes. The experiments demonstrated that porcine endothelial cells respond differently to the stress factor demineralized water compared to human endothelial cells. Additionally, photonic exposure combined with sufficient stress led to a reduction in cell density. However, it remains unclear how cell density would develop over several days without additional stress or whether a higher dose at 254 nm would yield different outcomes.

Keywords: cornea irradiation, porcine cornea, transplant, tissue irradiation, 222 nm Far-UVC, 254 nm UVC

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1 Introduction

Vision is one of the most important human senses, enabling the visual perception of the environment. The cornea plays a crucial role in this process by serving as a protective barrier and being responsible for light refraction [1]. Corneal diseases, such as bacterial keratitis, keratoconus, or corneal dystrophies, can compromise corneal integrity and, in severe cases, lead to vision loss [2]. Particularly important for corneal clarity are the sensitive, non-regenerative endothelial cells, which pump fluid out of the cornea against the pressure gradient of the anterior chamber [3].

In order to restore vision, keratoplasty (cornea transplantation) is frequently performed. This procedure represents the most successful transplantation method worldwide and can be conducted as either penetrating or selective lamellar keratoplasty. However, due to the increasing demand for donor corneas, there is a shortage of suitable tissue donations. To ensure the integrity of the transplant, strict quality criteria are essential, as microbial contamination is a common cause of corneal rejection or the need for additional treatments [4,5].

A promising approach to reducing microbial contamination is Far-UVC irradiation in the spectral range of 200 – 230 nm. While conventional UVC radiation at 254 nm penetrates deeply into tissue (Figure 1a) and may cause potential damage, Far-UVC radiation at 222 nm is considered safer, as it penetrates less deeply while still effectively inactivating microorganisms [6,7].

Preliminary studies on corneal epithelial cells from both humans and animals have demonstrated that Far-UVC irradiation does not result in significant damage or alterations in structural integrity [6,8,9]. The present study investigates the effects of Far-UVC irradiation on corneal endothelial cells in comparison to 254 nm irradiation, with a view to rendering contaminated corneas suitable for transplantation. Porcine corneas (pCornea) are used for irradiation experiments to assess differences in cell density. The non-regenerative nature

of endothelial cells, which is similar in pigs and humans, is used to ascertain the number of endothelial cells remaining after irradiation. The underlying principle is that, upon perishing, non-regenerative endothelial cells are spatially replaced by the expansion of adjacent cells. This results in a decrease in cell density, while maintaining endothelial integrity [10]. The findings from this study aim to contribute to the development of new methods to improve the availability of corneal transplants.

2 Material and Methods

2.1 Irradiation Setup

For the irradiation of the endothelial cell layer of porcine corneas (Figure 1b), the samples were placed in Petri dishes under dry conditions, without the addition of liquid media. Irradiation was performed using a 222 nm Ushio Care 222 B1 Far-UVC lamp (Ushio, Tokyo, Japan) and a 254 nm mercury vapor lamp type 3UVTM-36 (Analytik Jena, Jena, Germany). Both lamps were switched on several minutes prior to the start of the irradiation to ensure stable radiation output.

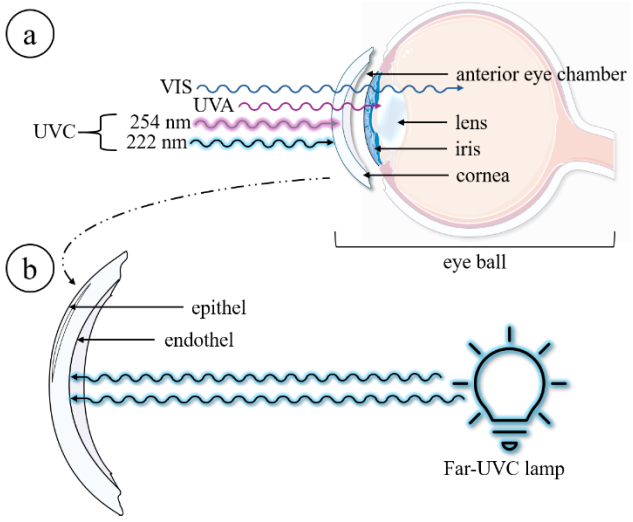

Figure 1: Irradiation setup with schematic representation of the eyeball and penetration depths of different wavelengths (a) and functional irradiation setup with irradiation on endothelial cells with Far-UVC (b). Figure 1 was created in part with Servier Medical Art and is licensed under CC BY 4.0. (Far-UVC: 200 – 230 nm; UVC: 100 – 280 nm; UVB: 280 – 315 nm, UVA: 315 – 400 nm; VIS: visible light)

The irradiance Ee was measured using an optometer type X1-5 in combination with a UV-3727 UV detector, both of Gigahertz Optik (Gigahertz Optik, Türkenfeld, Germany). To achieve an irradiance of 1 mW/cm², the distance between the lamps and the samples was adjusted using a laboratory lifting platform.

Following this adjustment, four pCorneas per Petri dish were positioned beneath the respective UVC or Far-UVC lamp for a period of 150 seconds. The exposure time was measured using a stopwatch. The resulting irradiation dose was calculated as 150 mJ/cm².

2.2 Design of experiments

In a preliminary experiment to validate the countability of cells after irradiation, DAPI staining (4′,6-diamidino-2-phenylindole, dilactate) (Invitrogen, Carlsbad, CA, USA) was evaluated as a suitable staining method. For this purpose, comparative tests were conducted, and cell densities obtained by DAPI staining were compared with those obtained using Live/Dead staining from the Viability/Cell Toxicity Assay Kit for Animal Live & Dead Cells (Biotium, Fremont, CA, USA).

A further experiment tested whether pCorneas stored in PBS (phosphate-buffered saline) change cell density after seven days. The pCorneas were stored at 4 °C, to prevent swelling, for seven days before analysis.

In all experimental trials, three different test groups were examined and compared:

- Non-irradiated porcine corneas served as control samples (non-irradiated, nirr).
- Porcine corneas irradiated with 222 nm at a radiation dose of 150 mJ/cm².
- Porcine corneas irradiated with 254 nm at a radiation dose of 150 mJ/cm².

The storage period of the porcine corneas was divided into four-hour intervals. The main factors analysed were wavelength and storage duration. To enhance the manifestation of actinic damage, storage was conducted in demineralised H_2O (H_2O DM) instead of PBS. The sequence of irradiation and storage duration was randomized to minimise bias. Corneal batches were consistently used across different sample groups to minimise systematic errors as far as possible.

For the investigation of extreme irradiation conditions, three porcine corneas were irradiated with an energy dose of 1500 mJ/cm². After a relaxation phase of approximately one hour in PBS, the endothelial cells were analysed.

2.3 Cell counting and statistics

To determine cell density, the irradiated corneas were trephined, and the respective sections were stained using DAPI

staining according to the manufacturer's instructions. Multiple images of each corneal section were captured using the Eclipse TE2000-U fluorescence microscope (Nikon, Gyoda, Japan). Following microscopy, the endothelial cell count was determined by counting the remaining cells within a defined area to calculate cell density.

For this purpose, DAPI-stained images captured with a 40× objective and a Nikon D5600 camera (Nikon, Gyoda, Japan) were analysed. The software ImageJ 1.53o [11] was calibrated using a Neubauer counting chamber to establish a reference length. A grid with an area of 0.005 mm² was overlaid on the image, and cell counting was performed manually using the Cell Counter plug-in [12]. For each cornea, two images containing four grids each were analysed. The average cell count was then calculated and multiplied by 200 to determine the endothelial cell number per mm².

The Kruskal-Wallis test and t-test were used for statistical analysis, as these non-parametric methods allow the comparison of mean values.

3 Results

The preliminary experiment demonstrated the applicability of DAPI staining for cell counting (Figure 2). In the irradiation experiments, the staining methods were validated after longer incubation times using live/dead staining and their application was checked considering the error rate, which corresponds to the standard deviation (SD).

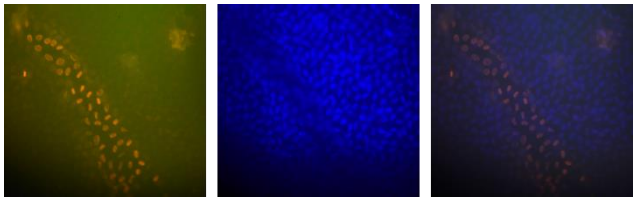

Figure 2: Images of live/dead (left) and DAPI stain (center) and a combination (right) of both to demonstrate countability.

The results of the storage test in PBS after seven days showed no significant change in endothelial cell density (3558 ± 189). The mean density of the reference value nirr-sample without storage pCorneas was considered equivalent to that of the seven days nirr-sample ($p = 0.099$).

Furthermore, no substantial alterations in epithelial cell density were demonstrated by nirr-samples pCorneas ($p = 0.715$), irrespective of the duration of storage or the presence of stress factors. Conversely, for irradiated samples stored in demineralized water, a significant decrease in cell density was observed after 16 hours of storage. The data, presented in graphical form, indicate that the cell density of the irradiated samples declined in a similar manner and followed a comparable time course.

The cell densities determined as a function of wavelength and storage duration are listed in Table 1 below. The cell density counts of non-irradiated pCorneas resulted in an average of well over 3500 cells/mm² with a SD of approximately 10%. As demonstrated in Figure 3, outliers and a decrease in the mean value, combined with a significantly increased SD, were only observed in irradiated samples after storage periods of 16 hours or longer.

Table 1: Results of the cell density counts after staining, divided into the irradiation groups and time intervals of storage. These results are presented as "cell density (cells/mm²) ± SD; sample size".

storage interval (h)	cell density non-irradiated (cells/mm²)	cell density 150 mJ/cm² at 222 nm (cells/mm²)	cell density 150 mJ/cm² at 254 nm (cells/mm²)
0 - 4	3779 ± 370; 12	3753 ± 258; 22	3835 ± 285; 18
4 - 8	3644 ± 338; 36	3830 ± 410; 35	3675 ± 427; 36
12 - 16	3680 ± 404; 23	4155 ± 919; 30	3522 ± 572; 24
16 - 20	3652 ± 378; 16	2959 ± 1464; 23	2414 ± 1146; 21
20 - 24	3575 ± 377; 90	1784 ± 1081; 11	2030 ± 1122; 11

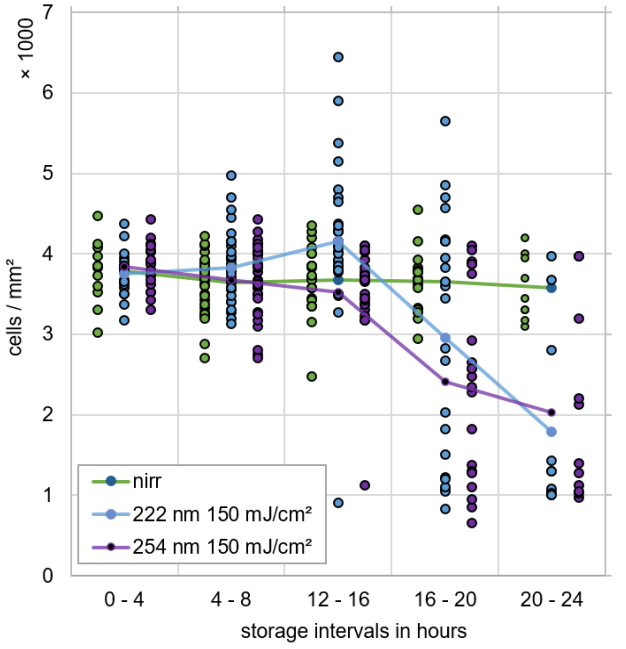

Figure 3: Endothelial cell concentrations grouped in timeslots with average regression line of the cell density in cells/mm².

Experiments involving increased exposure levels of 1500 mJ/cm² at 222 nm did not demonstrate any anomalous distribution of the stained nuclei. The study's sample

comprised three corneas, and the 3646 ± 335 cells/mm² measurement indicated no significant deviation from the nirr-sample without storage and 150 mJ/cm² at 222 nm irradiation sample without storage ($p = 0.614$). Furthermore, live/dead staining revealed no evidence of actinic reduction of the structural integrity of the endothelium.

4 Discussion

The study revealed that porcine corneas exhibited a high degree of resistance to stress. Furthermore, it was observed that cell densities remained fully quantifiable for several hours following irradiation. Although dead cells are also indicated by the staining, in the case of non-reproducible endothelial cells, these dead cells are displaced by intact cells and are therefore no longer detectable. This phenomenon introduces an element of inaccuracy into the method.

Despite the evident decrease in sensitive response to irradiation and stress exhibited by porcine corneas, the application of stress through irradiation and H_2O DM over time resulted in a substantial decline in cell density, particularly evident in the 222 nm and 254 nm samples. The desired clear effect in favour of one wavelength did not occur. Further investigations are required to elucidate the damage rate, for example by means of CPD staining or with a more effective factor, in order to better crystallize possible differences. The significantly higher exposure levels of up to 1500 mJ/cm² did not result in any detectable damage to the sample. The cell densities determined are consistent with the cell densities of other samples, which indicates that there was no indirect reaction. However, the long-lasting effect of death with the otherwise usually very harmful UVC radiation at 254 nm and the apparently similar effect of Far-UVC radiation, which should actually be less harmful, indicate that the main effect is not the irradiation but the storage in H_2O DM. These findings suggest that storage in H_2O DM is the primary factor contributing to the observed effects.

It is noteworthy that following relaxation periods of up to 16 hours, no discernible effect was observed on either wavelength. Further investigation, e.g., by CPD (cyclobutane pyrimidine dimer) staining, would be beneficial.

Author Statement

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