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REVIEW

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Corneal cross-linking (CXL)—A clinical study to evaluate CXL as a treatment in comparison with medical treatment for ulcerative keratitis in horses

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Abstract

Objective: Compare CXL treatment with medical treatment alone in horses with stromal, ulcerative keratitis. Animals studied: 24 horses (24 eyes) with stromal, ulcerative keratitis were included.

Procedure: 12 horses were initially treated with CXL, and 12 horses were given conventional medical treatment. Topical medical treatment was added to horses in the CXL group if necessary. Parameters including cytology, microbial growth, time to fluorescein negativity, and time to inhibition of stromal melting were evaluated.

Results: After the first day of treatments, a decrease in inflammatory signs and pain from the eye was observed in both groups. Stromal melting ceased within 24 hours regardless of treatment. CXL treatment alone was sufficient in 3 horses with noninfectious, superficial stromal ulcerations. Clinical signs of impaired wound healing were seen after 3-14 days in corneas with suspected or proven bacterial infection treated with CXL only, most likely because of insufficient elimination of bacteria deeper in the corneal stroma or because of re-infection from bacteria in the conjunctiva. The average decrease in stromal ulcer area per day after onset of treatment was almost identical between the groups, and no significant difference in time to fluorescein negativity was found.

Conclusions: We consider CXL a possible useful adjunct treatment of corneal stromal ulcers in horses, especially for melting ulcers and as a potential alternative to prophylactic antibiotic treatment for noninfected stromal ulcers. However, CXL should not be used alone for infected or suspected infected stromal ulcers, because topical antibiotics were required in all horses with proven infectious keratitis.

KEYWORDS

Corneal ulceration, Cross-linking, Equine, PACK-CXL, Riboflavin, UVA

1 | INTRODUCTION

Ulcerative keratitis is one of the major ophthalmic diseases of the horse.[1,2] Topical medical treatment of the equine patient suffering from stromal, ulcerative keratitis can be challenging, as frequent medication with eye drops to a painful eye requires considerable efforts, even when medication through a lavage system is used. The prognosis for retained vision for horses with infectious ulcerative keratitis depends on infection control and prevention of proteolytic activity.[2] Fulminating corneal breakdown, caused by an infectious agent and/or by release of enzymes from white blood cells, can rapidly result in severe complications.

For superficial noninfected corneal wounds in the horse, the healing time is normally short and epithelial migration is estimated at 0.6 mm/day.[3] Bacterial or fungal ulcerative keratitis can significantly prolong healing times. Wada et al [1] reported a healing time of 6.6 days for assumed uncomplicated ulcers and approximately 39 days for horses with positive bacterial culture. Serious keratopathogens as β-hemolytic Streptococci and Pseudomonas spp., as well as fungal organisms are reported to cause even longer healing times.[2,4-8]

Corneal cross-linking (CXL), usually a single-time, photochemical treatment using riboflavin and ultraviolet A-light (UVA), has been shown to be a feasible and safe adjunct treatment for stromal ulcers in horses.[9] CXL increases the bio-mechanical strength [10] and stabilizes the cornea by forming new cross-links in the corneal stroma between collagen fibers and between proteoglycan core proteins,[11,12] thus making it more resistant to melting and bacterial attack through steric hindrance.[13-15] Hence, it has been used to treat corneal melting in humans, cats, dogs, and horses.[9,16-20]

Laboratory research has demonstrated that UVA in combination with riboflavin can inactivate white blood cells and exert bactericidal effects [21-26] primarily through generation of reactive oxygen species produced in the photochemical reaction.[27-29] Several reports from in vitro studies have confirmed the antibacterial effect of CXL on different pathogens, including antibiotic-resistant bacteria.[21,23,24,26] Clinical studies in human patients have demonstrated that infectious, ulcerative keratitis can be successfully treated with CXL without adjunct topical antibiotics.[30] Therefore, it has been used for treating infectious and noninfectious ulcerative keratitis both in human and veterinary ophthalmology,[9,16-20,31,32] as well as for therapy-resistant keratitis in human patients.[33,34] The term "Photo Active Chromophore for Infectious Keratitis" (PACK-CXL) was introduced in 2013 to distinguish the use of CXL for keratitis from its primary indications for ectatic disorders.[35]

A photodynamic approach has several theoretical advantages compared to topical antibiotics which could facilitate management, reduce complications and allow therapy in antibiotic resistance. However, implementation of CXL as a solitary treatment without antimicrobial agents has not been adequately evaluated. This study aims to investigate if CXL can be used as a stand-alone treatment for corneal stromal ulcers in horses without using topical antibiotics, and if healing time of the cornea can be reduced.

2 | MATERIALS AND METHODS

2.1 | Horses

Twenty-four horses, aged 1 to 27 years (mean 9.3, median 7.5 years) were admitted for treatment of unilateral stromal ulcerative keratitis at two equine hospitals in Sweden. The horses were subdivided into two treatment groups (Tables 1 and 2). One group (12 eyes) was treated with CXL, the other group (12 eyes) served as a control group and was treated with a standard medical, non-CXL protocol.

Horses with very thin corneas in the ulcerated area (estimated corneal thickness less than 400 μm) were excluded from the study. Excluded were also horses with ulcerations secondary to adnexal abnormalities (i.e, trichiasis, eyelid defects) and corneal foreign bodies.

The Regional Ethical Committee (D.nr 246-2010) approved the use of horses for this study and informed consent was obtained from all horse owners prior to treatment.

2.2 | Ophthalmic examination

Ophthalmic examination was carried out according to a standardized protocol and clinical signs were judged according to predetermined criteria. The two examiners (AHE and LS) harmonized their judgments before the study started and then met regularly throughout the study period to follow-up on their assessments.

The horses were sedated with 0.01 mg/kg detomidine (Domodin vet, 10 mg/mL, Novartis Animal Health, Copenhagen, Denmark) and 0.02 mg/kg butorphanol (Butador vet 10 mg/mL, Vetoquinol Scandinavia, Åstorp, Sweden). For akinesia of the eyelids, the auriculopalpebral nerve was blocked with 1 ml mepivacaine subcutaneously (Carbocain, 20 mg/mL, Astra Zeneca, Södertälje, Sweden). For topical anesthesia, two drops of oxibuprocaine (Oxibuprokain Chauvin, 20 mg/mL, Bausch & Lomb Nordic AB, Stockholm, Sweden) were instilled after bacterial sampling.

Sampling for bacterial culture, cytology, and PCR for equine herpesvirus (EHV-2) was accomplished before any other treatments in all horses. Two sites, the corneal ulcer margin, and the conjunctival cul-de-sac were sampled for bacteriology and antimicrobial susceptibility testing (AST) using a sterile swab (ESwab, Copan Innovation Ltd, Brescia, Italy). Fungal culture was requested when indicated by the cytology results. Corneal ulcers were considered to be infected when bacteria or fungi could be cultured from the wound margin. Sampling for cytology was obtained from the edge of the ulcer with a cytology brush (Cytology brush plus, Cooper Surgical Inc., Trumbull, USA). Specimens for cytology were stained using May Grünewald Giemsa and Gram staining.

External examination of the eye with focal light, as well as testing of pupillary light reflexes, dazzle reflexes, and menace responses was performed. Extent, length, and depth of corneal stromal vascularization were evaluated using a slit-lamp biomicroscope (Kowa SL15, Kowa Company Ltd, Nagoya, Japan). Edematous areas of the cornea were estimated in percent of the entire corneal surface area.

TABLE 1 Details of the 12 horses treated with CXL

Horse				Clinical examination day 0 stromal corneal defect				Cytology and microbial cultures				Medical treatment post-CXL		Wound healing
Nr	Breed	Sex	Age [years]	Stromal melting	Depth range [%]	Area [mm^2]	Volume [mm^3]	Cytology cornea d0 pre-CXL	Corneal culture d0 pre-CXL	Conjunctival culture d0 pre-CXL	Re-cultured d+x	Topical eye treatment	Systemic treatment	Fluoresc neg.
1	SWB	G	5	No	25	23.8	4.8	Abund. neut No bacteria	Negative	Negative	No	No	D 0 flunixin	D+2
2	WBT	F	4	No	20	49.0	7.8	Sparse neut No bacteria	Negative	Negative	No	No	D 0 flunixin	D+6
3	WBT	F	17	No	22	50,3	9.0	Sparse neut No bacteria	Negative	Negative	No	No	D 0 flunixin	D+7
4	SWB	F	23	No	25	45.3	9.1	Sparse neut No bacteria	Negative	Negative	D+3 negative	D+3 chloramph., serum, atropine	D 0 flunixin	D+11
5	Arabian cross	G	4	Yes	50-75	328.1	137.2	Abund. neut No bacteria	Negative	Negative	No	D 0 chloramph., serum, atropine	D 0 flunixin, penicillin	D+22
6	NewForest	G	2	Yes	25-50	169.6	50.9	Abund. neut No bacteria	Negative	Negative	D+8 negative	D 0 atropine. D+4 serum D+8 chloramph.	D 0 flunixin D+8 penicillin	D+22
7	Am. Quarter	G	7	No	25-50	36.1	10.8	Abund. neut Cocc. bact.	ß-hem. Streptococcus	ß-hem. Streptococcus	No	D 0 atropine D+4 penicillin, serum	D 0 flunixin D+4 penicillin	D+15
8	Islandic Horse	F	2	No	50	62.3	24.9	Abund. neut Cocc. bact.	ß-hem. Streptococcus	ß-hem. Streptococcus	D+4 ß-hem. Streptococcus	D 0 atropine D+4 chloramph., serum	D 0 flunixin D+4 penicillin	D+29
9	WBT	G	2	No	25-50	59.6	17.8	Abund. neut Cocc. bact.	ß-hem. Streptococcus	ß-hem. Streptococcus	D+5 ß-hem. Streptococcus	D 0 atropine D+5 chloramph., serum	D 0 flunixin D+5 penicillin	D+16
10	WBL	F	15	No	15	15.3	1.7	Abund. neut No bacteria	ß-hem. Streptococcus	ß-hem. Streptococcus	D+14 ß-hemo. Streptococcus	D+3 atropine D+14 penicillin	D 0 flunixin	D+21
11	SWB	G	4	Yes	25-50	35.1	10.5	Abund. neut Cocc. G+bact.	ß-hem. Streptococcus	ß-hem. Streptococcus	No	D 0 chloramph., serum, atropine	D 0 flunixin, penicillin	D+11
12	WBT	G	3	Yes	25-50	37.7	11.3	Abund. neut Cocc. G+bact.	ß-hem. Streptococcus	ß-hem. Streptococcus	No	D 0 penicillin, serum, atropine	D 0 flunixin, penicillin	D+21

White, noninfectious keratitis; orange, infectious keratitis; depth range, depth range of the ulcer in % of cornea adjacent to ulcer; area ≈ fluorescein positive area of the ulcer; volume, estimated volume of the stromal defect (depth x area); D 0, day for CXL treatment; d+x, days after CXL treatment; SWB, Swedish warmblood; WBT, warmblood trotter; TB, thoroughbred; F, female (mare); G, gelding; Abund. neut, abundant neutrophils; cocc; coccoid; G+ bact, gram-positive bacteria; ß-hem, ß-hemolyzing; chloramph, chloramphenicol; Fluoresc. neg., fluorescein negative.

TABLE 2 Details of the 12 horses in the Control group

Horse				Clinical examination day 0 Stromal corneal defect				Cytology and microbial cultures				Medical treatment from day 0		Wound healing
Nr	Breed	Sex	Age [years]	Stromal melting	Depth range [%]	Area [mm^2]	Volume [mm^3]	Cytology cornea d0	Corneal culture d0	Conjunctival culture d0	Re-cultured d+x	Topical eye treatment	Systemic treatment	Fluoresc. neg.
13	WBT	F	8	No	20-30	198.7	62.4	Abund. neut	Negative	Negative	D+3 negative	Chloram., serum, atropine	Flunixin, penicillin	D+10
14	Mixed pony	G	27	No	25-50	39.0	11.7	Abund. neut	Negative	Negative	D+3 negative	Ciprofloxacin, serum, atropine	Flunixin	D+6
15	WBT	S	16	No	25-50	26.7	8.0	Neutrophils	Negative	Negative	D+4 negative	Ciprofloxacin, serum, atropine	Flunixin, penicillin	D+6
16	SWB	G	5	Yes	25-50	91.0	27.3	Abund. neut Rod. bacteria	Mixed bacteria	Mixed bacteria	D+3 negative	Ciprofloxacin, serum, atropine D+1 tobram., serum, atropine	Flunixin, penicillin	D+21
17	Islandic horse	F	9	No	25-50	59.4	17.8	Neutrophils Cocc. bacteria	ß-hem. Streptococcus	ß-hem. Streptococcus	D+3 ß-hem. Streptococcus	Ciprofloxacin, serum, atropine	Flunixin D+7 penicillin	D+32
18	Islandic horse	F	11	No	25-50	103.5	31.0	Abund. neut Cocc. bacteria	α-hem. Streptococcus	Mixed bacteria	D+3 negative	Ciprofloxacin, serum, atropine D+1 suppl. with penicillin D+6 chloram, penicillin, atropine	Flunixin, penicillin	D+29
19	TB	F	10	No	25	23.5	4.7	Neutrophils Cocc. bacteria	Moraxella	Mixed bacteria	D+2 negative	Chloram, serum, atropine	Flunixin	D+15
20	WBT	G	5	Yes	25-50	79.4	23.8	Neutrophils	Pseudomonas	Pseudomonas	D+3 sparse Pseudomonas	Tobramycin, penicillin, serum, atropine	Flunixin, penicillin	D+7
21	WBT	F	1	Yes	25	19.1	3.8	Abund. neut Cocc. bacteria	Moraxella	Moraxella	No	Ciprofloxacin, serum, atropine	Flunixin, penicillin	D+21
22	SWB	G	20	No	25-50	98.9	29.7	Abund. neut Cocc. bacteria	ß-hem. Streptococcus	ß-hem. Streptococcus	D+3 negative	Chloram, serum, atropine	Flunixin, penicillin	D+13
23	Conne-mara	F	11	Yes	20-60	235.3	59.0	Abund. neut Cocc. & rods	Actinobacillus	Negative	No	Ciprofloxacin, penicillin, serum, atropine	Flunixin, penicillin	D+35
24	Islandic horse	F	16	No	10-15	143.0	14.3	Neutrophils Fungal hyphae	Aspergillus	Negative	No	Chloram, natamycin, serum, atropine	Flunixin, penicillin	D+42

White, noninfectious keratitis; orange, infectious keratitis; depth range, depth range of the ulcer in % of cornea adjacent to ulcer; area ≈ fluorescein positive area of the ulcer; volume, estimated volume of the stromal defect (depth x area); D 0, day for CXL treatment; d+x, days after CXL treatment; SWB, Swedish warmblood; WBT, warmblood trotter; TB, thoroughbred; F, female (mare); G, gelding; Abund. neut, abundant neutrophils; cocc, coccoid; G+ bact, gram-positive bacteria; ß-hem, ß-hemolyzing; chloramph, chloramphenicol; Fluoresc. neg., fluorescein negative.

Furthermore, biomicroscopic examination of ulcerations was performed both with white light and with cobalt blue light after fluorescein staining (Bio-Glo fluorescein strips, 1 mg, Hub Pharmaceuticals, USA). The depth of the ulcers was estimated biomicroscopically in percent of the adjacent nonulcerated cornea. The size and density of stromal cell infiltration were assessed and was listed as mild, moderate or heavy. Corneal melting was diagnosed as stromal liquefaction and malacia at the site of the ulcer. Findings were documented by photography and measurement of ulcer diameter was also performed in all horses. Signs of anterior uveitis were also assessed. Frequent re-examinations were made until a steady progression of the corneal wound healing was observed and then at longer intervals. A corneal ulcer was considered to be healed when no fluorescein staining of the cornea was visible at slit-lamp examination with cobalt blue light.

2.3 | Treatment protocol for the CXL group

2.3.1 | Sedation, local and topical anesthesia

The horses were sedated with 0.01 mg/kg detomidine (Domodin vet, 10 mg/mL, Novartis Animal Health, Copenhagen, Denmark) and 0.02 mg/kg butorphanol (Butador vet 10 mg/mL, Vetoquinol Scandinavia, Åstorp, Sweden). The sedation was maintained throughout the CXL procedure by a continuous infusion of 1 mg detomidine per 100 mL physiologic saline solution (Natriumklorid, Fresenius Kabi, Uppsala, Sweden) given symptomatically at a rate of 1-2 mL/kg/h. The infusion rate was increased if the horse was moving making the CXL treatment difficult to perform. For akinesia of the eyelids, the auriculopalpebral nerve was blocked with 1 mL mepivacaine subcutaneously (Carbocain, 20 mg/mL, Astra Zeneca, Södertälje, Sweden). Two drops of oxibuprocaine (Oxibuprokain Chauvin, 20 mg/mL, Bausch & Lomb Nordic AB, Stockholm, Sweden) were instilled for topical corneal anesthesia before debriding 1-2 mm of the epithelium surrounding the ulcer, as well as removing debris in the ulcerated area with a scalpel.

2.3.2 | Corneal cross-linking

A modified "Dresden" protocol (Table 3) was performed as presented in our previous report.[9] The UVA lamp (CCL 365, Peschke Meditrade Huenenberg, Switzerland) used was calibrated according to manufacturer's instructions before each treatment.

Topical isotonic riboflavin eye drops (Medio-cross D, riboflavin ≥ 0.1% in dextran 500, 20%, Medio-cross GmbH, Neudorf, Germany) was administered every 2nd minute for 30 minutes. Thereafter, the de-epithelialized part of the cornea was irradiated with 365 nm UVA light at 3.0 mW/cm^2 for 30 minutes from a distance of 5 cm to the corneal surface, which implied a total fluence of 5.4 J/cm^2. During the UV-illumination, riboflavin was instilled every 5 minutes. The diameter of the beam was adjusted to the size of the corneal ulcer. For ulcers larger than 11 mm, the light beam had to be moved continuously over the ulcerated area. A lid-speculum was normally used to keep the eyelids open, although the eyelids in some horses were simply kept open manually by the surgeon.

TABLE 3 A brief description of the CXL protocol used in the CXL group

Treatment target	Ulcer
Epithelium status	off
Soak time and interval (minutes)	30 (q2)
Chromophore	Riboflavin (Medio-cross)
Chromophore carrier	Dextran 500
Chromophore osmolarity	Iso-osmolar
Chromophore concentration	0.1%
Light source	Peschke CCL365
Irradiation mode	Continuous
Fluence (J/cm2)	5.4
Intensity (mW)	3
Treatment time (minutes)	30

2.3.3 | Adjunct, topical medical treatment

Topical medical treatment with atropine and antibiotics was initially only initiated in the CXL group if clinical signs indicated abnormal wound healing, such as increasing ulcer size, more severe signs of corneal and concomitant anterior uveal inflammation. Because medical treatment had to be added in 6 out of the first 7 horses treated with CXL alone, we could not justify CXL alone in additional horses with signs of corneal infection on cytology or bacterial culture. Therefore, CXL was combined with topical antibiotics in these eyes. The topical treatments were administered as described below for the control group. Eyes with noninfected ulcerative keratitis were still treated with CXL only throughout the study.

2.4 | Treatment protocol for the Control group

All ulcers were treated prophylactically or therapeutically with topical antibiotics (Table 2 and 4). Infected ulcers were initially treated empirically with topical antibiotics and therapy was later adjusted according to susceptibility testing, if needed. Topical anti-protease treatment (1.8 mg EDTA/mL autologous serum) was used to inhibit enzymatic melting. A continuous delivery system (Infu-Disc, San Diego, CA) and a subpalpebral

TABLE 4 Description of topical antibiotic treatment of the eyes

Antibiotic	Concentration	Frequency
Chloramphenicol	2.5 mg/mL	continuous 0.35 mg/h
Penicillin	100 mg/mL	continuous 14 mg/h
Ciprofloxacin	1.5 mg/mL	continuous 0.21 mg/h
Tobramycin	3 mg/mL	q. 8-12

catheter (Mila subpalpebral lavage system, Mila International Inc., Florence, KY) were used to deliver the topical medical treatment at a rate of 0.14 mL/h (i.e, a mixture of antibiotics and atropine diluted in serum to a total volume of 10 mL). The delivery system was checked twice a day, and the pump was replaced if any problems were observed.

2.5 | Anti-inflammatory medication

Concurrent uveitis was treated with systemic flunixin 1.1 mg/kg (Cronyxin vet 50 mg/mL, Ceva Animal Health, Lund, Sweden) and topical atropine (Isopto-Atropin 1%, S.A. Alcon-Couvreur N.V., Belgium) when needed, in both the CXL and the control groups (Table 1 and 2).

2.6 | Assessment of area and volume of ulcers

A millimeter scale was positioned under the horse's eye when photographs were taken. From the photographs, the area of the ulcer was calculated using standard image processing software (Adobe Photoshop CS6) (Figure 1). The measurements from the photos were compared with the measurements made during the clinical examinations. To obtain a rough estimate of the volume of the ulcer, the area was multiplied by the depth of the ulcer in its center. In eyes where the depth of the ulcer clearly differed between different parts of the ulcer, the volume was calculated independently for each part of the ulcer and the results were then added up to a total volume for the entire ulcer. All images in both the CXL and Control groups were analyzed by the same author.

2.7 | Statistical processing

Means ± standard deviations were used as descriptive statistics. The Wilcoxon signed-rank test was used to compare results from the Control and CXL groups. $P < 0.05$ were considered significant.

3 | RESULTS

Both groups included horses with infectious and noninfectious keratitis, as well as patients with signs of stromal melting (Table 1 and 2). All horses were presented with stromal defects and inflammatory infiltrates. The mean corneal ulcer area was larger in the control group and the mean volume was larger in the CXL group, but differences were not significant (Table 5). A decrease in pain and inflammatory signs including blepharospasm, eyelid swelling and presence of aqueous flare in the anterior chamber was observed in all eyes in the CXL group and in 9 of 12 eyes in the Control group one day after initiation of treatment. Stromal melting was subjectively judged to be more extensive in the CXL group than in the control horses before treatment, but melting stopped within 24 hours after treatment regardless of treatment protocol. No surgical interventions, such as conjunctival flap placements, had to be performed in either group and all eyes treated in both groups retained vision. All horses in both groups were negative for EHV-2 on PCR.

3.1 | CXL group

All 12 eyes with stromal ulcers healed and were fluorescein negative within 2 to 29 days (Table 1).

Six eyes were negative for bacteria and fungus on initial microbiological cultures and cytology. Three of the six noninfected ulcers healed in 2 to 7 days after CXL treatment alone without additional topical treatment (Figure 2). In the additional three horses, it was decided to add topical antibiotics to prevent secondary infection as a safety measure. One of these horses, no. 5, presented with extensive stromal

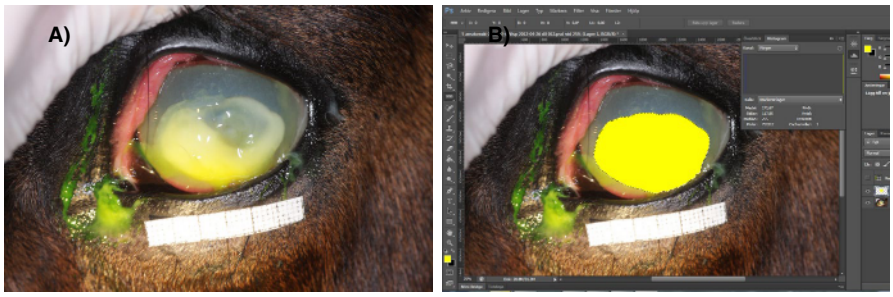

FIGURE 1 Processing of images to assess the area and volume of the ulcer, here in horse no. 5 (A-B). The millimeter scale placed under the eye is used as a reference to convert pixels (px) to millimeters as shown in the digital picture. The ulcer is filled with color to assess the number of pixels in the image, and the area in mm^2 is calculated (B)

TABLE 5 Comparison between corneal ulcer area and volume, time for fluorescein negativity and mean reduction in wound area in the CXL and the Control groups. Results are given as mean ± std

	CXL group	Control group	-value
Wound area [mm^2]	76.1 ± 88.5	93.1 ± 69.4	0.34
Wound volume [mm^3]	25.4 ± 39.8	22.0 ± 15.6	0.36
Days to fluorescein negativity	15.2 ± 8.0	19.7 ± 12.3	0.56
Reduction in wound area [mm^2/day]	5.7 ± 4.4	6.0 ± 5.3	1.0

tissue damage (Figure 1A), whereas the two other horses (nos. 4 and 6) were started on topical antibiotics when clinical signs indicated impaired wound healing 3 and 8 days after CXL, respectively. However, re-sampling was negative in both eyes.

In four out of six eyes with bacterial infection, topical antibiotics were added after 4-14 days due to poor wound healing and increased inflammatory signs in the eye (Figure 3). Interestingly, in three of these four eyes (horses nos. 8-10), β-hemolytic Streptococci were found at sampling both before CXL and when clinical signs showed deterioration, indicating either insufficient antimicrobial effect of CXL or re-infection due to bacterial contamination of the ulcers. Therefore, topical antibiotics were initiated together with CXL treatment in the remaining eyes where bacteria were detected on initial examination.

Already at the end of the CXL treatment, the stromal surface of the four eyes with stromal melting had turned from a sticky glue-like consistency to a firmer jelly when touched with a sterile cotton swab, indicating stabilization of the tissue. One eye with a deep stromal ulcer and severe melting (horse no.6) was treated with CXL and atropine only the first 4 days without any other anti-protease therapy (Figure 4). Three of the four melting eyes in the CXL group were given topical autologous serum with EDTA together with topical antibiotics from day 0 due to the change in treatment protocol for the CXL group mentioned above.

3.2 | Control group

In summary, all corneal ulcers in the control group healed within 6-42 days (Table 2). Eight out of 12 eyes were positive on both microbial culture and cytology and one eye on culture only. One eye was infected with Aspergillus sp. and the other eight with bacteria.

3.3 | Comparison between the CXL group and the Control group

Table 5 shows that the mean wound area was larger in the control group, but the mean time to fluorescein negativity was shorter and the estimated total volume of the ulcers was on average larger in the CXL group. Neither of these differences was statistically significant. The average decrease in wound area per day after initiated treatment was almost identical between the groups.

4 | DISCUSSION

4.1 | Time to healing of ulcers

We found no significant difference in time to fluorescein negativity in equine corneas treated with CXL ± topical antibiotics compared to eyes receiving only conventional medical treatment. Although the mean time until re-epithelization of the ulcer was approximately 5 days shorter in the CXL treated eyes, the mean area of the ulcers in the control group was larger and also included one case of fungal keratitis. For the superficial, stromal, nonmelting/noninfectious ulcers with mild cell infiltration and relatively small amounts of neutrophils on cytology in both groups, the time for epithelial healing complies with the results of studies by Neaderland et al and Wada et al[1,3] The average time to healing of infectious ulcers was shorter in both the CXL group and the Control group (19 and 24 days respectively), compared to the 39 days reported by Wada et al. and close to 45 days for corneal ulcers infected by streptococci reported by Brooks et al[5] Andrew et al [4] reported a median treatment time of 48 days for horses with ulcerative keratomycosis.

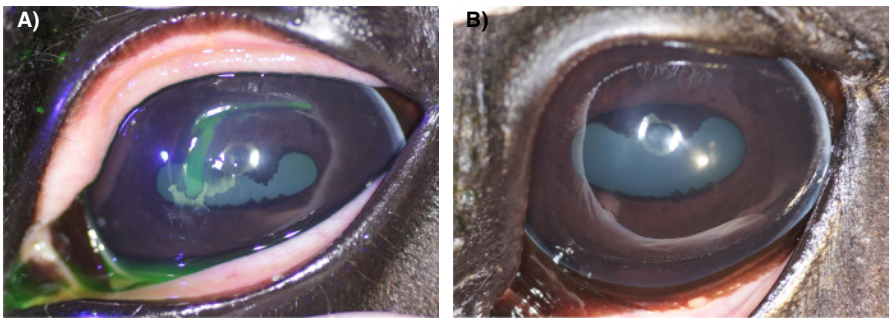

FIGURE 2 A noninfectious stromal ulcer treated with CXL only (horse no. 1). The eye was fluorescein positive before CXL treatment (A). The same eye was fluorescein negative after 2 days (B)

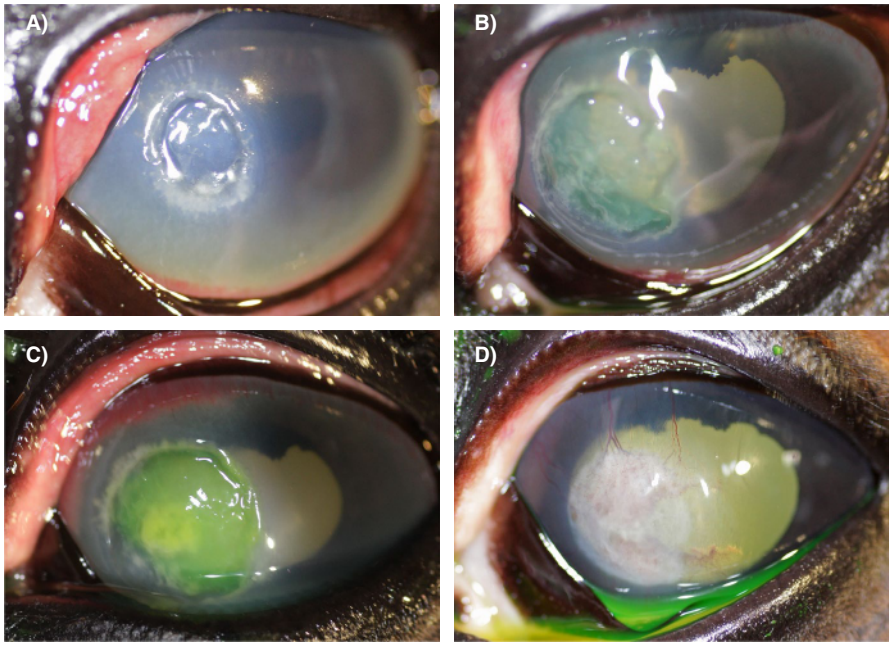

FIGURE 3 Images showing the ulcer healing of a CXL-treated eye with a Streptococcus infection (horse no. 9). Microbial cultures from both the ulcer and conjunctiva were positive pre-CXL and 5 days after CXL. Antibiotic treatment was initiated on day 5 when healing of the ulcer did not progress as anticipated (A-D). Pre-CXL (A), 3 days post-CXL (B), 5 days post-CXL(C), and 22 days post-CXL (D)

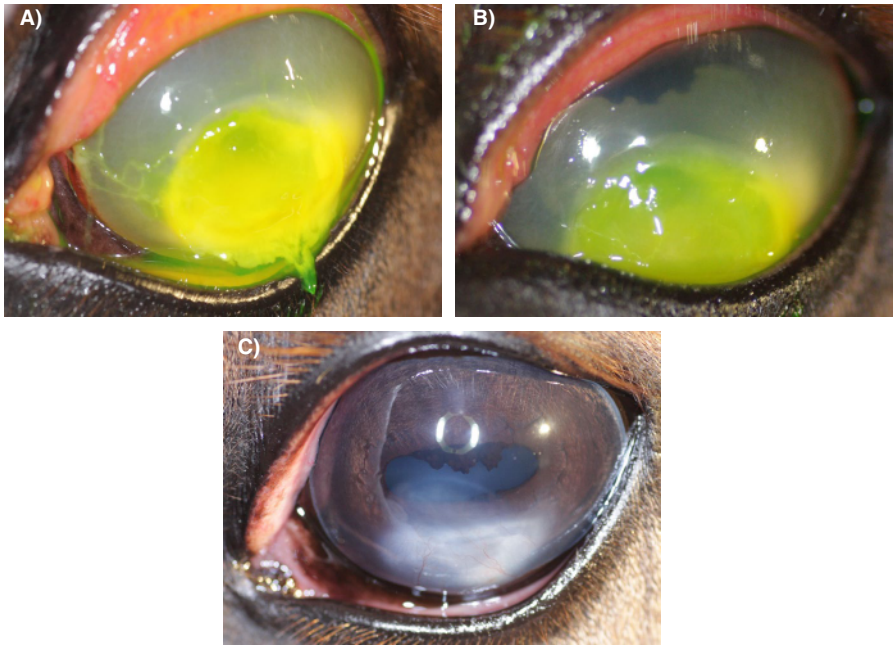

FIGURE 4 Images showing the ulcer healing of a CXL-treated melting cornea (horse no. 6) (A-C). The melting ulcer before treatment with CXL and atropine (A). The same eye 2 days after CXL treatment (B). The same eye 8 months after CXL treatment (C)

All eyes treated in both groups retained vision. Da Silva Curiel et al [6] reported that 2/3 of ulcerated corneas infected with Streptococci spp. healed with retained vision following medical treatment and approximately 75% after medical and/or surgical treatment. Sweeney and Irby [7] also reported approximately 75% of eyes infected with Pseudomonas spp. retaining vision after medical and/or surgical therapy.

Calculations of ulcer area reduction per day revealed that wound healing progressed almost identically in both groups. The study thus indicates that CXL is probably not associated with adverse effects on wound healing rates in the horse cornea, which is in agreement with our previous study.[9]

4.2 | Antibacterial effect

Despite numerous clinical reports with promising results after use of PACK-CXL, there are few controlled trials regarding the outcome of CXL treatment compared to antibiotic treatment alone for infectious keratitis.[36] CXL research in animals has mainly been performed experimentally, Tal et al [37] compared CXL treatment alone, medical treatment

alone, CXL + topical antibiotics to no treatment in rabbits with corneal ulcers infected with Staphylococcus aureus. The authors concluded that CXL had a positive effect both as stand-alone treatment and in combination with medical therapy. In contrast, Cosar et al [38] did not observe a significant or positive effect of CXL treatment alone on colony-forming unit counts and ulcer scores in rabbits with Pseudomonas-infected ulcers, whereas the greatest post-treatment reduction in ulcer scores was seen when CXL and medical treatment were combined.

In the study presented here, insufficient elimination of bacteria or bacterial re-infection was observed in three of the photochemically treated equine corneas. In these eyes, Streptococci were detected both before CXL and at 4-14 days post-CXL. In most eyes in both groups where specific bacteria could be identified on culture, the same genus was detected in both conjunctiva and at the ulcer margin. It is possible that the conjunctiva served as a reservoir for bacteria in the cross-linked ulcer, because the UV-light was only directed to the de-epithelialized part of the cornea. It is also plausible that the CXL treatment in some patients with deep infiltrates and extensive tissue destruction did not penetrate deep enough to neutralize the infection. Gallhoefer et al [39] found a median penetration of CXL in the ex vivo horse cornea of 173 μm or 9% of the total stroma. The penetration in infected corneas in vivo is still to be studied. Furthermore, there is not much known about the riboflavin concentration after pre-CXL instillation in the equine corneal stroma. In porcine and human corneas, the majority of the riboflavin solution is absorbed in the outer layer of the cornea and the concentration deeper than 200 μm is limited.[40] It is likely that the distribution of riboflavin is similar also in equine corneas. Thus, deeper infiltrates with bacteria may not be laden with riboflavin and absorb a sufficient amount of the UVA-irradiation.

4.3 | Stromal melting

Signs of melting were absent the day after initiated treatment in both groups. The ability of CXL to stop melting processes in the cornea is consistent with results from previous clinical studies in humans, dogs, and cats.[16,17,19,20,31,32] A few clinical reports describe the use of CXL in animals as adjunct to medical therapy for melting keratitis. Pot et al,[20] studied the stabilizing effect of CXL on melting keratitis in 49 dogs and cats, comparing CXL + medical treatment with medical treatment only in a nonrandomized study. The authors concluded that CXL could be a useful adjunctive therapy for corneal melting in dogs and cats. Famose [16,17] described a satisfactory outcome in two separate studies where melting keratitis in seven dogs and ten cats, respectively, was treated using accelerated CXL + tobramycin but no anti-protease medications.

In this current study, only one of the four equine eyes with melting ulcers in the CXL group was treated with CXL alone during the first days. Thus, it is not possible to draw any conclusions whether CXL is superior to medical treatment of stromal melting from this study. However, the immediate appearance of stabilization of the melting tissue in the cornea after CXL was performed indicates that the use of riboflavin and UV-A (365 nm UV-A light at 3.0 mW/cm^2 for 30 minutes) has a repressive effect on stromal melting in the horse cornea.

4.4 | Inflammatory response

The inflammatory response and pain were reduced for all eyes in both groups. Systemic NSAID medication used in both groups in the study usually gives a significant relief of pain and inflammatory symptoms, but also riboflavin and ultraviolet light have been shown to inactivate residual leukocytes in blood products used for transfusion (Mirasol Pathogen Reduction Technology) [28] and to inhibit the immunologic response mediated by leukocytes.[41] Furthermore, the reduction in pain from the cross-linked cornea may also be caused by disappearance of the corneal subepithelial nerve plexus and anterior midstromal nerve fibers, as shown by Mazotta et al[42] Hence, part of both reduction in inflammation and pain relief may be attributed to the CXL treatment.

4.5 | Use of fluorescein

All eyes were stained with fluorescein in the diagnostic procedure of the ophthalmic examination prior to treatment in both groups. Although the corneas were rinsed and the ulcer margins debrided and cleaned from debris as preparation for the CXL, we cannot exclude that traces of fluorescein may have affected the outcome of the CXL treatment, as it may interfere with the UV-A absorption of riboflavin during irradiation.[43] Fluorescein present in the superficial layers of the stroma absorbs UV-A thus preventing the absorption of UV- A by riboflavin and impeding the antimicrobial effect.

4.6 | Large ulcers

For ulcers larger than 11 mm, the light beam had to be moved back and forth to cover the entire ulcerated area. It is not known how this lack of full-field illumination affected the cross-linking. In this study, we could not see any obvious negative effect on the two eyes with large ulcers.

4.7 | Scarring

Subjectively, CXL-treated corneas healed with less residual scarring than anticipated from the initial appearance (superior portion of the corneal scar shown in Figure 4C). Since

the amount of scarring was not followed up over time, the authors cannot determine whether a significant difference between CXL-treated and conventionally treated eyes was present.

5 | CONCLUSION

Uninfected corneal ulcers healed after CXL alone without complications. However, clinical signs of impaired wound healing were seen after 3-14 days in the eyes with suspected or proven bacterial infection treated with CXL only, most likely because of insufficient elimination of bacteria deeper in the corneal stroma or because of re-infection from bacteria in the conjunctiva. For ethical reasons, we therefore decided to combine the CXL treatment with topical antibiotic treatment when infection of the stromal ulcer was suspected in subsequent horses in the study. Hence, we consider that CXL should not be used as stand-alone treatment for infected or suspected infectious ulcerative keratitis in horses.

Time to fluorescein negativity was not significantly different between horses treated medically only and horses receiving CXL treatment, which confirms that CXL has no adverse effect on wound healing rate in the horse corneas. It therefore seems to be safe and feasible to perform CXL on standing, sedated horses as also shown in a previous study. [9]

In summary, we find CXL to be a possible useful adjunct treatment of corneal stromal ulcers in horses, especially for melting ulcers and as a potential alternative to prophylactic antibiotic treatment for noninfectious stromal ulcers with active inflammation indicated by stromal infiltrates and neutrophils on cytology. Further research, including both experimental and clinical comparative studies, is needed to evaluate the efficacy of PACK-CXL in the treatment of ulcerative keratitis, as well as to refine the method for use in the horse cornea.

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