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Research Article

Accelerated Cross-Linking for the Treatment of Chronic Corneal Ulcers and Evaluation of Healing with Optical Coherence Tomography (OCT)

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Abstract

Purpose: To assess the epithelial-stromal healing process after corneal phototherapy with riboflavin and UV-A of corneal ulcers, which were resistant to medical therapy, by means of Anterior Segment Optical Coherence Tomography (OCT; iVue optovue).

Procedure: 14 dogs (14 eyes), with corneal ulcer involving at least 1/3 of the stroma and not responding to medical therapy for at least 30 days, were included in this study. The corneal phototherapy consisted in the instillation of 0.1% riboflavin ophthalmic so lution on to the cornea for 20 minutes, followed by UV-A irradiation at 30 mW/cm2 for 3 minutes. Subjects underwent a complete ophthalmological examination on the day of treatment, after 7 days and then at an interval between 7 and 20 days until the resolution of the ulcer for a follow-up period up to 3 months. At each follow-up examination, the stromal and epithelial thickness of both the ulcerated area and the surrounding healthy areas were measured with OCT in order to evaluate the epithelial-stromal healing process.

Results: Healing of the ulcerative corneal lesion was achieved in 12.5 ± 4 days after corneal phototherapy in all cases. The OCT assessment of corneal lesions was valuable to assess objectively the course of the healing process.

Conclusions: OCT assessment provided useful information on corneal wound healing process after corneal phototherapy. The treatment has proven to be a safe and valid therapeutic option for chronic corneal ulcers no responders to medical therapy.

Keywords: Corneal Phototherapy; Corneal Cross-Linking; UV-A; Riboflavin; Corneal Ulcers; OCT

Abbreviations

OCT: Optical Coherence Tomography; SCCED: Spontaneous Chronic Corneal Epithelial Defects; CXL: Accelerated Corneal Cross-Linking; UV-A: Ultraviolet A Light; EOG: General Objective Examination; IOP: Intraocular Pressure; STT: Shirmer Tear Test

Introduction

Corneal ulcers consist of a loss of substance of the corneal tissue. They can be classified according to the etiology and depth of corneal involvement. Corneal ulcers can be caused by primary ocular surface disorders, such as SCCED (Spontaneous Chronic Corneal Epithelial Defects), or secondary ocular surface disorders, such as keratitis. Keratitis are generally caused by physical, chemical or microbiological noxae, but can also be induced by pathological processes that have arisen primarily in other eye districts. The prognosis is correlated with the cause and the functional outcomes, which are in turn related to the location and density of the corneal scar that may occur. The restoration of

transparency is due to the healing process which, depending on the cause and intensity of the insult, can be avascular or vascular [1]. Diagnosis of corneal ulcer is made on the basis of clinical signs and corneal fluorescein retention. Therapy of corneal ulcers aims at eradicating the cause and at reducing both the inflammation and the ocular immune response in order to preserve the transparency of the tissue as much as possible. In case of treatment failure within a week, further investigation should be considered, for example using microbiological and antibiogram tests as well as a cytological examination to undertake specific antimicrobial therapy, in order to contain the evolution of the antibiotic resistance phenomenon [2]. In this study, we aimed to assess a new method for the treatment of corneal ulcers, namely corneal phototherapy, also known as accelerated corneal cross-linking [3] (CXL), term borrowed from human ophthalmology. The CXL is a photodynamic therapy which consists in the administration of an ophthalmic solution enriched with riboflavin (vitamin B2) followed by irradiation of the cornea with ultraviolet A light (UV-A). The CXL was introduced in the late 1990s for the treatment of keratoconus [4,5] and corneal ectasia and later proposed as a modality for the treatment of corneal ulcers and bullous keratopathy [6]. In the veterinary field, the procedure has been used to treat several disorders, such as corneal abscess [7], refractory keratitis [8], bullous keratitis [9-10], and keratitis with corneal melting [11-14].

The main mechanism of action of CXL includes the photo-oxidation, which generates reactive oxygen species, which in turn determine the effects of the treatment, such as the generation of additional corneal cross-links among stromal proteins and the peroxidation of cell membranes (inducing apoptosis) of keratocytes and microorganisms. The overall treatment effects of CXL include the following

- The increase of the corneal stiffness.
- Increase of the thickness of collagen fibers.
- Increase of resistance to enzymatic degradation.
- Decrease of the edema of the stromal matrix.
- Induction of the lipoperoxidation of cell membranes.

The first CXL protocol used for the treatment of keratoconus, known as *Dresden protocol*, made use of 3 mW/cm^2 UV-A irradiation for 30 minutes for a total UV-A energy dose of 5.4 J/cm^2 to the corneal tissue. Further treatment protocols have been developed that vary for the emitted UV-A power density, the UV-A irradiation time and, more recently, the UV-A energy dose [13-15]. Based on the Bunsen-Roscoe law of photochemical reciprocity, the same photochemical effect could be obtained by keeping the total energy dose constant while reducing the irradiation time and increasing the irradiation density [16]. The corneal phototherapy protocol used in this study was validated in the laboratory for exclusive use in veterinary medicine [17] and consisted of 30mW/cm^2 UV-A power irradiance for 3 minutes with a total emitted energy dose of 5.4 J/cm^2 (VET-CXL® protocol).

The aim of this study was to evaluate the epithelial-stromal healing process in corneal ulcers resistant to topical antimicrobial therapy treated with corneal phototherapy (VET-CXL protocol) by means of OCT.

Materials and Methods

This was a prospective study for consecutive cases conducted at the Veterinary Medicine and Surgery Department of the University of Perugia.

The inclusion criteria included the presence of a corneal ulcer, with or without melting, involving at least 1/3 of the stroma and that was no responder to previous topical medical therapy for at least one month. Patients were included in the study after obtaining owner's consent.

Medical history was collected from the owner of each study participant, which then underwent a general objective examination (EOG) and an ophthalmological examination. All clinical data were recorded in a standardized medical record, which was also used for all post-treatment examinations.

The ocular surface of each participant was examined with a slit lamp before and after corneal fluorescein staining. Since fluorescein has a similar absorbance peak as riboflavin, it can interfere with the effectiveness of the treatment, and for this reason it should not be used immediately before corneal phototherapy. Generally, it is preferred to apply fluorescein at least one hour before the procedure and, in the event that the treatment must be carried out urgently, fluorescein can only be used as a post-treatment control.

A slit-lamp based clinical score was defined (absence/presence: mild moderate or severe), for each of the following signs:

- Mucopurulent material,
- Corneal edema,

- Corneal vascularization,
- Conjunctivitis,
- Blepharospasm,
- Prostration,
- Aggression,
- Photophobia,
- Ocular itching,
- Uveitis.

Photographs of the ocular surface were taken at each visit by all participants in order to keep records.

The following tests were also performed

- Intraocular pressure (IOP) measurement using a rebound.
- Schirmer-test (STT) to assess whether the ulcer could be associated with keratoconjunctivitis sicca.
- Threat reflex and pupillary reflex, to highlight any neurological pathology.

A corneal swab was performed in all cases in order to detect and identify any pathogen that could be responsible for ulcer; the swab could also be repeated during follow-up, when necessary. In all of our cases, there was a suspicion that a (bacterial or mycological) infection was accompanying the lesion. However, it is not always possible to highlight it. As for the bacteriological and mycological examination, the results that emerged were largely negative, due to the previous local antibiotic therapy; cases 1 and 10 were exceptions, where a positivity to Staphylococcus spp. and to Pseudomonas Aeruginosa were observed, which were resistant to the antibiotic treatment.

Based on the medical history provided by the owner and based on the appearance of the ulcer, 4 causes have been identified that determine the lesion: melting ulcer, ulcer of traumatic origin, ulcer in bullous keratopathy and indolent ulcer.

Assessment of the corneal wound healing process after corneal phototherapy was carried out objectively using an Anterior Segment OCT (OCT; iVue optovue). The OCT provided objective assessment of the corneal tissue and corresponding pathologies that cannot be provided by slit lamp examination [18]. In this view, OCT imaging may play an important role both for detection and for tracking disease progression; in addition, by measuring corneal thickness, it allowed for better planning of surgery. Both corneal and epithelial thickness were measured through OCT, comparing the ulcerated portion with the healthy one; measurements are repeated throughout the follow-up. When pachymetry (Figure 1) cannot be obtained, measurements are performed by hand through the program's measurement tool. This examination also highlights the possible presence of edema and bubbles (Figure 2). Using OCT, we measured the point of least thickness of the cornea, evaluating it from the day of treatment until the resolution of the ulcer. Through the OCT and the clinical visit, we assessed the presence/absence of the ulcer, the affected layers, the shape, cell infiltrates and malacia.

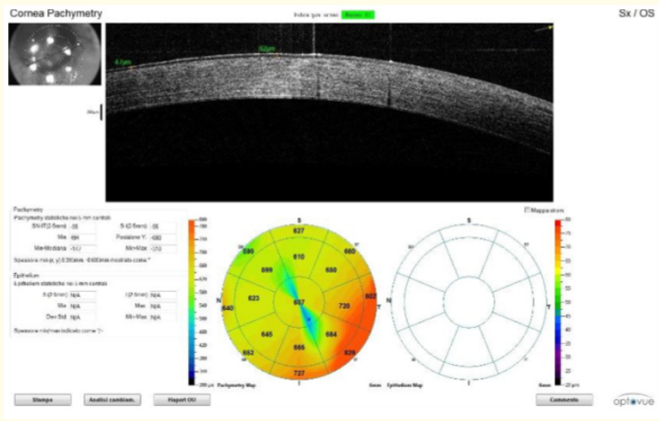


Figure 1: Corneal pachymetry in study case 7.

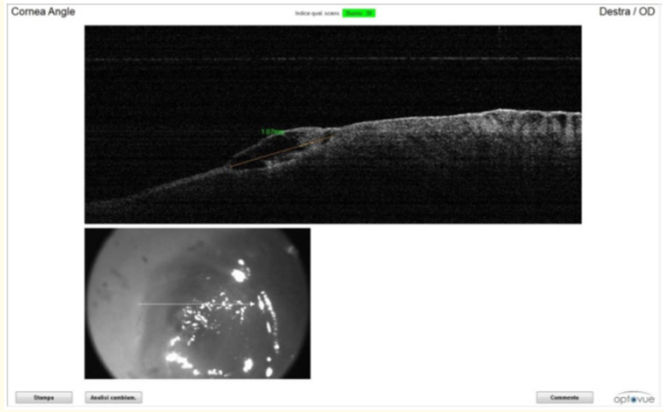


Figure 2: Corneal OCT scan in patient 11.

During the healing process we evaluated the evolutionary aspect of the cornea, therefore the edema and the formation of bubbles in the cornea, evaluating the size of the latter.

At the end of the healing process, we compared, again using OCT, the healthy portion with the healed portion to describe any discrepancies and any side effects.

All these diagnostic methods were performed in both eyes on the day of treatment, except for the measurement of IOP and pupillary reflex, which were detected only in follow-up visits, in the ulcerated eye. Corneal fluorescein staining was made one hour prior to corneal phototherapy in order not to interfere with treatment safety and efficacy. In case of a negative Schirmer test in the pre-treatment visit, the test was not repeated in follow-up visits.

The first post-treatment examination was performed 7 days after corneal phototherapy in all participants. Following visits (3 or 4 per patient) were performed at an interval between 7 days and 20 days from the first follow-up visit based on the owner's availability. The visits were repeated until the corneal ulcer was completely resolved and the symptoms remitted.

Corneal phototherapy (VET-CXL protocol)

All patients underwent only one corneal phototherapy treatment.

Patients were sedated with medetomidine or dexmedetomidine (3-5 µg/kg) and butorphanol (0.1-0.2 mg/kg) or ketamine (5 mg/kg) and placed in lateral decubitus.

After instilling topical oxybuprocaine hydrochloride, the edges of the ulcer were cleaned with a sterile scalpel blade in order to remove cellular debris that can affect homogeneous diffusion of riboflavin into the stroma and may interfere with the photo-activation of riboflavin, which is essential for repair.

The patient's head was placed onto a pillow in order to place the eye parallel to the table and apply riboflavin eye drops (0.1% riboflavin hypotonic ophthalmic solution: Visioflavin®, Vision Engineering Italy srl, Rome, Italy) using a 1 ml syringe every 20 seconds for 20 minutes. After application of the eye drops, the eye was irradiated with a point-of-care UV-A device (Vetuvir®, Vision Engineering Italy srl, Rome, Italy) at 30 mW/cm^2 for 3 minutes for a total dose of 5.4 J/cm^2. Before UV-A irradiation, the penetration of riboflavin into the stroma was confirmed by clinical examination using a blue light slit lamp, even if it was visible to the naked eye. The UV-A light beam, with a diameter of approximately 10 mm, was easily focused onto the ulcerative lesion (Figure 3) by using the focalizer, which is connected to the device by an optic fiber. Overall, the corneal phototherapy protocol had a maximum duration of 20 - 30 minutes.

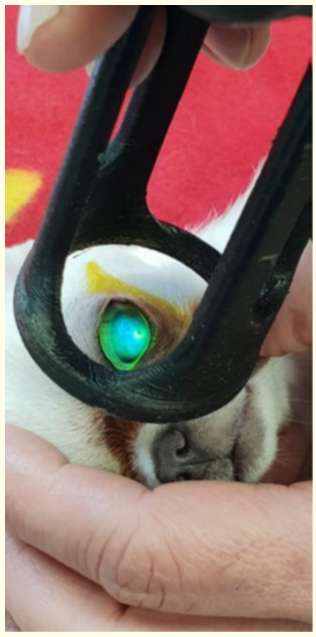


Figure 3: UV-A irradiation of the cornea with point-of-care device for exclusive use in veterinary medicine.

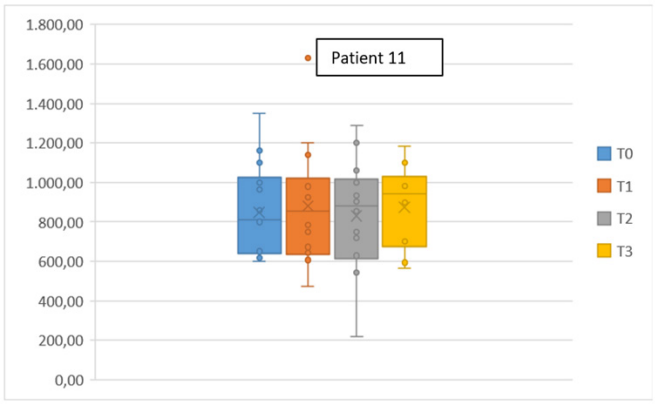
Statistical analysis

Data were evaluated by a non-parametric Mann-Whitney test. The Mann-Whitney test was used to assess frequency of species, race, sex, clinical score, shape, infiltration and malacia.

We used these values to highlight the homogeneity of the subjects, defining their inclusion criteria.

The statistical power was calculated using the Post-hoc Power Calculator, taking the data obtained from the previous article as the reference value of success from standard-of-care treatments [17].

The tables from 1 to 4, obtained using the Excel program, describe the values measured by OCT of the corneal and epithelial portion, ulcerated and healthy, of all patients, from the day of treatment to the last visit, highlighting the process ulcer healing.



Light blue colour T 0 (day of treatment)

Red colour T 1 (7 days after the treatment)

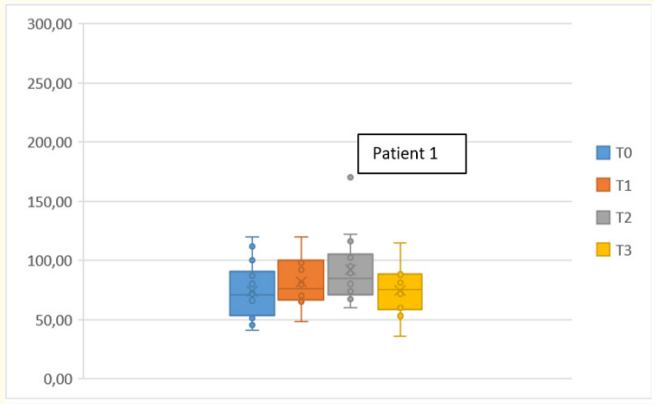
Grey colour T 2 (14 days after the treatment)

Yellow colour T3 (21 days after the treatment).

Graph 1: Healthy portion corneal thickness.

Corneal Thickess	T0	T1	T2	T3
Media	847,9286	881,5	829,6429	873,52
Mediana	811	855	879,5	940,5
Dev. Sta.	236,3317	302,5358	283,8428	217,7698

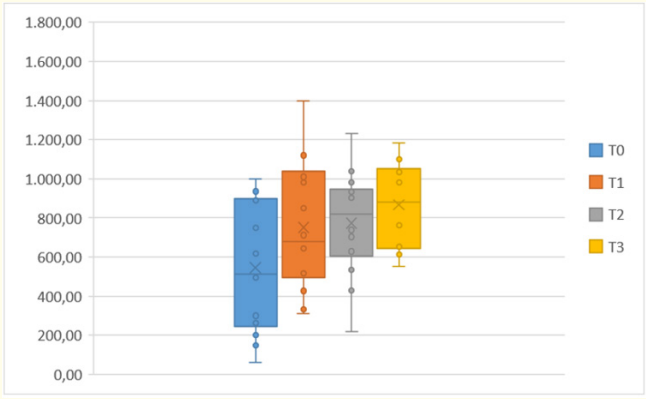
Table 1



Graph 2: Healthy portion epithelial thickness.

Epithelial Thickness	T0	T1	T2	T3
Media	73,82143	81,92857	91,89286	74,5
Mediana	70,5	76	85	75
Dev. Sta.	24,46788	19,85123	28,90713	21,77282

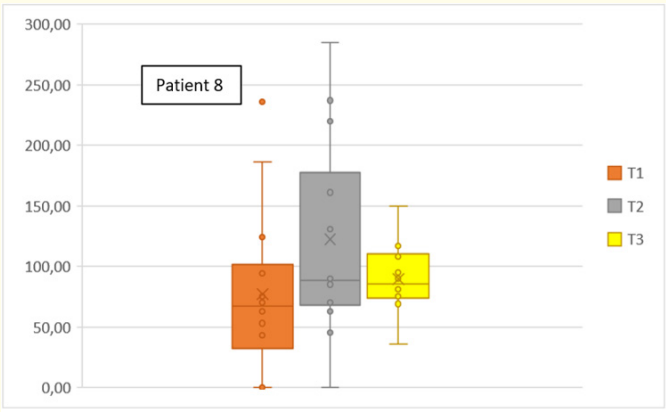
Table 2



Graph 3: Ulcerated portion corneal thickness.

Corneal Thickness of Ulcer	T0	T1	T2	T3
Media	544,4286	750,7143	773,7143	867,3
Mediana	511,5	677,5	820,5	883
Dev. Sta.	320,6939	336,681	268,4335	221,9365

Table 3



Graph 4: Ulcerated portion epithelial thickness.

Epithelial Thickness of Ulcer	T1	T2	T3
Media	77,21429	122,1786	89,6
Mediana	67,5	88,5	85,5
Dev. Sta.	67,70772	81,16877	30,84801

Table 4

Results

14 dogs (14 eyes) were enrolled in the study and were treated during May 2018 and June 2020.

Table 5 provides information on study participants, including race, age, the affected eye and medical therapy used before inclusion in the study and corneal phototherapy.

Patient no.	Species	Race	Sex	Age (yrs)	Topical therapy before VET-CXL	Affected eye
1	dog	Pekingese	F	6	Netilmicin Sulphate	Left
2	dog	Labrador retriever	M	7	Chloramphenicol/Sodium colistimethate/Tetracycline Hydrochloride and Acetylcysteine	Right
3	dog	crossbred	F	15	Hyaluronic acid/Cross-linked sodic salt/Carboxymethyl beta-glucan	Right
4	dog	Chihuahua	F	0.3	Ciprofloxacin hydrochloride and cross-linked hyaluronic acid 0.20%/Aloe gel 0.10%/EDTA disodium salt/N-hydroxymethylglycinate/Buffered solution at pH 7.2	Left
5	dog	crossbred	Fs	14	Ciprofloxacin hydrochloride and cross-linked hyaluronic acid 0.20%/Aloe gel 0.10%/EDTA disodium salt/N-hydroxymethylglycinate/Buffered solution at pH 7.2	Left
6	dog	crossbred	Fs	15	Ciprofloxacin hydrochloride and cross-linked hyaluronic acid 0.20%/Aloe gel 0.10%/EDTA disodium salt/N-hydroxymethylglycinate/Buffered solution at pH 7.2	Right
7	dog	Beagle	Fs	9	Ciprofloxacin hydrochloride and cross-linked hyaluronic acid 0.20%/Aloe gel 0.10%/EDTA disodium salt/N-hydroxymethylglycinate/Buffered solution at pH 7.2	Left
8	dog	French Bulldog	M	1.6	first Netilmicin Sulphate and Hyaluronic Acid sodium salt 20mg then Chloramphenicol/Sodium Colistimethate/Tetracycline Hydrochloride and Acetylcysteine	Right
9	dog	crossbred	Fs	10	Netilmicin Sulphate	Right
10	dog	crossbred	F	11	Chloramphenicol/Sodium Colistimethate/Tetracycline Hydrochloride and Hydroxypropylmethylcellulose 0.3%/Cetrimide 0.010%/dexpanthenol/disodium edetate/potassium dihydrogen phosphate/biphasic sodium phosphate/purified water	Right
11	dog	Jack Russell	M	8	Ofloxacin	Right
12	dog	French Bulldog	Fs	6	Netilmicin Sulphate	Left
13	dog	crossbred	F	6	Tobramycin and Diclofenac Sodium	Right
14	dog	French Bulldog	M	9	Tobramycin/Dexamethasone	Left

Table 5: Information on study participants.

All eyes included in the study had a positive history of persistent corneal ulcer involving 1/3 of the corneal thickness. The characteristics of the ulcer, in terms of interested layers (epithelium, half superficial stroma, half deep stroma and endothelium), shape (circular, elliptical and irregular), and presence of infiltrators and malacia were described for all eyes and summarized in table 6.

Threat reflex, physiological lacrimation and positive corneal fluorescein staining were found in all cases at enrolment in the study. As for the bacteriological and mycological examination, the results were negative in all cases, likely due to the previous local antibiotic therapy, except for case 1 and case 10, which were positive to Staphylococcus spp. and Pseudomonas Aeruginosa respectively.

Characteristics of the corneal ulcer					
Patient no.	**presence/absence**	**Affected layers**	**Shape**	**Cellular infiltrates**	**Malacia**
1	present	Epithelium, superficial and deep stroma	Circular	Yes	Yes
2	present	Epithelium, superficial and deep stroma	Circular	No	Yes
3	present	Epithelium, superficial stroma	Elliptical	No	Yes
4	present	Epithelium, superficial and deep stroma	Circular	No	No
5	present	Epithelium	Circular	No	No
6	present	Epithelium	Circular	No	No
7	present	Epithelium	Circular	No	No
8	present	Epithelium, superficial and deep stroma	Circular	Yes	Yes
9	present	Epithelium and superficial stroma	Circular	Yes	Yes
10	present	Epithelium, superficial and deep stroma	Circular	Yes	Yes
11	present	Epithelium, superficial and deep stroma	Circular	Yes	Yes
12	present	Epithelium, superficial and deep stroma	Circular	Yes	Yes
13	present	Epithelium, and superficial stroma	Circular	No	Yes
14	present	Epithelium and superficial stroma	Elliptical	Yes	No

Table 6

The clinical score of all cases at enrolment in the study was summarized in table 7.

All demographics obtained with the Mann-Whitney test are listed in table 8.

Based on the eye examination, the corneal ulcers in study participants have been classified as follows

- In patients 1, 4 and 12 ulcer melting.
- In patients 2 ulcer of traumatic origin.
- In patients 3, 8, 9, 11 and 14 ulcers in bullous keratopathy;
- In patients 5, 6, 7, 10 and 13 indolent ulcers.

At the end of the ophthalmological examination, OCT imaging was performed without sedation in all cases.

Clinical signs diminished immediately after corneal phototherapy in all cases. The clinical score decreased significantly by first follow-up (day 7) and complete epithelial wound healing was achieved between 7 and 21 days after corneal phototherapy in all study participants (average: 12.5 ± 4 days). The evolution of the healing process using photographic images is documented in figure 4.

In cases n. 1, 6, 7 and 10, clinical resolution of the ulcer was found at day 7; in cases n.2, 3, 4, 8, 9, 11, 12, 13 and 14, complete

Patient no.	Clinical score
1	Severe
2	Severe
3	Moderate
4	Severe
5	Mild
6	Moderate
7	Moderate
8	Severe
9	Mild
10	Mild
11	Moderate
12	Severe
13	Moderate
14	Moderate

Table 7: Slit lamp based clinical score.

DEMOGRAPHICS	
Breed frequency	7,1 % Beagle 21,4 % Bulldog 7,1 % Chihuahua 7,1 % Jack Russel 7.1 % Labrador 7,1 % Pekingese 42,9 % Half-breed
Sex frequency	35,7 % Females 35,7 % Spayed females 28,6 % Males 0 % Spayed males
Clinical-score frequency	21,4 % Mild 42,9 % Moderate 35,7 % Severe
Frequency: shape	85,7 % circular 14,3 % elliptical 0 % irregular
Frequency: infiltrates	50 % no 50 % yes
Frequency: malacia	35,7 % no 64,3 % yes

Table 8

Figure 4: Patient 1 before corneal phototherapy (A). Patient 1 at the last follow-up visit (B).

wound closure was found at day 14 day. Case n.5 had complete healing at day 21 after treatment. No treatment complications were recorded.

Corneal swabs were repeated during follow-up and were negative in all patients, including case 1 and case 10.

Effects obtained

- Increase in the biomechanical and biochemical stability of the cornea.
- Repair of chronic ulcers.
- Reduction of corneal inflammation.
- Increase in collagen density.
- Reduction of edema.
- Reduction of pressure.
- Bactericidal effect.

Concomitant treatments following corneal phototherapy

Upon clinical evaluation, eye drops were used during follow-up. Table 9 summarized the topical treatment used in study participants. An antibiotic eye drop was used in only one case (n.10) on the basis of antibiogram data available. In patient 10 the following active ingredient was used

Chloramphenicol / Sodium Colistimethate/ Tetracycline Hydrochloride, also used before treatment with VET-CXL. As the fluorescein staining was negative, the topical treatment was interrupted (7 days after the treatment).

Topical therapy after VET-CXL		
Patient n.	**Day 0 (VET-CXL treatment)**	**Treatment at first follow-up visit**
1	-	-
2	-	Threalose, TSP, Euphrasia
3	Ozonized vegetable oil eye drop	-
4	Ozonized vegetable oil eye drop	-
5	-	-
6	-	-
7	Sodium hyaluronate eye drop	-
8	-	-
9	-	-
10	Chloramphenicol, tetracycline, colistimethate	-
11	-	-
12	-	-
13	-	-
14	-	-

Table 9: Topical therapy used after VET-CXL.

Clinical signs not related to OCT

Vascularization was evaluated in the 1st post-treatment checkup and it was essential for the healing process (Figure 5); it was present on the periphery of the ulcer, advancing to the center of the lesion from the seventh day onwards. When the ulcer was resolved, the vascularity was reduced. It should also be noted that vascularization was never exuberant, so we can deduce a reduction in inflammation due to treatment with VET-CXL. Just in one case the healing was reached by mitosis and not by vascular nourishment, in patient 10.

The edema was reduced thanks to the bonds between the collagen fibers obtained by VET-CXL, and the effect of the reduction, as well as by OCT as we will see later, it was possible to evaluate it by measuring the pressure at the corneal level, which was reduced for all patients from the first post-treatment checkup.

Another aspect to consider is post-treatment fibrosis. Fibrosis that developed following treatment was variable: it is not known whether it can be attributed to the treatment, to the previous disease or to both. Fibrosis in all cases went into remission during follow-up. To stop fibrosis we used corticosteroid eye drops after wound closure as needed, in almost all patients expect for 1 and 4.

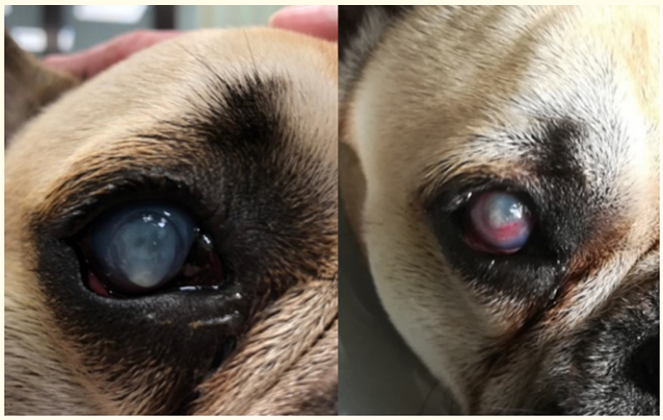


Figure 5: Corneal vascularization before (A) and after (B) VET-CXL in patient 8.

OCT data

OCT scanning provided the following information useful to track response to corneal phototherapy during follow-up

- Evolution of the corneal ulcer by measuring the corneal and epithelial thickness of the ulcerated and non-ulcerated portion
- Different corneal density between anterior and posterior stroma

- Reduction of corneal edema
- Presence of bubbles in the cornea during the healing process
- Corneal ulcer healing

Through the OCT we obtained sequential images of the ulcer healing process (Figure 6).

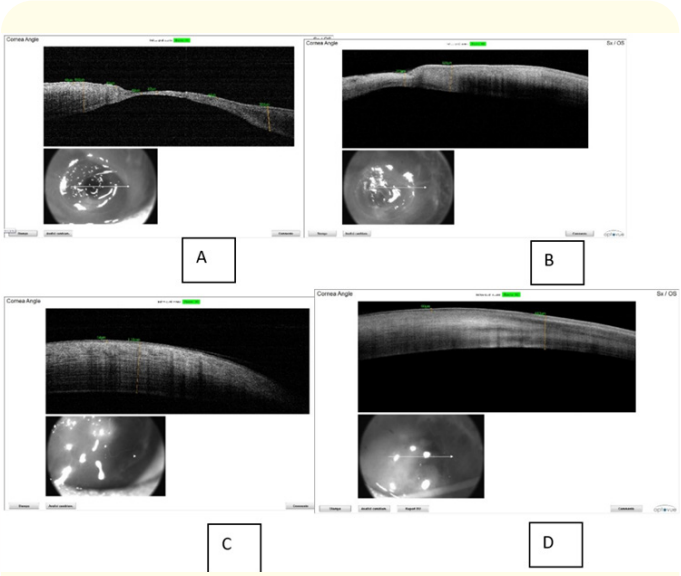


Figure 6: Corneal OCT scan (patient 11) showing hyper-reflection of the anterior stroma, which is an imaging biomarker of VET-CXL therapeutic effect.

The tables from 1 to 4 indicate the homogeneity of the group for both the corneal and epithelial thickness of both the healthy and ulcerated portion.

Evaluation of the data obtained

Through the OCT we have highlighted the recovery time for each patient. In some subjects (1, 6, 7 and 10) it was evident that the resolution of the ulcer occurred at the 1st follow-up visit, in almost all cased (2, 3, 4, 8, 9, 11, 12, 13 and 14), it was found at the 2nd follow-up visit. However, it should be pointed out that in one case, in patient number 5, the 3rd check-up had to be waited for to have an effective recovery.

In the OCT images it was also possible to highlight a distinction between the stroma in which the VET-CXL has an effect and the underlying stroma, in fact a different density was observed (Figure 7). The anterior stroma was more reflective than the posterior one; this increase of the corneal intensity, highlighted by the OCT, was due to an increase in the biomechanical and biochemical stability of the cornea obtained through the VET-CXL.

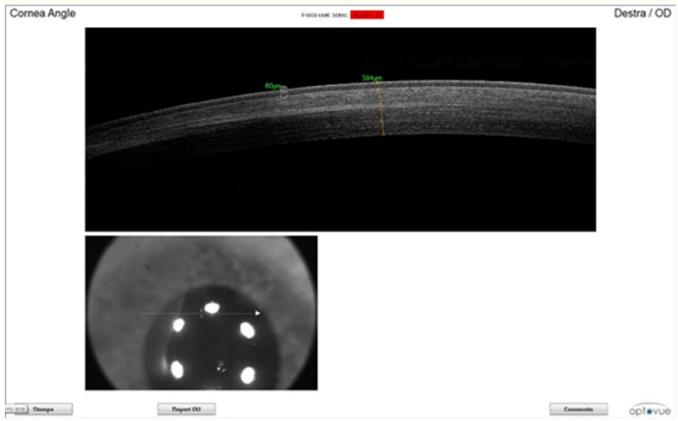


Figure 7: Corneal OCT scan (patient 2) showing corneal oedema.

Stromal edema, which was confirmed in all cases at enrolment by OCT scanning gradually decreased towards physiological values during follow-up. In a few cases (in 3 of them, in patients 2, 8 and 11), corneal edema was so massive to make it difficult to measure the stromal thickness through the OCT (Figure 8). Despite this, we were able to assess the thickness of all patients. Edema in patient 2 was reduced already after one week of treatment, while in patients 8 and 11 it remained elevated even after the resolution of the ulcer, up to the third control.

During follow-up, it was of note to identify the presence of inner stromal bubbles in several cases. In patients 1 and 12 these bubbles, at the level of the cornea, developed in the healing process, while in patients 3, 8, 9, 11 and 14, being bullous keratitis, they were present before treatment with CXL. Bubbles in these 5 patients were also maintained in the healing process but were smaller in size thanks to the effect of the CXL, which were only evident through OCT (Figure 9).

During the healing process with the reduction of oedema, bubbles were formed, which likely originated from cavitation in the granulation tissue in which the pre-existing fluid was collected. This phenomenon indicated the reparative process.

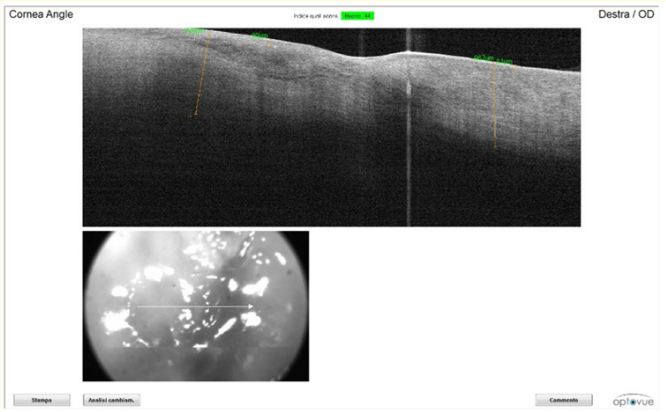


Figure 8: Corneal OCT scan (patient 14) showing bubbles across the most anterior stroma.

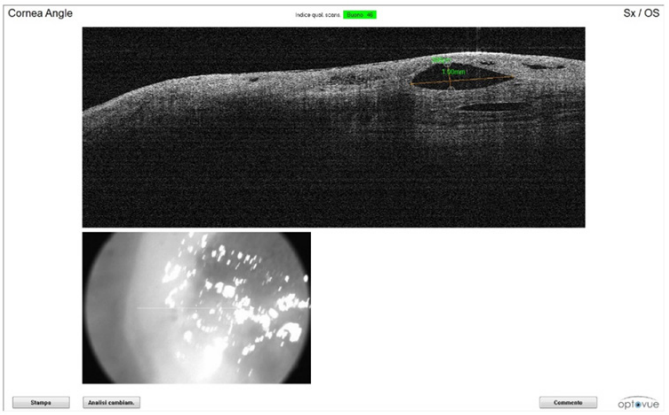


Figure 9: Sequence of images of patient 4 from time 0 to time 3.

When the loss of substance was considerable, as in the case of patient 4, 9, 10 and 13, a step is highlighted. With time the thickness is recovered.

In cases where the lost substance was significant (patient 4) and in cases where the lost substance was limited (as in patients 9, 10, 13) through the OCT a step has been highlighted, caused or scar tissue that is not particularly excessive or a slow-developing vascular tissue, which with time has shrunk and disappeared.

As the epithelial wound closed, epithelial thickness returned to physiological levels. The epithelial thickness underwent variations, as it first increases and then decreases. Patient 5 had a delay in reducing epithelial thickness as the resolution of the ulcer occurs only at the 3rd control.

In table 1 the corneal thickness of patient 11 at T1 has an above average corneal thickness. It has a very high starting thickness, which then decreases during subsequent checks.

In table 2 patient 1 at T2 has an epithelial thickness much above the group average. This very high thickness then decreases in subsequent checks.

In table 4 the epithelial thickness of patient 8 at time 1 is much higher than the average. This very high thickness then decreases in subsequent checks.

Through the tables from 1 to 4 obtained during the statistical study through the values measured with the OCT, both the corneal and epithelial values of the group were compared to evaluate their homogeneity, and it was possible to highlight the presence of patients who differ from the average of the group to which they belong as regards the values of the corneal but also epithelial thickness.

As for the corneal thickness, patient 11 had an increase in corneal thickness in the healthy portion at time T1, so there was a delay in the reduction of edema despite the treatment.

As regards the epithelium, it was possible to highlight the subjects who came out of the homogeneity of their respective groups. Among these subjects there is patient 8 at time T1 in the ulcerated portion has a greater thickness than the other members of the group, this condition then resolved over time. This exaggerated value, determined by the reparative process, could be a characteristic of the subject. Another non-conformity is given by patient 1 who in the healthy portion has a greater thickness at time T3 than in the group, this could be due to individual variability.

Adverse events

We found possible adverse events in 4 cases, however not influencing positive clinical resolution of corneal lesion. The adverse effects found were:

- Endothelial thickening (in patient 4).
- Epithelial thickening with pigmentation (in patients 3 and 14).
- Iridocorneal adherence (in patient 2).

In case 4, endothelial thickening was found by OCT scanning; in this case, corneal stroma was considerably thin at enrolment. However, it did not pose a problem to the clinical case. This occurrence, however, did not develop in cases 1 and 12, which had significant thin corneal stroma at enrolment [13]. Indeed, since case 4 had excessive edema at enrolment, we were unable to visualize the endothelium clearly before VET-CXL, so we could not exclude that it was a pre-existing condition.

Corneal pigmentation was found in case 3 and case 14 through OCT, which is one of the complications present in the literature [17]. Due to corneal edema masking tissue details at enrolment, it was unclear whether it developed prior to or following treatment. It is unclear whether it developed from the disease prior to or following treatment. Pigmentation is a condition that develops very frequently in dogs of brachycephalic breeds, as in case 14.

In case 2, irido-corneal adhesion was found by OCT scanning. Due to the presence of edema, we do not know if this condition was pre-existing. This condition, however, has not been reported as an undesirable effect in the literature. This event did not cause a problem to the clinical case and it was not caused by VET-CXL.

Discussion and Conclusions

The procedure used in this prospective clinical study is a specific application of corneal cross-linking for the treatment of corneal ulcers in veterinary medicine, called corneal phototherapy or VET-CXL® protocol. This procedure was validated in the laboratory for treatment of corneal ulcers in veterinary medicine [5] and was based on the use of higher UV-A power density than previously published treatment protocols in veterinary medicine [12].

In this study, OCT imaging was used to assess the changes of the corneal tissue after corneal phototherapy for the treatment of chronic corneal ulcers, which were no responders to topical medical therapy for at least 30 days. The clinical resolution of both signs and symptoms were direct indicators of treatment success compared to previous medical therapy procedures.

OCT imaging was useful to track response to corneal phototherapy during follow-up by demonstrating

- Reduction of corneal inflammation
- Closure of the corneal epithelium
- Physiological remodelling of the corneal stroma.

As for the last parameter, OCT imaging was also useful to underline the differential postoperative changes across the anterior and posterior stroma providing evidence of inducing more tissue compactness across the anterior stroma than preoperatively. During follow-up, CXL decreased corneal edema and improved corneal tissue transparency, thus improving visual capacity.

Efficacy of corneal phototherapy depended on the absorption of UV-A light rays by riboflavin-soaked tissue, so the saturation of the cornea with riboflavin was crucial. The result consisted of an increase in the biomechanical and biochemical stability of the cornea, which supported the epithelial wound closure, an increase in collagen density, and a reduction in tissue inflammation and swelling. The decrease in corneal inflammation could be determined by anterior stromal keratocytes apoptosis and sterilization of corneal wound infection. Infection by microorganisms was indeed associated with stromal infiltration by inflammatory cells, which release cytokines, degrade collagen causing tissue necrosis; for this reason, in addition to using antimicrobial therapies, collagen must be made resistant to the action of these enzymes; corneal phototherapy was found to provide both evidences, even in cases that did not respond to medical treatments.

This study highlighted how VET-CXL could be effective for the treatment of chronic corneal ulcers, which were no responders to topical antimicrobials for at least 30 days. In these cases, treatment response was quite fast, with decreased corneal inflammation and vascularization by 7 days postoperatively.

The golden standard for the treatment of corneal infections was topical antimicrobial therapy, nevertheless in 50% of cases, isolated Staphylococci and Streptococci (2/3 of all corneal infections) have been shown to be resistant, so the results of topical therapy were variable and often ineffective [19]. Antimicrobial activity of CXL has been demonstrated against numerous bacterial [11-20], but also fungal [21] and parasitic species under experimental conditions [22]. The greatest beneficial effects were on superficial bacterial corneal ulcers. The bactericidal effect could be explained by the generation of reactive oxygen species following the photoactivation of riboflavin, the UV-A and riboflavin association caused damage to cell membranes due to lipoperoxidation of the cell membranes of microorganisms. In order to determine corneal

phototherapy treatment efficacy, we enrolled in the present study only those cases which were no responder to common medical therapy for a long period of observation. Medical therapy failure could be explained by the presence of antibiotic-resistant bacteria, although the swabs were positive in 2 cases only. It was likely that swabs were false negatives due to previous antibiotic treatments. The antimicrobial effect of corneal phototherapy could be somewhat confirmed by negative swabs, which were repeated during follow-up. However, it must be considered that the patients treated had already been subjected to antibiotic therapy, somewhat distorting the result of the swabs.

We found three possible adverse effects during follow-up, which included iridocorneal adhesion (case 2), endothelial thickening (case 4) and corneal pigmentation (cases 3 and 14) [23]. These conditions, however, did not affect positive clinical resolution of the corneal lesion. Based on current literature, risks associated with the use of UV-A can develop if

- Corneal thickness is thinner than 400µm [24].
- Corneal soaking with riboflavin is not adequate.
- UV-A light is not precisely focused onto the area of the cornea to treat.

In this study, endothelial thickening was highlighted in patient 4, which had a very thin cornea (60 µm) at enrolment. Due to excessive edema, we were unable to visualize the endothelium clearly before CXL, so we could not exclude that it was a pre-existing condition. For a sake of completeness, cases 1 and 12 had thin corneas (with a thickness of 120 µm at the centre of the injury in patient 1, and of about 112 µm in patient 12) before corneal phototherapy, however any endothelial damage was found during follow-up. Corneal pigmentation was found in case 3 and case 14. Due to corneal edema masking tissue details at enrolment, it was unclear whether it developed prior to or following treatment. Pigmentation is a condition that develops very frequently in dogs of brachycephalic breeds, as case 14. An irido-corneal adhesion was found in case 2. No previous case has been found in the available literature. Long-term adverse effects are not known in pets; in the human field, the adverse events of CXL, rare, are essentially recorded during the first days after surgery.

The results described in this study are in agreement with the current basis of the scientific literature [14-17,25]; therefore, the CXL can be considered an adjunct therapy for the treatment of ulcers, including colliquative, in the veterinary field. All cases had the same clinical outcome regardless of the presence or absence of infectious agents and regardless of the duration of the condition prior to treatment with CXL.

The possibility that a bacterial infection may have played a role, at least in some of our patients, cannot be completely ruled out despite the negative results of the swabs, as, prior to CXL, patients had undergone treatments with topical antibiotics. So as in the articles cited above, despite the suspicion of microbial infection, it has not always been possible to prove it.

Although the bactericidal effect of the treatment has been demonstrated experimentally and is used in transfusion medicine, it has not yet been clinically proven. Unlike the study by Spiess., *et al.* in ours we can identify CXL as the only treatment for ulcers, melting and not, of an infectious nature, as the patients recruited did not undergo treatments with post CXL antibiotic eye drops. The use of Chloramphenicol/Sodium Colistimethate/Tetracycline Hydrochloride in patient 10 may affect the results, however the postoperative evolution was the same as in the other cases and the use of the drug did not show any added value.

The use of CXL should also be considered to reduce antibiotic resistance. As Famose also points out, CXL could be considered the primary treatment for keratitis with or without the use of antibiotics.

Limitations of the study include

- A series of 14 cases, although treatment efficacy was 100%
- The patients were brought to follow-up visits according to owners' availability, which created a temporal variability in the clinical follow-up.

A factor that did not create limitations in the study conducted is the following: the animals, although the ulcers were of a different nature, have a homogeneous ulcer appearance.

The results described in this study suggest that the VET-CXL® protocol may represent a cost-effective, adjunctive or replacement therapy for chronic corneal ulcers; further studies recruiting more cases are needed to increase clinical and scientific evidence on this new protocol specifically designed for veterinary medicine.

The results of this study encouraged the continued use of this procedure. In fact, this technique allowed corneal healing to be obtained without being invasive for the animal and without the aid of antibiotics. In particular, the latter aspect is currently desirable due to the high onset of antibiotic resistance. Also considering that surgical interventions do not always lead to resolution of the ulcer and very often lead to a reduction in functionality and an aesthetic deficit, despite anatomical repair, the use of corneal phototherapy can represent a valid therapeutic option for the treatment of severe corneal ulcers.

In conclusion, corneal phototherapy with riboflavin and UV-A was shown to be safe and effective for the treatment of chronic corneal ulcer. OCT imaging provided useful information on corneal tissue response to therapy during follow-up.

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