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Riboflavin/UV-A corneal phototherapy as stand-alone management of ulcerative keratitis in dogs

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Abstract: Corneal ulcers are one of the most common ocular disorders in veterinary ophthalmology and several factors can negatively influence the efficacy of the currently available therapeutic options, leading to a loss of corneal transparency and, thus, vision. Twenty-five dogs with clinical signs of corneal ulcers were randomised to receive either corneal phototherapy (16 dogs; study group) or topical standard medical therapy (9 dogs; control group). The riboflavin/UV-A corneal phototherapy (PACK-CXL) consisted in the application of a riboflavin ophthalmic solution (Visioflavin®; Vision Engineering Italy srl, Rome, Italy) onto the cornea for 20 min followed by 30 mW/cm^2 UV-A irradiance for 3 min using a point-of-care UV-A device (Vetuvir®; Vision Engineering Italy srl, Rome, Italy). The complete healing of the ulcerative lesion was defined as the complete restoration of the corneal epithelial integrity with negative fluorescein staining. The corneal phototherapy achieved complete corneal healing in all the dogs by 20.5 ± 7.8 days. In the control group, only two dogs achieved complete healing by 21.5 ± 15.6 days. This intervention may represent a valid option to hasten corneal wound healing and a clinical resolution of ulcerative keratitis in dogs.

Keywords: corneal melting; corneal ulcer; cross-linking; keratitis; riboflavin; UV-A

Corneal ulcers are one of the most common ocular disorders in veterinary ophthalmology (Murphy et al. 1978; Ollivier 2003; Ledbetter et al. 2009; Spiess et al. 2014; Kim et al. 2019) and melting keratitis is a serious complication of corneal ulcerations that occurs with relative frequency (Ollivier et al. 2003; Wang et al. 2008; Brejchova et al. 2010; Pot et al. 2014) lastly resulting in stromal liquefaction

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and vision loss (Fini et al. 1998; Ollivier et al. 2007; Famose 2015). Topical medical management of corneal melting includes the administration of antimicrobials and protease inhibitors to counteract the collagenolytic processes; despite this, highly variable outcomes have been reported in the literature (Brooks and Ollivier 2004; Ollivier et al. 2007; Famose 2015; Ion et al. 2015; Gallhoefer et al. 2016). Several factors, such as the advanced state of the disease at the time of referral, the inability of owners to adequately administer the topical therapy as well as antibiotic drug resistance can negatively influence the course of the disease and often limit the efficacy of the currently available therapeutic options (Spiess et al. 2014). Surgery is indicated in advanced corneal melting to avoid vision loss caused by keratomalacia and corneal perforation (Famose 2015; Ion et al. 2015). Such surgical procedures include conjunctival or biomaterial grafts, which lead to variable degrees of corneal opacity and, therefore, are unable to restore corneal transparency and vision (Hollingsworth 2003; Goulle 2012).

In recent years, the need for effective alternatives to the current therapies for the management of corneal ulcers has become more critical (Varges et al. 2009; Cain 2013; De Briyne et al. 2014; Spadea et al. 2018; Marchegiani et al. 2019). Photoactivated chromophor for keratitis corneal crosslinking (PACK-CXL) is a procedure based on the UV-A illumination (365 nm) of the cornea enriched with a photosensitiser, such as riboflavin; although the mechanism of action has not been fully elucidated upon, it causes the generation of riboflavin triplets and reactive oxygen species (ROS), which, in turn, generate additional covalent bonds between the stromal proteins and the peroxidation of lipids in cell membranes (Spoerl et al. 2007; Hayes et al. 2013; Tabibian et al. 2016). In laboratory studies, the use of high UV-A irradiance (30 mW/cm^2) has been shown to induce more rapid photodegradation of riboflavin and reactive oxygen species (ROS) generation in the corneal stroma than lower UV-A irradiance (3 mW/cm^2) and further to support the epithelial healing and stromal compaction (Spoerl et al. 2004; Sondergaard et al. 2013; Lombardo et al. 2015; Pot et al. 2015; Perazzi et al. 2018; Lombardo and Lombardo 2019; Perazzi et al. 2020). The overall treatment effect is characterised by the lipid peroxidation of the microbial cell membranes across the irradiated area, thus promoting a corneal wound healing response (Kumar et al. 2004; Wollensak et al. 2004; Martins et al. 2008). Few clinical reports have provided preliminary results on the efficacy of PACK-CXL for treating infectious and non-infectious ulcerative keratitis in dogs, cats and horses (Hellander-Edman et al. 2013; Famose 2014a; Pot et al. 2014; Spiess et al. 2014; Famose 2015). To the best of our knowledge, there is no randomised controlled clinical trial aimed at evaluating the efficacy of corneal phototherapy in comparison with topical medical treatment for the management of ulcerative and melting keratitis. The purpose of this original paper is to report the outcomes of PACK-CXL as a stand-alone intervention for ulcerative keratitis in dogs in comparison with a topical medical treatment.

MATERIAL AND METHODS

This prospective, randomised controlled clinical trial has been submitted to the University of Camerino Institutional Animal Care and Use Committee (Reference No. E81AC.11/A) and complies with European Directive 2010/63/EU on the protection of animals used for scientific purposes; a written informed consent was obtained from all the owners of the participants before enrolment in the study. The only inclusion criterion was the clinical diagnosis of ulcerative or melting keratitis. Corneal perforation, descemetocele, leishmaniasis, endocrine or metabolic disorders, allergic pruritus, neoplasia or a kidney malfunction represented the exclusion criteria. A bacterial culture and sensitivity testing were performed from the corneal swabs collected from all the participants at enrolment. Thereafter, the participants were randomly allocated to the study or control group (blocking randomisation generated with Microsoft Excel® using a 2 : 1 ratio) by an author who was completely blind about the medical examination. Only one eye per participant was enrolled in the study. The dogs allocated in the study group received a single corneal phototherapy application and neither topical nor systemic additional therapies were given to these participants.

The dogs enrolled in the control group were treated with topical antibiotics on the basis of susceptibility testing and topical collagenase inhibitors (*N*-acetylcysteine 5%) which were both administered five times per day.

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All the participants underwent a complete ophthalmological examination (including a slit-lamp examination and corneal fluorescein staining) performed by a board-certified ophthalmologist at enrolment and then at 1-, 3-, 7-, 14-, 21-, 28-, 35-, 42-, and 49-days after enrolment. Corneal pachymetry was performed at enrolment with an iVue Optical Coherence Tomography (OCT) device (Optovue, Fremont, CA, USA), as described by Famose (2016) and measurements were obtained by focusing on the centre of the corneal lesion. The corneal lesions were evaluated using a modified version of a scale previously described by Famose (2016). Briefly, the mucopurulent discharge, corneal oedema, corneal vascularisation, conjunctivitis, blepharitis, and uveitis were assessed accordingly to a 0–3 point scale (0 = absent; 1 = mild; 2 = moderate; 3 = severe), with a total score ranging between 0 and 18. The diagnosis of corneal melting was based on a subjective evaluation, as previously described (Pot et al. 2014), including the stromal stability/melting activity, the presence of cellular infiltrates, the perceived stability of the stroma, the presence of changes in the corneal contour and ulcer depth, as well as the presence of malacic corneal material in the ulcer area. The clinical score was used to determine any improvement or worsening of the lesion during follow-up.

PACK-CXL intervention

A corneal phototherapy was performed at enrolment, under sedation or general anaesthesia, depending on the patient's temperament. The dogs were sedated with 2 µg/kg of dexmedetomidine (Dexdomitor; Vetoquinol Italia, Bertinoro, Italy) and 5 mg/kg of ketamine (Ketavet 100; MSD Animal Health Italia, Segrate, MI, Italy); when anaesthesia was required, induction was obtained with intravenous injection of 1 mg/kg of propofol (Propovet; Zoetis Italia, Rome, Italy) followed by mixture of 2% isoflurane (Isoflo; Zoetis Italia, Rome, Italy) in oxygen after endotracheal intubation. PACK-CXL was performed after application of oxybuprocaine eye drops (Novesina®; Novartis Farma S.p.A., Origgio, Italy) onto the cornea. A hypotonic 0.1% riboflavin ophthalmic solution (Visioflavin®; Vision Engineering Italy, Rome, Italy) was applied for 20 min onto the cornea using a biocompatible ring applicator. Thereafter, a UV-A light beam generated by a point-of-care device (Vetuvir®; Vision Engineering Italy, Rome, Italy) was focused for 3 min onto the corneal lesion at 30 mW/cm^2 UV-A irradiance (wavelength: 365 nm; total energy dose: 5.4 J/cm^2) over a 10 mm treatment area. All the dogs enrolled in study group received a single corneal phototherapy treatment; no additional topical therapy was added to these dogs.

Statistical analysis

The primary efficacy outcome of the study was the complete corneal wound healing, defined as the disappearance of the corneal ulceration and negative fluorescein staining. All the data were reported as the mean ± standard deviation**.** The differences in the complete corneal wound healing and clinical score between groups were evaluated using the Wilcoxon rank sum (Mann-Whitney *U*) test for unpaired data. The dogs' age, gender, and corneal defect laterality were evaluated using Fisher's exact test. All the statistical analyses were run with GraphPad Prism v8 for Windows (GraphPad Software, USA).

RESULTS

Twenty-five eyes (25 dogs) were enrolled in the study, of which 16 dogs received the corneal phototherapy and 9 dogs received the topical medical treatment. The signalment data of the enrolled dogs are summarised in Table 1.

The average age was 8.3 ± 2.6 years and 7.7 ± 3.7 years in the study and control group, respectively; no significant differences were found between the groups regarding the age; in addition, the ulcer localisation and depth at enrolment did not differ between the groups (Table 2).

All the participants, except for one in the control group that was withdrawn by the owner due to non-compliance with the scheduled visits, completed the study. Microbial swabs were obtained from all the patients and twenty-two were positive for bacteria (*Staphylococcus* spp., *Enterococcus* spp., and *Pseudomonas aeruginosa*) as shown in Table 1. All the isolated bacteria were sensible to tobramycin (3 mg/ml); two dogs from the control group had sterile ulcerative lesions and received only topical collagenase inhibitors five times a day.

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Table 1. Signalment of the enrolled dogs and isolated bacteria

Dogs treated by PACK-CXL at enrolment (study group)					
Code	breed	age (years)	sex	eye to treat	isolated bacteria
S1	Chihuahua	6	female	right	*Enterococcus* spp.
S2	Chihuahua	7	male	left	*Staphylococcus* spp.
S3	French Bulldog	6	female	left	*Pseudomonas aeruginosa*; *Staphylococcus* spp.
S4	French Bulldog	7	male	left	*Enterococcus* spp.
S5	Maltese	8	female	right	*Staphylococcus* spp.; *Enterococcus* spp.
S6	Mixed breed	15	female	left	*Enterococcus* spp.
S7	Mixed breed	9	female	right	*Staphylococcus* spp.; *Enterococcus* spp.
S8	Mixed breed	11	female	left	*Enterococcus* spp.
S9	Mixed breed	11	female	right	*Enterococcus* spp.
S10	Mixed breed	10	female	right	*Staphylococcus* spp.
S11	Pug	6	male	left	*Pseudomonas aeruginosa*
S12	Pug	7	female	left	*Staphylococcus* spp.
S13	Shih-Tzu	9	male	left	*Staphylococcus* spp.
S14	Shih-Tzu	4	female	left	*Staphylococcus* spp.; *Enterococcus* spp.
S15	Shih-Tzu	8	male	right	negative
S16	Shih-Tzu	9	male	right	*Pseudomonas aeruginosa*
Dogs treated by topical medical therapy (control group)					
Code	breed	age (years)	sex	eye to treat	isolated bacteria
C1	Beagle	9	female	left	*Staphylococcus* spp.
C2	Boxer	6	male	right	*Staphylococcus* spp.
C3	Chihuahua	3	male	right	*Pseudomonas aeruginosa*
C4	French Bulldog	2	male	right	negative
C5	Golden Retriever	7	male	left	*Enterococcus* spp.
C6	Mixed breed	14	male	left	negative
C7	Mixed breed	9	male	right	*Staphylococcus* spp.
C8	Mixed breed	11	female	right	*Enterococcus* spp.
C9	Mixed breed[†]	8	female	left	*Pseudomonas aeruginosa*

[†]Withdrawn by the owner at day 14

Table 2. Clinical data at presentation

Parameter	Dogs treated by PACK-CXL (study group)	Dogs treated by topical medical therapy (control group)
Number of eyes	16	9
Ulcer depth	50% (range from 10% to 75%)	40% (range from 25% to 70%)
Ulcer area	20 mm^2 (range 7 mm^2 to 32 mm^2)	18 mm^2 (range 6 mm^2 to 34 mm^2)
Stromal stability/melting activity	14/16	7/9
Cellular infiltrates	16/16	6/9
Malacic corneal material	10/16	3/9
Schirmer tear test	18 ± 2 mm	19 ± 2 mm
Tonometry	14 ± 3 mmHg	16 ± 2 mmHg
Eye to treat	9 left and 7 right	4 left and 5 right

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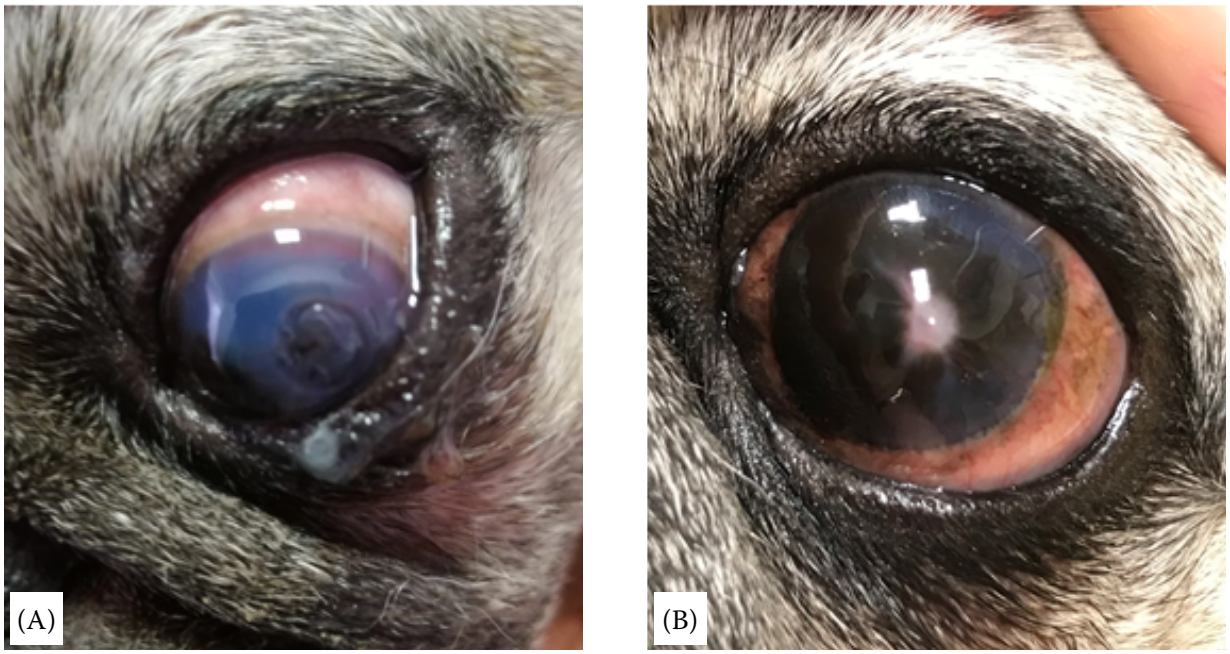

Figure 1. (A) Photograph of the cornea of a dog before undergoing PACK-CXL. The culture and sensitivity analysis revealed the presence of multi-resistant *Staphylococcus* spp. and *Enterococcus* spp. (B) At day 21, the corneal ulcer was filled with granulation tissue and the corneal fluorescein staining was negative. The corneal tissue surrounding the lesion was clear

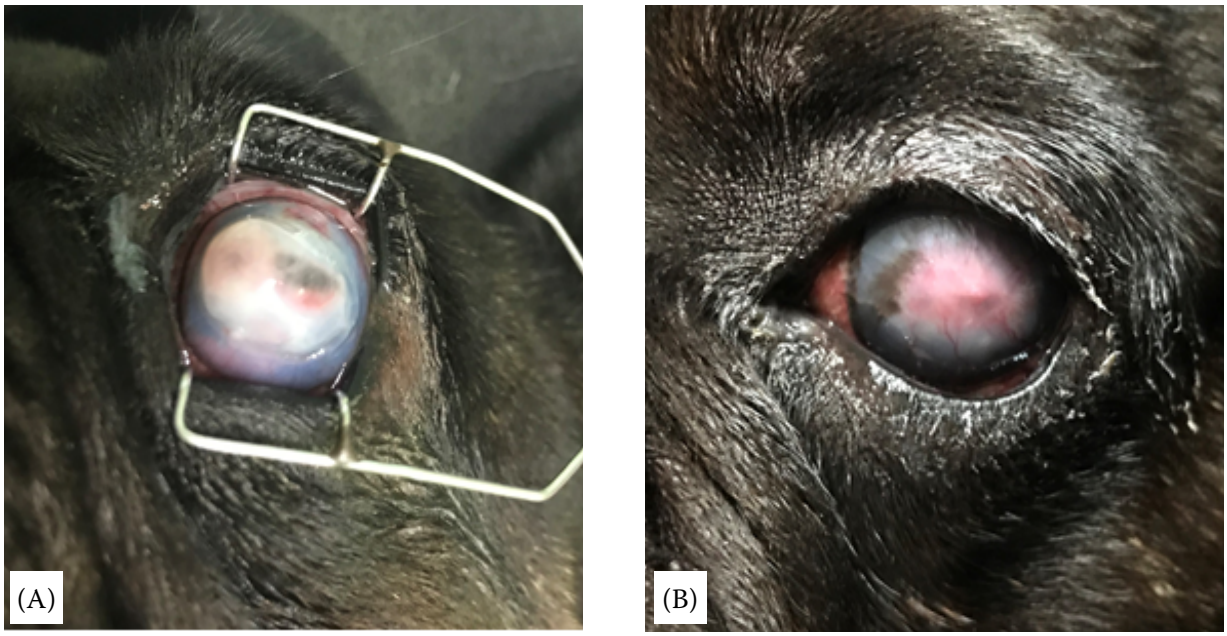

Figure 2. (A) Photograph of the cornea of a dog before PACK-CXL. The culture and sensitivity analysis revealed the presence of *Staphylococcus* spp. (B) At day 14, the corneal ulcer was filled with granulation tissue and the fluorescein staining was negative. The corneal tissue surrounding the lesion was clear

In the study group (Figures 1 and 2), all the dogs achieved complete corneal healing by 20.5 ± 7.8 days ($P < 0.01$) with a single corneal phototherapy application; no recurrence was observed during the follow-up.

In the control group, only two out of eight dogs achieved a clinical resolution by day 10 and day 32. The mean clinical score in both groups during the first 14 days of follow-up is shown in Table 3 and Figure 3.

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Table 3. Clinical score in all the enrolled dogs

Clinical score	Dogs treated by PACK-CXL (study group)	Dogs treated by topical medical therapy (control group)	*P*-value
Enrolment	9.00 ± 3.12	12.14 ± 3.18	0.08
Day 1	5.25 ± 4.68	11.86 ± 2.85	< 0.01
Day 3	2.13 ± 2.75	12.29 ± 3.04	< 0.01
Day 7	1.50 ± 1.77	9.71 ± 2.06	< 0.01
Day 14	0.00	9.57 ± 2.57	< 0.01

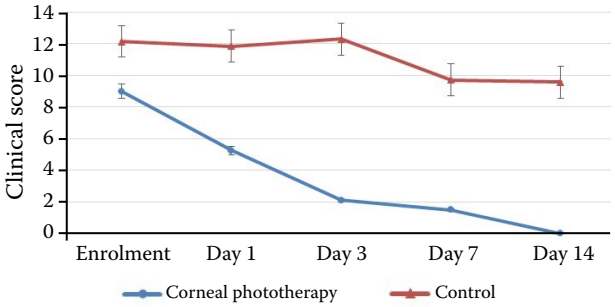

Figure 3. Mean clinical score in the study (blue) and control (red) group in the first 14 days of the follow up
Bars represent ± SD. In the study group, the clinical score, on average, decreased by 4 times (from 9.0 to 2.1) during the first three days after treatment; no changes were reported in the control group in the same period (from 12.1 to 12.3). At day 14, the mean clinical score was 0.0 and 9.6 ± 2.6 in the study and control group, respectively

At enrolment, no statistical difference was found between the study and control group ($P = 0.08$). The mean clinical score significantly improved 24 h after the corneal phototherapy and at day 7, the clinical score decreased by 84% in the said group. In the control group, the mean clinical score was statistically significantly higher than the study group ($P < 0.01$) from day 1 onwards and the mean clinical score did not change in six out of eight participants.

The corneal thickness at the ulcer level ranged between 494 and 574 µm at enrolment and did not differ significantly between the groups (522 ± 18 µm and 532 ± 22 µm in the study and control group, respectively). In the study group, cellular infiltration of the cornea was found at enrolment and disappeared seven days after treatment in all the cases. The mucopurulent discharge and corneal oedema disappeared 3 to 7 days after the corneal phototherapy; corneal vascularisation was present at enrolment and disappeared between day 7 and day 30 after treatment in all the dogs. It is worth noting that the corneal phototherapy was responsible for the appearance of corneal neoangiogenesis, which resolved by itself after epithelial closure, within 3 days after treatment. No endothelial phototoxicity damage was found after the corneal phototherapy in any case.

DISCUSSION

In this study, PACK-CXL has been shown to provide a successful treatment outcome for treating corneal ulcers in dogs. The standardised UV-A irradiation protocol consisting of 30 mW/cm^2 for 3 min (delivering a total energy dose of 5.4 J/cm^2) after enriching the cornea with a hypotonic riboflavin solution, was effective in all the treated cases (22/22), including those previously randomised to receive the topical medical therapy (6/6).

The PACK-CXL protocol used in this study has been validated in pre-clinical studies (Lombardo et al. 2015; Lombardo et al. 2016; Lombardo and Lombardo 2019). Experimental data have shown that it is imperative to fully enrich the corneal tissue with riboflavin before the UV-A irradiation in order to make the treatment become effective (Lombardo et al. 2015; Lombardo et al. 2016; Lombardo and Lombardo 2019). In addition, the 30 mW/cm^2 UV-A irradiance for 3 min (5.4 J/cm^2 energy dose) has been shown to be effective for the treatment of corneal ulcers in *ex vivo* animal models (Perazzi et al. 2018; Perazzi et al. 2020). In addition, the PACK-CXL treatment was effective irrespective of the presence of bacteria or not.

As a limit of the present study, no validated scale was used to evaluate the corneal ulcers in the dogs and a scale developed by Famose (2016) to assess the bullous keratopathy was adapted for the scope of this study. The presence of a thick cellular infiltration or corneal oedema did not interfere with the PACK-CXL treatment. In addition, the PACK-CXL

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treatment has also shown to stimulate corneal neoangiogenesis, accelerating and promoting wound healing that subsequently disappears once the corneal epithelium was restored. The PACK-CXL treatment did not require any additional topical or surgical therapy and no endothelial decompensation was observed during follow-up. In laboratory studies, enriching the cornea with riboflavin has been shown to be most important factor in improving the treatment efficacy and protecting the endothelium from hazardous UV-A energy levels (Lombardo et al. 2015; Lombardo et al. 2016; Lombardo and Lombardo 2019).

Previous studies have demonstrated the effect of riboflavin/UV-A therapy on bacterial cultures and corneal disorders both in experimental and clinical settings (Martins et al. 2008; Kamaev et al. 2012; Pot et al. 2015; Famose 2016). Pot et al. (2014) have compared the effect of a PACK-CXL therapy on melting keratitis in comparison with a medical therapy in forty-nine eyes of both dogs and cats; at enrolment, the ulcer depth was about 50% of the corneal thickness in all the cases. The riboflavin/UV-A therapy protocol was the same that was previously used by Spiess et al. (2014) and consisted of the application of 0.1% riboflavin drops onto the cornea for 30 min followed by 3 mW/cm^2 UV-A irradiance for 30 min (total energy dose 5.4 J/cm^2). Due to similar failure rates in the control and PACK-CXL therapy groups, the authors concluded that such a therapy may be considered as an adjunctive therapy for melting keratitis. In the present study, the corneal ulcers at enrolment had a similar clinical presentation as those in Pot et al. (2014) in terms of the ulcer depth and size, stromal stability and melting activity. Famose (2016) have also used a high-irradiance riboflavin/UV-A therapy protocol to treat corneal ulcers in eight dogs; the protocol included the application of a dextran enriched riboflavin solution for 30 min followed by UV-A irradiation at 30 mW/cm^2 for 3 min (total energy dose 5.4 J/cm^2), as previously used for treatment of bullous keratopathy by the same authors (Famose et al. 2014b). The authors achieved complete corneal healing in all the cases by 15 days.

The results of the present study are in good ageeement with previous studies regarding the effect of a PACK-CXL treatment to inactivate ocular surface pathogens and confirmed the ability of a high UV-A irradiance corneal phototherapy protocol to halt the progression of melting and to promote corneal stabilisation and wound healing (Hellander-Edman et al. 2013; Pot et al. 2014; Spiess et al. 2014; Famose 2015; Pot et al. 2015).

Further clinical studies are needed in order to confirm the efficacy of the PACK-CXL treatment in treating corneal ulcers in dogs and establish whether it may represent a valid stand-alone option for the management of these severe conditions (Pot et al. 2014; Famose 2015; Pot et al. 2015).

Riboflavin/UV-A corneal phototherapy may represent a valid stand-alone therapy for corneal ulcers in veterinary medicine and can be considered as an alternative to the current medical options. PACK-CXL treatments promote the appropriate use of antimicrobials and relieve owners of the frequent administration of topical or systemic therapies.

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Conflict of interest

Giuseppe Lombardo and Marco Lombardo are co-founders and shareholders of Vision Engineering Italy srl.

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