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CORNEA

Crosslinking and corneal cryotherapy in acanthamoeba keratitis — a histological study

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Abstract
Purpose Acanthamoeba keratitis is rare, but difficult to treat. Penetrating keratoplasty is performed in therapy-resistant cases. Nevertheless, subsequent recurrences occur in 40 % of the cases. In addition to triple-topical therapy (polyhexamid, propamidinisoethionat, neomycin), treatment alternatives are corneal cryotherapy and/or crosslinking (CXL). The aim of our present histological study was to analyze the persistence of acanthamoebatrophozoites and cysts, the persistence of bacteria, and activation of keratocytes in corneas of acanthamoeba keratitis patients following corneal cryotherapy and/or CXL.
Patients and methods We analyzed histologically corneal buttons (from penetrating keratoplasties) of nine patients with acanthamoeba keratitis, following corneal cryotherapy (two patients) or a combination of crosslinking and corneal cryotherapy (seven patients), using haematoxilin–eosin, periodic acid Schiff (PAS), Gram and alpha-smooth muscle actin (alpha-SMA) stainings.
Results Acanthamoeba trophozoites persisted in three corneas after cryotherapy and CXL. Cysts persisted in one of two corneas following corneal cryotherapy and in six of seven corneas after a combination of CXL and cryotherapy. One cornea showed positive Gram staining, but there were no alpha-SMA positive keratocytes in any of the corneas.
Conclusions Crosslinking and corneal cryotherapy have only limited impact on killing of acanthamoeba trophozoites, cysts, or bacteria. Corneal cryotherapy and CXL did not stimulate myofibroblastic transformation of keratocytes.

Keywords Acanthamoeba · Keratoplasty · Crosslinking · Cryotherapy

Introduction

The incidence of acanthamoeba keratitis is 1 per 30,000 contact lens wearers [1]. Acanthamoeba has two forms: a metabolically active trophozoite, and the inactive cyst which is present under adverse living conditions [2]. Topical triple therapy of acanthamoeba keratitis includes diamidins, biguanids, and neomycin. Diamidins kill acanthamoeba cysts and trophozoites [3]. Biguanids inhibit the respiratory enzyme of acanthamoeba [4], and neomycin kills trophozoites and bacteria as a nutritional source [5].Even with this triple therapy, treatment may take 6 months or more, but 90 % of patients can expect to retain visual acuity of 6/12 or better [6].Topical biguanides are the only effective therapy for the resistant encysted form of the organism in vitro [6]. But acanthamoeba cysts may be resistant against topical conservative therapy [7]. Although primarily a corneal disease, acanthamoeba can expand extracorneally and initiate a sclerokeratitis which can end up with loss of the eye [8].

Therefore, in therapy-resistant cases, therapeutic keratoplasty might be necessary to preserve the eye [9]. Another problem is the fact that correct diagnosis is often delayed by up to 1 year [10]. In a study on keratoplasty in 50 patients suffering from acanthamoeba keratitis, 37 were initially misdiagnosed as having herpes simplex keratitis [11].

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Nevertheless, recurrence of acanthamoeba keratitis occurs in 41 % of cases after penetrating keratoplasty [12]. To reduce microbial load of the cornea, corneal cryotherapy was introduced [13]. Moreover, some authors reported a dose-dependent inhibition of trophozoites and cysts using photodynamic therapy [14, 15]. Other authors were not able to reproduce these data [16]. Khan et al. reported three cases with therapy-resistant acanthamoeba keratitis successfully treated by corneal crosslinking [17].

The purpose of our present histological study was to analyze the persistence of acanthamoeba trophozoites and cysts, the persistence of bacteria, and activation of keratocytes in the cornea of acanthamoeba keratitis patients following corneal cryotherapy and/or crosslinking (CXL).

Patients and methods

We included nine corneas of nine patients (mean age: 36.9± 12.5 years) in our study. All patients gave their informed consent to participate in this study. Patients' data before keratoplasty are displayed in Table 1. Four of the patients presented with ring of Wessely and five with non-transparent corneal infiltrates or scars; two of them had both ring of Wessely and corneal infiltrates/scar. Acanthamoeba keratitis was diagnosed using polymerase chain reaction (PCR) (six eyes) or diagnostic keratectomy (histological analysis) (four eyes). The time between first ocular symptoms (treated not at our Institution) and diagnosis of acanthamoeba keratitis at our department was 6.1±10.3 months. The subgroups of different acanthamoeba species were not further analyzed by the microbiological or histological departments.

All patients received topical triple-therapy (polyhexamid, propamidinisoethionat, neomycin) from the time of diagnosis to the moment of penetrating keratoplasty.

In all analyzed eyes, overlapping corneal cryotherapy was used circularly (−80 °C for 2–3 seconds) at the planned trephination margin directly before corneal trephination (penetrating keratoplasty).

Seven of these eyes underwent crosslinking therapy [18] using the CCL 365 System (Peschke, Waldshut–Tiengen, Germany) 3–58 days before penetrating keratoplasty. We used 0.1 % riboflavin eye drops (Mediocross, PeschkeGmbh, Huenenberg, Switzerland) every 2–3 minutes for 30 minutes during crosslinking therapy, following total corneal abrasion. While we further applied 0.1 % riboflavin eye drops every minute, a 5-minute (patients 3 and 9; 18 mW) or 30-minute (patients 4–8; 108 mW) UVA illumination (5400 mJ or 32400 mJ) with 11.5 mm diameter was used.

Of the seven patients with CXL therapy, six underwent CXL 3 to 7 days (patients 4–9) before penetrating keratoplasty (PKP) [19] and one patient 58 days before PKP (patient 3). The central corneal thickness (PENTACAM, Oculus, Wetzlar, Germany) of these seven patients ranged from 515 to 985 μm (median 642.3 μm ± 169.7 μm) before CXL.

Histological analysis

The excised corneal buttons of all nine patients were analyzed histologically as follows:

After formaline-fixation and paraffin wax-embedding of the patients' corneal buttons, sections of 3 μm thickness were cut using a standard microtome and transferred onto microscope slides (SuperFrost, Menzel–Gläser, Braunschweig, Germany). We performed serial sections to rule out affection of the corneal margin. The slides were dried at 37 °C overnight in the incubator. Standard haematoxylin–eosin, periodic acid Schiff (PAS) and Gram stainings were performed. Immunohistochemistry was performed on a tissue stainer (BenchMark ULTRA, Ventana Medical Systems, AZ, USA.) according to standard protocols for detection of alpha-smooth muscle actin (monoclonal antibody, Dako, Hamburg, Germany).

Using PAS staining, we analyzed presence/absence of trophozoites or cysts in our corneal buttons. If cysts or

Table 1 Results of PAS and Gram stainings in acanthamoeba keratitis patients after corneal cryotherapy (patients 1–2) and after corneal crosslinking (CXL) and cryotherapy (patients 3–9)

Patient number	CXL beforekeratoplasty	Central/maximal corneal thickness before CXL (μm)	Trophozoites in PAS	Cysts in PAS	Gram staining
1	−	Not measurable	−	−	−
2	−	532	+	+	−
3	+	515	−	−	−
4	+	529	−	+	−
5	+	985	+	+	−
6	+	598	−	+	−
7	+	742	−	+	+
8	+	595	+	+	−
9	+	Not measurable	−	+	−

The results of periodic acid Schiff (PAS) and Gram stainings are indicated as "+" if positive or "–" if negative

trophozoites were present, they were photodocumented at 40x magnification. Their distance to the margin was measured using caliper software.Additionally, the distance of the trophozoites/cysts in the corneal center from the corneal surface and from the endothelium, and their distance from the incision margins was also measured using caliper software.

Results

Data of the analysed patients are summarised in Table 1.

From the two patients who received cryotherapy only, there were acanthamoeba cysts and trophozoitesin; the central cornea of one patient was without presence of cysts or trophozoites at the corneal margins.

In one patient 58 days after CXL and cryotherapy, neither cysts nor trophozoites could be detected. However, PAS staining revealed persistence of cysts in six patients after CXL and cryotherapy, and the additional presence of trophozoites in two patients (Fig. 1). The distance between corneal surface and cysts was 6.1–297.6 μm (median 37.2 μm) and between corneal endothelium and deepest stromal cyst 55–428 μm (median 192.6 μm). Three patients had acanthamoeba cysts up to the peripheral margin of the explant.Trophozoites could not be detected in these patients. The distance of acanthamoeba cysts from the trephination margins was 11.4–37.2 μm (median 20.7 μm).

Gram staining was positive in one single patient following CXL and cryotherapy. There were no alpha-smooth muscle actin-positive corneas in our samples (Fig. 2).

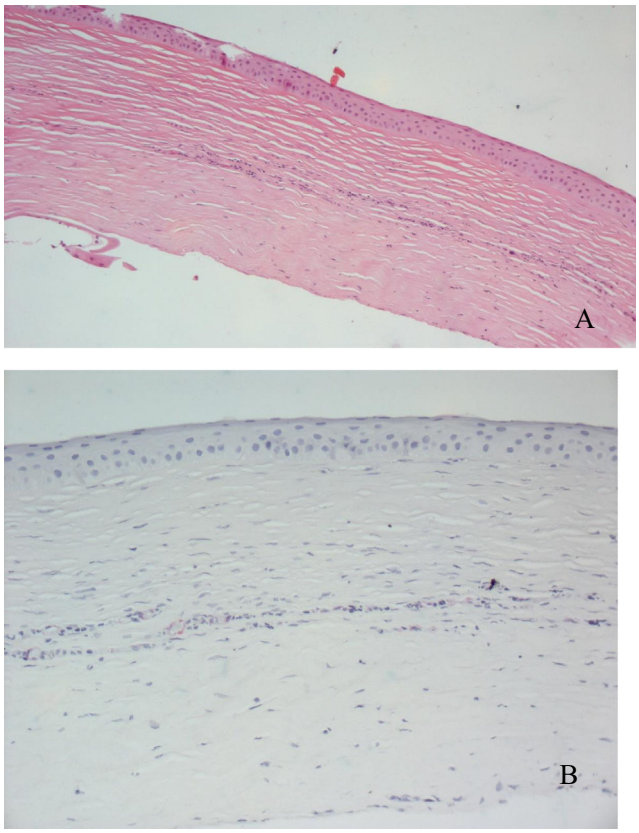

Fig. 2 Haematoxilin–eosin (**a**) and alpha-smooth actin (**b**) stainings of the cornea 6 weeks after corneal crosslinking and after cryotherapy (patient 3). There were no acanthamoeba cysts or trophozoites in the cornea (**a**).There were no alpha smooth muscle actin-positive cells (**b**). Original magnification 10× (**a**) and 20× (**b**)

Discussion

The most conspicuous finding of our study is that acanthamoeba cysts and trophozoites persist in the cornea following crosslinking and/or corneal cryotherapy.We discussed two different therapeutical approaches with the three patients in whom the histological examination showed persisting cysts in the marginal excised cornea: re-penetrating keratoplasty with larger diameter to try further reduction of cysts, or topical triple therapy for 6 months. All patients opted for a continuation of triple therapy.

Using CXL, the photosensitizer riboflavin is first excited through UVA light illumination. Following relaxation, free oxygen radicals and singlet oxygen are produced, and may

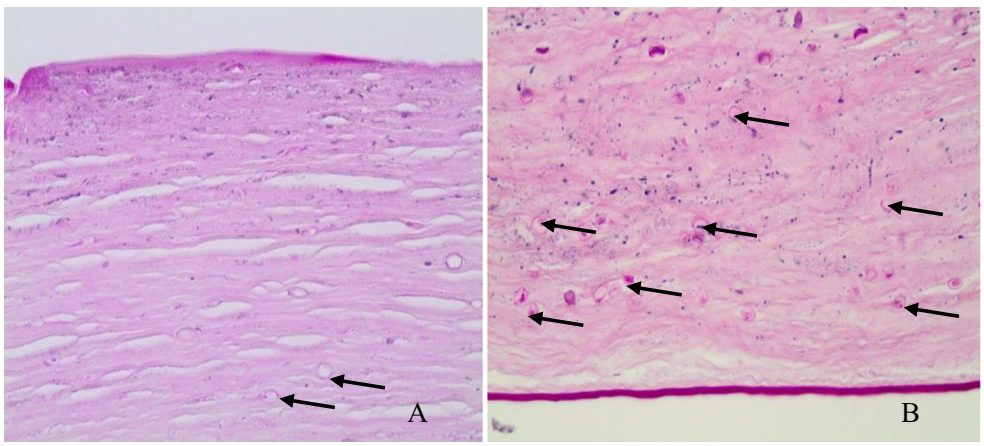

Fig. 1 PAS staining of the corneal stroma following corneal crosslinking and cryotherapy. Acanthamoeba cysts are indicated with *arrows*. **a** Patient no. 4: The corneal epithelium is absent due to prior corneal crosslinking. **b** Patient no. 8: acanthamoeba cysts can reach close to the endothelium. Original magnification 40×

cause damage of acanthamoeba cysts and trophozoites. The diffusion of riboflavin into the cornea is time-dependent. Highest concentration is reached in the anterior 100 μm of the cornea after 30 minutes [20]. However, we detected acanthamoebatrophozoites and cysts not only in the posterior, but also within the first 100 μm of the anterior stroma after CXL. Therefore, our results do not support the hypothesis that CXL and/or corneal cryotherapy are beneficial in destruction of acanthamoeba trophozoites or cysts. The fact that we found cysts close to Descemet's membrane prevents us from performing a deep lamellar keratoplasty in these eyes.

Due to tissue shrinking during embedding and staining of the corneal buttons, the values given for the distances of cysts to the corneal epithelium and endothelium represent only an approximation, and cannot be understood as real and representative values. An additional limitation is that viability of the remaining acanthamoeba cysts could not be analyzed.

Another limitation of our study is that we cannot separate the effect of corneal cryotherapy and crosslinking in our study. The killing effect of corneal cryotherapy and crosslinking may even be additive at the corneal interface in the case of application of both techniques. A study with a higher number of acanthamoeba keratitis patients with cryotherapy only, crosslinking only, or a combination of both techniques prior to keratoplasty would give additional information.

Corneal infiltrates/scars may inhibit penetration of UVA-light during CXL to the cornea. Most interestingly, patient 8 without corneal infiltrates/scars or ring of Wessely was positive for acanthamoeba cysts and trophozoites after treatment. Presence of trophozoites and cysts could also be shown in patient 3 with ring of Wessely only, and in patient 6 with ring of Wessely only we were also able to detect acanthamoeba cysts. Properties and protective mechanisms of different acanthamoeba subspecies should be further analyzed.

Bacteria are known to be a nutritional source for acanthamoeba [5]. Some authors describe healing of bacterial keratitis after crosslinking without the use of topical antibiotics [9]. We found one patient with positive Gram staining following CXL and cryotherapy. Therefore, in our opinion, CXL is not effective in elimination of all bacteria causing keratitis — at least in our case series.Nevertheless, we have to remark that the effect of CXL in this single patient has been limited due to corneal infiltrates/scars which inhibited penetration of UVA-light to the cornea during treatment.

Interestingly, myofibroblasts (alpha-smooth muscle actin positivity) could not be detected after CXL and/or cryotherapy in the analysed corneal buttons. This is in accordance with a previous study by Messmer et al. [21] who could not verify alpha-smooth actin-positive keratocytes in the cornea 5 to 30 months after CXL, and demonstrated a decreased number of keratocytes in the entire cornea. In addition, our previous experiments on keratocyte cell cultures have also shown decreased alpha-smooth actin expression of keratocytes parallel to an increased CD34 marker positivity 24 hours after photodynamic therapy [22].

Free oxygen radicals and singlet oxygen have a short half-life-time; therefore, their effect on acanthamoeba cysts or trophozoites must happen promptly after CXL therapy. In our opinion, killing of acanthamoeba cysts and trophozoites happens before the 3rd day after CXL, and we should not expect a further killing effect of the reactive oxygen species. This is why the auhors performed penetrating keratoplasty even 3 days after CXL. However, the activated multipotenshaematopoetic stem cells could have an impact on the microorganisms, even over a longer time-period. This should be analysed in further prospective studies.

The fact that the only cornea free from cysts and trophozoites was the one with penetrating keratoplasty 58 days after CXL could show us the importance and positive effect of the longer-term triple topical therapy in treatment of acanthamoeba keratitis. Nevertheless, patient 2 following 4-month-long triple topical therapy and corneal cryotherapy still had cysts and trophozoites in the cornea. Patient 5 following 3-month-long and patient 7 after 32-month-long triple topical therapy also had cysts in the cornea following a combination of corneal CXL and cryotherapy. Nevertheless, patient 7 was free from trophozoites.

In contrast, we also found trophozoites and cysts in the cornea of patient 8, who received an early diagnosis (9 days after first symptoms) and early triple topical therapy of acanthamoeba keratitis. In our opinion, further prospective studies with a higher number of patients should even more effectively analyze the effect of topical steroid use parallel to triple topical therapy, and the decisive protective or microorganism growth supporting properties of the different acanthamoeba subspecies causing keratitis.The use of topical steroids is controversial, but probably beneficial for the management of severe corneal inflammatory complications that have not responded to topical biguanides alone [6]. Robaei et al. found out in a large cohort study on 196 patients that corticosteroid use before diagnosis of acanthamoeba keratitis is highly predictive of poorer visual outcome [11].

In conclusion, CXL and corneal cryotherapy are limited in killing acanthamoebatrophozoites, cysts or bacteria. Corneal cryotherapy and crosslinking did not stimulate myofibroblastic transformation of keratocytes.

Compliance with ethical standards

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Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing

arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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