A Rabbit Model of *Acanthamoeba* Keratitis: Use of Infected Soft Contact Lenses After Corneal Epithelium Debridement With a Diamond Burr

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Purpose. To develop a rabbit model of *Acanthamoeba* keratitis (AK) as the best method to reproduce the natural course of this disease.

Methods. To induce AK, infected contact lenses (1000 amoebae/mm^2, 90% trophozoites) were placed over the previously debrided corneal surface, in combination with a temporary tarsorrhaphy. Environmental and clinical strains of *Acanthamoeba* spp. (genotype T4) were used. Three groups (1L, $n = 32$; 2L–21d, $n = 5$; 2L–3d, $n = 23$) were established according to the number of contact lenses used (1L, 1 lens; 2L–21d and 2L–3d, 2 lenses) and the placement day of these (1L, day 1; 2L–21d, days 1 and 21; 2L–3d, days 1 and 3). The infection was quantified by a clinical score system and confirmed using corneal cytology and culture, polymerase chain reaction and histopathologic analysis.

Results. The infection rate obtained was high (1L, 87.5%; 2L–21d, 100%; 2L–3d, 82.6%), although no clinical signs were observed in the 50% of the infected animals in group 1L. Among groups, group 2L–3d showed more cases of moderate and severe infection. Among strains, no statistically significant differences were found in the infection rate. In the control eyes, cross infection was confirmed when a sterile contact lens was placed in the previously debrided corneas but not if the eye remained intact.

Conclusions. The combination of two infected contact lenses after corneal debridement seems to be an alternative model, clinically and histopathologically similar to its human counterpart, to induce the different AK stages and reproduce the course of the disease in rabbits.

Keywords: *Acanthamoeba*, corneal infection, keratitis, animal model, rabbit

Most research about *Acanthamoeba* keratitis (AK) has been performed in vitro;[1–3] however, the conclusions obtained cannot always be extrapolated to clinical situations. The development, validation and use of an in vivo experimental animal model similar to the course of this disease, is essential for a knowledge improvement of different key aspects, such as its risks factors, the clinical, immunologic, biological, and pathologic characteristics, the diagnostic possibilities, and especially for in vivo testing of new therapeutic agents.[3,4]

In vivo AK models have been described using species, such as rabbit, rat, mouse, hamster, cat, and pig (Font RL, et al. *IOVS* 1981;20:ARVO Abstract 8; Ledbetter EC, et al. *IOVS* 2012;53:ARVO E-Abstract 6146).[5–12] To induce AK, intrastromal injection is a fast method to achieve high infection rates but with severe signs and complications, such as endophthalmitis and even death (Font RL, et al. *IOVS* 1981;20:ARVO Abstract 8).[7,9,11–15] Other techniques, including subconjunctival injection, irrigation of abraded cornea with parasite-rich inocula, deposition of parasite suspensions into the conjunctival cul-de-sac after tarsorrhaphy, and microinjections between the corneal epithelium and Bowman's layer, have been reported (Ledbetter EC, et al. *IOVS* 2012;53:ARVO E-Abstract 6146).[6,9,12,13] Nevertheless, considering the contact lens wear as the main risk factor, animal models induced through contaminated contact lenses over previously abraded or scratched corneas seem to be the most realistic method and this has been widely used (Ledbetter EC, et al. *IOVS* 2012;53:ARVO E-Abstract 6146).[4,5,9,10,12,16–30]

The aim of this study was to develop a rabbit model of AK as the best method to reproduce the natural course of this disease, allowing further pathogenicity studies and assess new therapeutic strategies. Bandage contact lenses infected with *Acanthamoeba* trophozoites and cysts were placed onto the ocular surface after epithelial debridement with a diamond burr unit.

Methods

Animals and Ethics Requirements

Male New Zealand white rabbits (1.8–2.2 kg) obtained from the Animal Experimentation Service of the University of Zaragoza

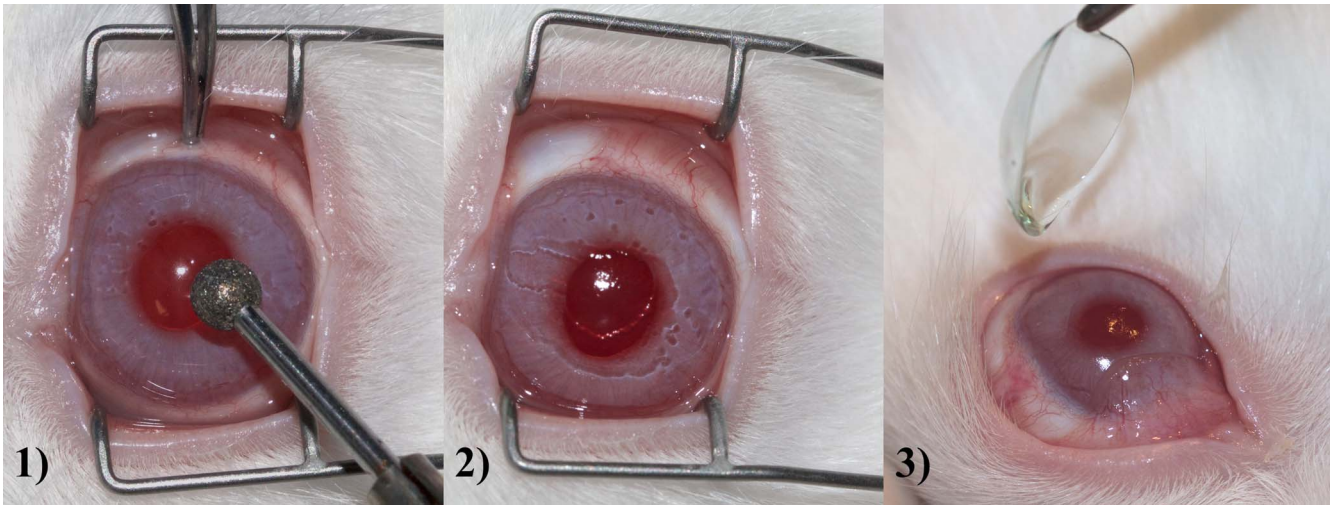

FIGURE 1. Development process of *Acanthamoeba* keratitis in a rabbit model: (**1**) epithelial debridement using a diamond burr unit, (**2**) corneal epithelium partially debrided, and (**3**) placement of the parasite-laden lens onto the corneal surface.

were used. All animals were healthy and free of clinically observable ocular diseases. This research was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were performed under Project License 15/13 approved by the in-house Ethics Committee for Animal Experiments of the University of Zaragoza. The care and use of the animals were performed in accordance with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Acanthamoeba Isolates

Three strains were used: *P31* (GenBank accession No. KY038362) as environmental amoeba isolated from recreational water, and *G* (GenBank accession No. KY038361) and *L* (GenBank accession No. KY038363) as clinical strains isolated via corneal scraping from two patients. All strains were previously identified as belonged to genotype T4.

Culture and Infected Soft Contact Lens Preparation

A 7-day axenic culture in PYG medium was centrifuged (3300*g*; 10 minutes) and the pellet was resuspended in new PYG medium up to a concentration of 5×10^5 cells/ml. The number and percentage of trophozoites and cysts were determined using a Neubauer chamber.

One milliliter of a dilution to obtain 100 (3.8×10^4 cells/ml) or 1000 (3.8×10^5 cells/ml) amoebae/mm^2, with 90% trophozoites, was deposited on the inner surface of a silicone-hydrogel bandage contact lens (Air Optix Night & Day Aqua; Alcon Cusi, Barcelona, Spain) previously washed with PBS and stored at 27°C to 30°C until its placement. Control lenses were only washed with PBS.

Development of the *Acanthamoeba* Keratitis Model

Corneal Infection. Each animal was intramuscularly anesthetized with medetomidine (0.14 mg/kg, Medeson; Uranovet, Barcelona, Spain), ketamine (20 mg/kg, Imalgene 100 mg/ml; Merial Laboratorios, Barcelona, Spain), and butorphanol (0.4 mg/kg, Torbugesic; Fort Dodge Veterinaria, Girona, Spain), and topically with 0.1% tetracaine hydrochloride and 0.4% oxybuprocaine eye drops (Colircusi anestésico doble; Alcon Cusi). The left eyes were considered as control with debridement and noninfected soft contact lens, or intact without debridement, and the right eyes were infected. An eyelid speculum was placed in the fornix and the corneal epithelium was completely debrided using a battery-operated handheld diamond burr unit (AlgerBrush II; The Alger Company, Lago Vista, TX, USA) with 3.5 mm burr medium grit. Following the aseptic placement of the control or the parasite-laden lens onto the corneal surface, the eyelids were closed by a temporary central tarsorrhaphy (6/0 nonabsorbable monofilament polyamide, Dafilon; B-Braun VetCare, Barcelona, Spain; Fig. 1). An Elizabethan collar was placed up to the tarsorrhaphy and contact lenses were removed after 3 days.

A total of 64 animals was divided randomly into 7 experiments. An order was followed (Table 1), modifying the model according to the results previously obtained. The different experiments were grouped by the amoebae concentration contained in the contact lenses (100 or 1000 amoebae/mm^2), the infected contact lenses used (1 or 2), and their placement day (day 1, days 1 and 21, or days 1 and 3) into 4 groups (0, 1L, 2L–21d, and 2L–3d).

Clinical Evaluation. All eyes were monitored by slit-lamp examination (SL-8Z; Topcon, Barcelona, Spain) every 3 days. Digital photographs were taken with a reflex digital camera (EOS 1000D; Canon, Tokyo, Japan) using a macro objective (SP 90 mm F/2.8 Di VC USD 1:1; Tamron, Tokyo, Japan). The degree and severity of the corneal infection was scored according to the criteria described by Van Klink et al.[12] and Ren et al.[9] The clinical score system was based on 4 clinical signs: epithelial defects, corneal edema, neovascularization, and opacity/infiltration. These were graded on a 4-point rating scale: 1 (if $\leq$25% cornea was involved), 2 (if $>$25% but $\leq$50%), 3 (if $>$50% but $<$ 75%), and 4 (if $\geq$75%). Higher clinical scores were related with a worse clinical status and healthy corneas were given a score of 0 in each category. A masked observer evaluated the extent of keratitis. After removing the contact lens, the scores from 4 categories were checked every 2 to 10 days for each eye to yield a possible total score, ranging from 0 to 16: $\leq$5 was classified as mild infection, 6 to 10 as moderate, and $\geq$11 as severe.

Acanthamoeba Keratitis Confirmation

Corneal Cytology and Culture. For confirming the *Acanthamoeba* presence, two corneal cytologies and cultures

Table 1. Animal Group Distribution and Order Followed in the Experimental Model According to the Results Previously Obtained

Group	Experiment	Number of Rabbits (Strain)	Eye	Epithelial Debridement/ Amoebae Concentration (SCL Type and Placement Day)	Total Duration, d
0	1	2 (*P31*); 2 (*G*)	Control	DB + nSCL	15
			Infected	DB + 1 iSCL 100 amoebae/mm^2 (day 1)	
1L	2	1 (*P31*); 1 (*G*)	Control	Intact	9
			Infected	DB + 1 iSCL 1000 amoebae/mm^2 (day 1)	
	3	5 (*P31*); 5 (*G*)	Control	DB + nSCL	15
			Infected	DB + 1 iSCL 1000 amoebae/mm^2 (day 1)	
	4	10 (*P31*); 10 (*G*)	Control	Intact	28
			Infected	DB + 1 iSCL 1000 amoebae/mm^2 (day 1)	
2L–21d	5	2 (*P31*); 3 (*G*)	Control	Intact	44
			Infected	DB + 2 iSCL 1000 amoebae/mm^2 (days 1 and 21)	
2L–3d	6	4 (*P31*); 4 (*G*); 2 (*L*)	Control	Intact	24
			Infected	DB + 2 iSCL 1000 amoebae/mm^2 (days 1 and 3)	
	7	13 (*P31*)	Control	Intact	28
			Infected	DB + 2 iSCL 1000 amoebae/mm^2 (days 1 and 3)	

DB, diamond burr; SCL, soft contact lens; iSCL, infected soft contact lens; nSCL, noninfected soft contact lens.

of each eye were performed at different times: immediately after removing the tarsorrhaphy and just after the euthanasia. A milliliter of 0.9% NaCl was instilled in the infected and control eyes to collect the ocular discharge. A scraping of the corneas with epithelial defects or infiltrates was carefully performed using a cytobrush and the sample was mixed with the previous 0.9% NaCl solution. Following its centrifugation (9200*g*; 5 minutes), a sediment drop was wet mounted with 10% potassium hydroxide and examined by light microscopy (AZ-100; Nikon, Kanagawa, Japan). Another drop was deposited in a plate of nonnutritive agar with *Escherichia coli* and incubated at 30°C for 30 days. The evaluation of amoeba viability was based on the daily observation of trophozoites and cysts in the agar plate by light microscopy. The presence of trophozoites beyond the limits of the seeding area indicated growth.

Polymerase Chain Reaction (PCR). A corneal scraping was resuspended in 200 µl of lysis buffer containing 0.2 mg of proteinase and incubated overnight at 56°C. The DNA of this suspension was extracted using a commercial kit (Stool DNA isolation kit; Norgen Biotek, Ontario, Canada). An *Acanthamoeba* species–specific PCR, including primer pair JDP1/JDP2 targeted toward 18S ribosomal DNA stretch ASA, was performed to detect *Acanthamoeba* DNA.[31] Polymerase chain reaction products were electrophoresed on 1.5% agarose gel stained with a solution of ethidium bromide and visualized under UV light.

Histopathologic Analysis. All animals were humanely euthanized with intravenous sodium pentobarbital (150 mg/kg, Dolethal; Vétoquinol, Madrid, Spain) at different times (Table 1). The whole globes were harvested and under a dissecting microscope, corneas were excised and fixed in 10% neutral-buffered formalin. Paraffin sections 5 µm thin were stained with hematoxylin-eosin and periodic acid-Schiff according to the standard methods, analyzed by light microscopy and photographed (CapturePro 2.5; JENOPTIK Laser Technology, Jena, Germany).

Statistical Analysis

The quantitative variables were described with the mean, standard deviation, median, interquartile range, minimum, and maximum values, and the qualitative variables with the number of animals and percentages. A normal distribution was tested with the Shapiro-Wilk normality test. The clinical signs between groups at the different days were compared with the unpaired Kruskal-Wallis and Mann-Whitney *U* tests, and between infected and control eyes with the paired Wilcoxon test. The Pearson's χ^2 or Fisher's exact tests were used to correlate the strains and infection variables. A survival analysis allowed assessing the disappearance of the clinical signs. The cumulative survival probability was calculated by the Kaplan-Meier method, and the Breslow Tarone test was used to compare the survival curves between groups and between infected and control eyes. $P < 0.05$ was considered as significant level. All statistical analyses were performed using SPSS 22.0 (SPSS, Chicago, IL, USA).

Results

Infected Eyes

The infection was confirmed based on AK signs, corneal cytology and culture, PCR and histopathologic study (Fig. 2). The infection rate was high and no statistically significant differences were observed between groups for the infected and noninfected animals (1L vs. 2L–21d, $P = 0.544$; 1L vs. 2L–3d, $P = 0.447$; 2L–21d vs. 2L–3d, $P = 0.432$; Table 2). In group 1L, 50% of infected animals showed no clinical signs. Regarding the severity of these, more moderate and severe infection cases were observed in group 2L–3d, including two cases of keratomalacia and endophthalmitis.

Pathogenicity of the *Acanthamoeba* Strains. There were no statistically significant differences in the number of infected animals with each strain (group 1L, $P = 0.700$; in group 2L–21d all animals were infected; and group 2L–3d, $P = 0.426$). Furthermore, no statistically significant differences were observed between infected animals with and without clinical signs (group 1L, $P = 0.127$; group 2L–21d, $P = 1.000$; in group 2L–3d all animals showed clinical signs). The infection rate obtained is shown in the Table 3.

Clinical Signs of the AK. Figure 3 shows nine representative AK cases. The progress over time and score of the four signs checked, and the mean (standard deviation), median (interquartile range), minimum, and maximum values corresponding to each group are shown in Figure 4 and Table 4, respectively.

A cumulative survival analysis was performed for each group. When the signs were assessed together, in group 1L half of the animals presented no signs after 20 days (95%

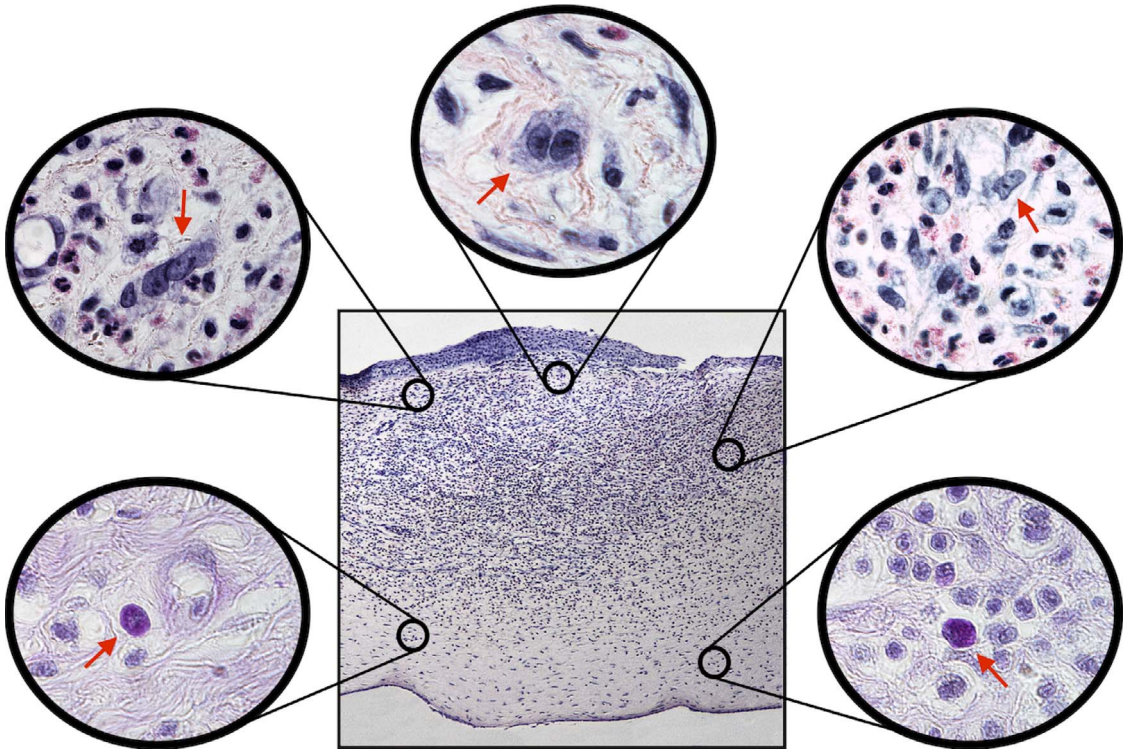

Figure 2. Histologic images of a severe case of *Acanthamoeba* keratitis. In the *center*, a general view of the cornea shows a great cellular infiltrate, mainly in the anterior stroma (×40). Additionally, *arrows* indicate the protozoal organisms in amplified sections stained with hematoxylin-eosin (*three upper images*) and periodic acid-Schiff (*two lower images*; ×800). Marked eosinophilic and neutrophilic infiltrates are evident in all sections.

confidence interval [CI95%]: 17.9–22.1), 8 days before the total sample analyzed. In group 2L–21d and 2L–3d, the median time for the four signs to disappear would be longer than in group 1L (Fig. 5), and this difference was statistically significant (Breslow = 15.75, $P < 0.001$). In these groups, the signs persisted at the end of the experiment. Figure 4 confirms this fact, with a high value after placing each infected contact lens due to the epithelial lesion induced. This also was detected on the days 24 and 6 of groups 2L–21d and 2L–3d, respectively, 3 days before placing the second contact lens. While in most of the group 1L animals the clinical signs eventually disappeared, these persisted in the last day in the other groups. When the signs were assessed separately, in all groups the trend was the same (epithelial defects: Breslow = 8.96, $P = 0.011$; corneal edema: Breslow = 28.74, $P < 0.001$; corneal neovascularization: Breslow = 26.89, $P < 0.001$; corneal opacity/infiltration: Breslow = 23.84, $P < 0.001$).

Table 2. Infection Rate (Number and Percentage of Animals) in Each Group of the Rabbit Model of AK

	Group 1L		Group 2L–21d		Group 2L–3d	
	n	%	*n*	%	*n*	%
Noninfected	4	12.5	0	0	4	17.4
Infected without clinical signs*	16	50	1	20	0	0
Infected with mild infection†	9	28.1	2	40	4	17.4
Infected with moderate infection‡	3	9.4	2	40	11	47.8
Infected with severe infection§	0	0	0	0	4	17.4
Total	32	100	5	100	23	100

* Total score = 0.
† Total score ≤5.
‡ Total score = 6 to 10.
§ Total score ≥11.

Control Eyes

No clinical signs were observed in the intact control eyes and the laboratorial diagnosis was negative for all. Experiment 1 was not used for avoiding statistical errors (its infected contact lens contained 100 amoebae/mm^2). Therefore, only the control (epithelial debridement and noninfected contact lenses) and infected (epithelial debridement and infected contact lenses with 1000 amoebae/mm^2) eyes of experiment 3 were considered (Fig. 6; Table 5), being the clinical signs assessed on days 3, 9, and 15. In the infected and control eyes, the distributions of the infection and clinical signs were homogeneous with the two strains (Fischer's exact test; $P = 1.000$). Cross infection was confirmed in the control eyes (Fig. 7). The infection rate obtained in each eye is shown in Table 6.

The cumulative survival analysis for all clinical signs together showed that in the infected eye, half of the animals presented no signs after 9 days, whereas in the control eye this was not observed at the end of the experiment, and the difference was statistically significant (Breslow = 4.91, $P = 0.026$; Fig. 8).

Discussion

All experiments in the present study were performed to develop a rabbit model of AK using infected contact lenses. The contact lens wear and presence of corneal injury are

Table 3. Infection Rate (Number and Percentage of Animals) Depending on the Strain in Each Group of the Rabbit Model of AK

	Group 1L				Group 2L–21d				Group 2L–3d					
	G		*P31*		*G*		*P31*		*G*		*P31*		*L*	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Noninfected	2	12.4	2	12.4	0	0	0	0	0	0	4	23.5	0	0
Infected without clinical signs	6	37.6	10	62.6	1	33.3	0	0	0	0	0	0	0	0
Infected with clinical signs	8	50	4	25	2	66.7	2	100	4	100	13	76.5	2	100
Total	16	100	16	100	3	100	2	100	4	100	17	100	2	100

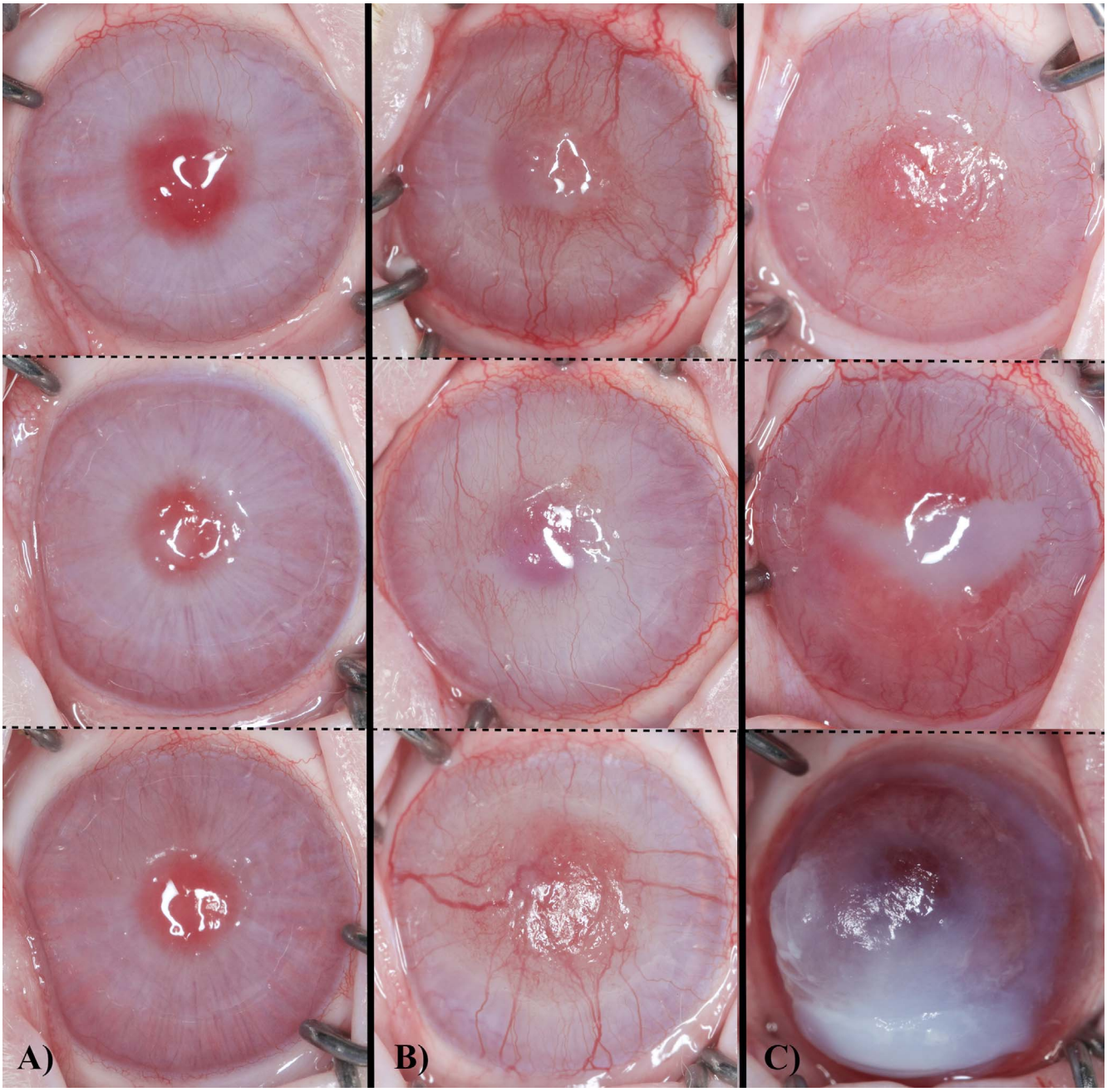

Figure 3. Representative cases of AK (in columns): (**A**) group 1L with mild infection ($n = 3$, 28th day), (**B**) group 2L–21d with moderate infection ($n = 3$, 44th day), and (**C**) group 2L–3d with severe infection ($n = 3$, 28th day). Group 2L–3d showed the highest clinical scores of epithelial defect, corneal edema, neovascularization, and opacity/infiltration, with the only two cases of keratomalacia (*lower right corner*) and endophthalmitis noted.

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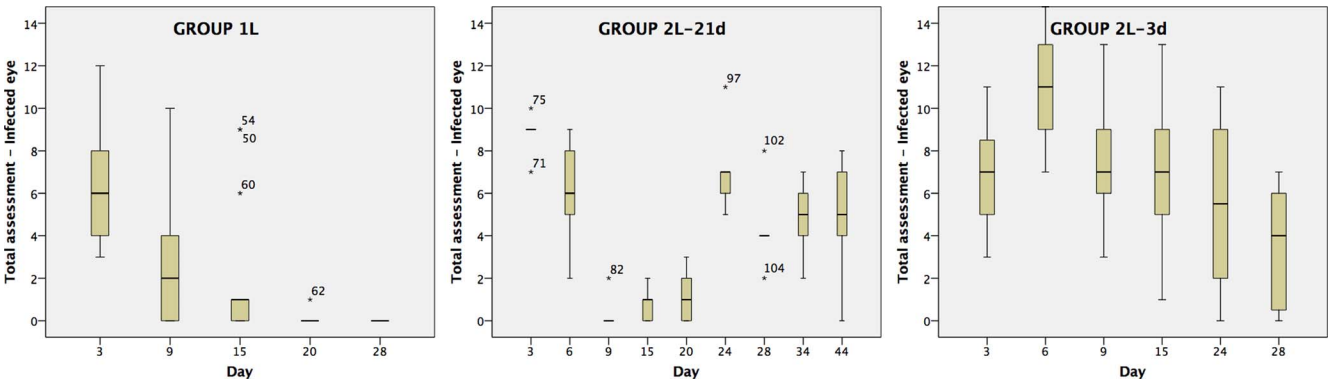

FIGURE 4. Box plots representing the distributions. Median (interquartile range [IQR]), minimum (Min), and maximum (Max) of the four clinical signs assessed (infected eyes) at different days. In all groups, the total value was initially higher (epithelial debridement and placement of the contact lens), including on the 24th and 6th day of groups 2L–21d and 2L–3d, respectively (3 days before the placement of the second infected contact lens).

important risk factors.[1–3,9,12,16] The animal models induced through contaminated contact lenses have been developed with hamsters, rats, mice, pigs, cats, and rabbits (Ledbetter EC, et al. *IOVS* 2012;53:ARVO E-Abstract 6146).[4,5,9,10,12,16–30] In the majority of them, the cornea was partially or completely abraded with a sterile cotton applicator, scratched with a syringe needle or scraped with a blade before placing the contact lens,[4,5,9,10,16–30] because van Klink et al.[12] and Ledbetter et al. demonstrated that this lesion was essential to allow the penetration of *Acanthamoeba* into the cornea (Ledbetter EC, et al. *IOVS* 2012;53:ARVO E-Abstract 6146). In this study, the epithelial debridement was performed with a diamond burr unit, used for removing the nonadherent epithelial tissue in the management of spontaneous chronic corneal epithelial defects in dogs and horses.[32,33]

Genotype T4 is the most commonly found in the environment and also the cause of AK.[2] This is used in the great majority of the AK models published, predominating clinical strains. To our knowledge, this is the first study where this disease has been developed successfully through an environmental strain. Regarding the L strain, this was included in two animals after it was isolated from a patient and received in our laboratory for its characterization and classification during the experiment.

The exposure time to *Acanthamoeba* also seems to be an important factor. In this study, the contact lenses were removed after 3 days exposure (6 days when two were placed), obtaining similar or better infection rates in all groups than in other studies. Nevertheless, these results could be influenced by a higher final concentration (2×1000 amoebae/mm^2) in groups 2L–21d and 2L–3d. Experimentally, van Klink et al.[12] described an increase of animals with AK signs after 7 or 10 days exposure to the lens. Others corroborated this in hamsters, cats, and pigs (Ledbetter EC, et al. *IOVS* 2012;53:ARVO E-Abstract 6146).[19,23,24,26–28,30] However, good results have been shown using exposure times of 24 hours in rats and mice, 4 to 5 days in pigs and 3 to 6 days in hamsters, but not with 4 to 5 days in rabbits.[4,5,9,10,16–18,20–22,25,29]

Contact lenses serve as a mechanical vector for transmitting trophozoites to the corneal surface, facilitating parasite binding to the epithelium. The adhesion is regulated mainly by mannosylated glycoproteins, and the use of contact lenses and presence of a previous abrasion or mild trauma have been correlated with an increased expression of these proteins on the epithelium, promoting the adhesion.[12,16] Different amoeba vehicles, such as dialysis membrane tubing, filter paper, or hydrophilic soft contact lenses, have been used in AK models (Ledbetter EC, et al. *IOVS* 2012;53:ARVO E-Abstract 6146).[4,5,9,10,12,16–30] In this study, commercial

TABLE 4. Mean (SD), Median (IQR), Minimum (Min), and Maximum (Max) of the Four Clinical Signs Assessed (Infected Eyes) at Different Days

Group	Day 3	Day 6	Day 9	Day 15	Day 20	Day 24	Day 28	Day 34	Day 44
1L									
Mean (SD)	6.1 (2.6)	Not evaluated	2.9 (4.1)	2.2 (3.5)	0.1 (0.4)	Not evaluated	0.0 (0.0)	Not evaluated	Not evaluated
Median (IQR)	6 (4)		2 (4)	1 (4)	0 (0)		0 (0)		
Min–max	3–12		0–9	0–9	0–1		0–0		
2L–21d									
Mean (SD)	8.8 (1.1)	6.0 (2.7)	0.4 (0.9)	0.8 (0.8)	1.2 (1.3)	7.2 (2.3)	4.4 (2.2)	4.8 (1.9)	4.8 (3.1)
Median (IQR)	9 (2)	6 (5)	0 (1)	1 (2)	1 (3)	7 (4)	4 (3)	5 (4)	5 (6)
Min–max	7–10	2–9	0–2	0–2	0–3	5–11	2–8	2–7	0–8
2L–3d									
Mean (SD)	7.5 (2.8)	11.0 (2.5)	7.8 (2.5)	6.9 (3.3)	Not evaluated	5.6 (3.8)	3.5 (2.9)	Not evaluated	Not evaluated
Median (IQR)	7 (4)	11 (4)	7 (3)	7 (5)		5.5 (8)	4 (6)		
Min–max	3–16	7–16	3–13	1–13		0–11	0–7		
P values	0.020*	0.003*	<0.001*	<0.001*	0.150	0.290	0.660	–	–

P values between groups for each day. Unpaired Kruskal-Wallis test was used on the days 3, 9, and 15. Unpaired Mann-Whitney *U* test was used on the days 6, 20, 24, and 28 day.

* Statistically significant values.

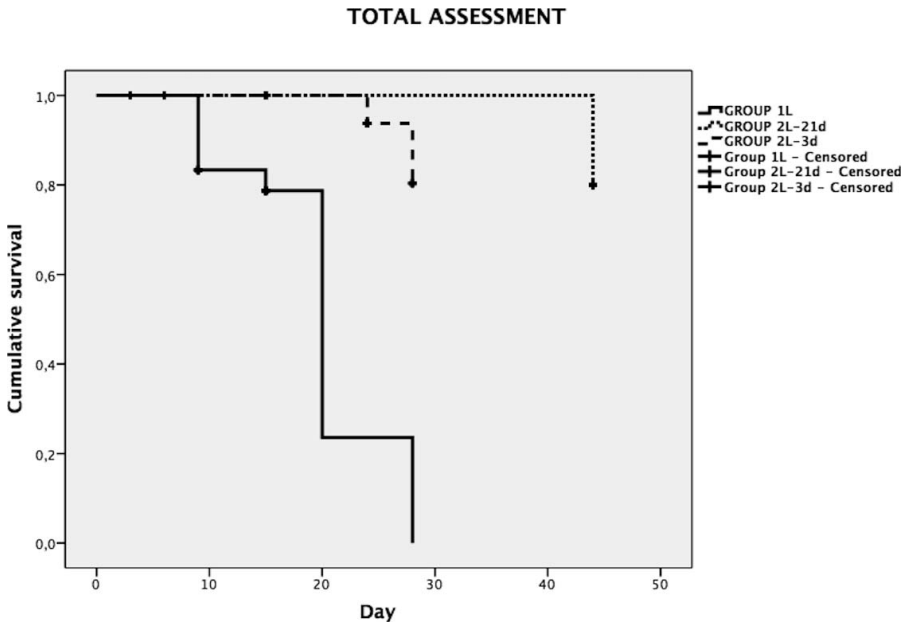

Figure 5. Cumulative survival of the clinical signs assessed (infected eyes) in each group at different days. In group 1L, the clinical signs disappeared completely on day 28; however, in both groups where two infected contact lenses were placed, the signs persisted at the end of the experiment (from days 44 and 28 in groups 2L–21d and 2L-3d, respectively; marked with "+"). In these groups, the clinical scores selected to perform the survival analysis were those observed after removing the second lens (from days 24 and 6 in groups 2L–21d and 2L-3d, respectively).

contact lenses, the most common vehicle in the natural course of the disease in humans, were selected.[1–3] Furthermore, a requirement for *Acanthamoeba* to establish keratitis is the ability to bind to, and invade, the hosts' corneal surface. Different in vitro studies investigating this fact have concluded that the parasite was more capable of binding to the human, hamster, pig, and rabbit corneas.[34–36] Moreover, subsequent studies have determined that this ability in vitro is correlated with in vivo susceptibility to the disease.[5,9,12] However, although this characteristic likely contributes to the rabbit's vulnerability to the naturally acquired AK and supports the use of this animal as a model, no previous successful reports using contact lenses have been published with this species.

To our knowledge, this is the first study where two infected contact lenses in the same eye are used to induce AK in rabbits. Previously, van Klink et al.[28] assessed the susceptibility of hamsters to reinfection in the noninfected contralateral eye, obtaining a 71% rate in the first infection (infected eye) and 75% in the reinfection (contralateral eye). Furthermore, Alizadeh et al.[29] and Leher et al.[30] rechallenged the infected eyes of pigs with a second or even a third contact lens, and in their control groups a 100% rate of infection was observed. In these studies, like in our group 2L–21d, the second lens was placed after the first complete keratitis resolution, but not in group 2L–3d, where it was performed in which the clinical signs still remained (sixth day). A similar infection rate was obtained using one (group 1L, 87.5%) or two (group 2L–21d, 100%, and group 2L–3d, 82.6%) lenses. However, unlike the fact desired in any infection model, the placement of only one lens was correlated with a higher percentage of infected animals without clinical signs (50%), considering these as asymptomatic carriers, and being more difficult to diagnose and susceptible to a recrudescence of active infection from viable cysts. This fact has been widely noted in humans, mainly as consequence of the failure to kill all cysts with anti-amoebic therapies.[2,3] In most of the AK models, confirmation of the infection was based on the

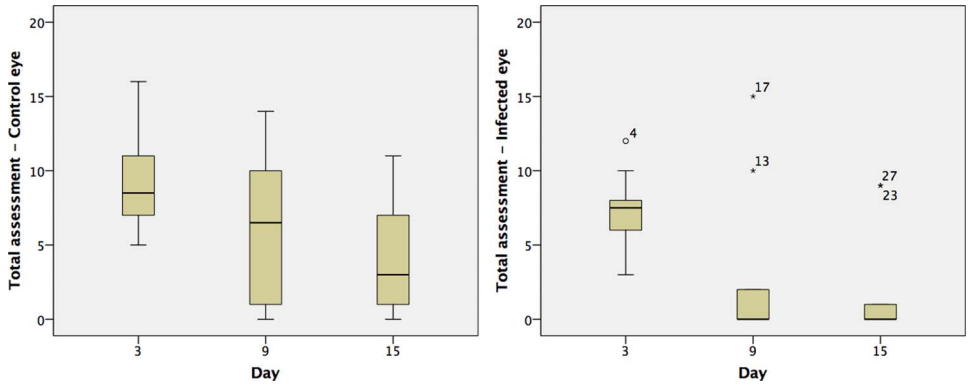

Figure 6. Box plots representing the distributions. Median (IQR), minimum (Min), and maximum (Max) of the four clinical signs assessed (control versus infected eyes) at different days. In both eyes, the total value was initially higher (epithelial debridement and placement of the contact lens), decreasing afterwards.

Table 5. Mean (SD), Median (IQR), Minimum (Min), and Maximum (Max) of the Four Clinical Signs Assessed (Infected Versus Control Eyes) at Different Days

	Day 3	Day 9	Day 15
Infected eye			
Mean (SD)	7.5 (2.4)	2.8 (5.3)	2.0 (3.7)
Median (IQR)	7.5 (3)	0 (4)	0 (3)
Min–max	3–12	0–15	0–9
Control eye			
Mean (SD)	9.2 (3.2)	6.1 (5.1)	4.1 (3.7)
Median (IQR)	8.5 (4)	6.5 (9)	3 (6)
Min–max	5–16	0–14	0–11
P values	0.137	0.213	0.100

P values between groups for each day. Paired Wilcoxon test was used.

Table 6. Infection Rate (Number and Percentage of Animals) Depending on the Strain in the Control and Infected Eyes of the Experiment 3

	Infected Eyes				Control Eyes			
	G		*P31*		*G*		*P31*	
	n	%	*n*	%	*n*	%	*n*	%
Noninfected	0	0	0	0	1	20	1	20
Infected without clinical signs	4	80	3	60	0	0	0	0
Infected with clinical signs	1	20	2	40	4	80	4	80
Total	5	100	5	100	5	100	5	100

presence and severity of the keratitis signs; no laboratorial confirmation of *Acanthamoeba* was performed or this was just done in those showing clinical signs.[4,10,12,16–28,30] Therefore, the results of this study are difficult to compare with these studies.

Regarding the infection rate using infected contact lenses with previous corneal injury, the published data are variable (70% in rats, 33.3%–80% in mice, 66.7%–100% in hamsters, and 100% in cats and pigs; Ledbetter EC, et al. *IOVS* 2012;53:ARVO E-Abstract 6146).[5,9,10,12,23,25,26,28–30] On the other hand, He et al.[5] were not capable to induce AK in rabbits. For all of these models, the total days of clinical evaluation were 21 days in rats, 15 to 21 days in mice, 14 to 35 days in hamsters, 21 days in cats, 28 to 84 days in pigs, and 30 days in rabbits. In this study, they were assessed for 39 to 44 days.

The severity of the clinical signs also is a widely analyzed factor. In group 1L, most of the infected cases with signs were mild (28.1%), in group 2L–21d mild and moderate (40% and 40%, respectively), and in group 2L–3d predominantly moderate (47.8%), including two severe cases of endophthalmitis (to date, not reported using contact lenses) and keratomalacia. The group 2L–3d results seemed to be more consistent with those desired when infection models are performed. The corneas of noninfected and asymptomatic animals returned to their normal appearance within several days after removing the tarsorrhaphy and contact lens. In general, similar clinical and pathologic characteristics also have been cited in previous AK models with contact lenses, regardless of the species used (Ledbetter EC, et al. *IOVS* 2012;53:ARVO E-Abstract 6146).[5,9,12,19,27] However, animals considered infected with clinical signs showed a reestablishment of the epithelial integrity, reduction of the edema and infiltration, and restoration of the corneal clarity up to almost or completely disappeared in many studies.[4,5,9,10,12,16–18,20–22,25,28–30] Therefore, these self-limiting models are controversial because they are far different from human beings. These were classified as asymptomatic although infected in this study, because no clinical signs were observed but the laboratorial diagnosis was positive. Among the infected animals, the severity of the signs also was variable. Most investigators described a moderate or severe keratitis in cats and hamsters; severe in pigs; and mild, moderate, or severe in rats and mice (Ledbetter EC, et al. *IOVS* 2012;53:ARVO E-Abstract 6146).[4,5,9,10,12,16–23,25,26,29,30] However, the clinical scoring criteria followed were not standardized.

In some AK models using infected contact lenses, another sterile lens was placed in the contralateral eye after abrading or not the cornea, or a different control group of animals was established receiving only corneal abrasion in one eye or *Acanthamoeba*-free contact lens after abrasion remaining the contralateral eye intact.[5,9,12,22,29] In most of them, the control eye remained intact and no other control groups were considered. Based on this, two types of control eyes were established. As expected, among those considered intact, no clinical signs were observed and the laboratorial diagnosis was negative. However, when a noninfected contact lens was placed after debriding the cornea, cross infection was confirmed. This fact has not been reported previously to our knowledge, but corroborates that the presence of corneal injury is correlated with the possibility of developing AK. This

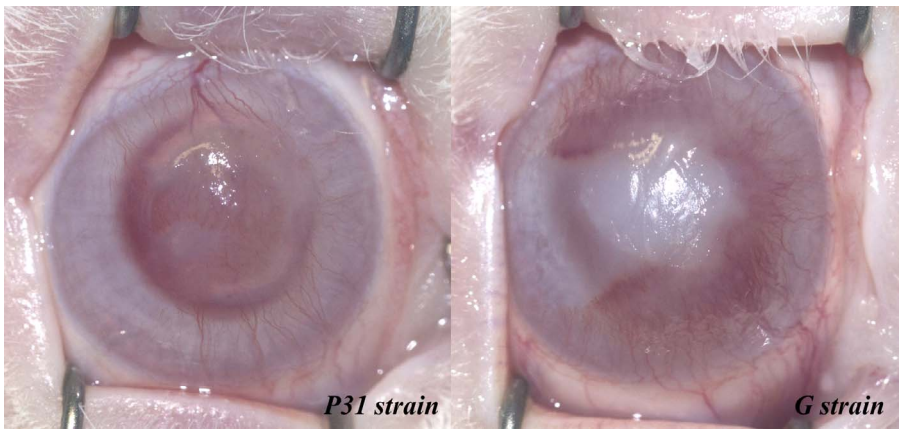

Figure 7. Control eyes after complete epithelial debridement and placement of a noninfected contact lens. Regardless of the *Acanthamoeba* strain, cross infection was confirmed in the right eyes (belonged to experiment 3 in group 1L) on day 15, showing clinical signs of keratitis similar to other infected eyes.

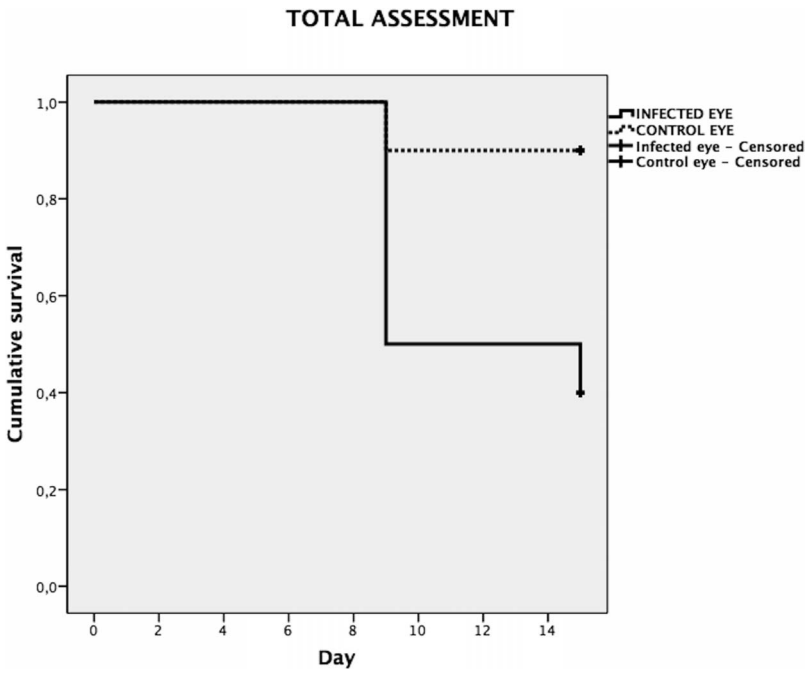

Figure 8. Cumulative survival of the clinical signs assessed (control versus infected eyes) in each group at different days. In both eyes, the signs persisted from day 15 (marked with "+").

cross infection could be due to the self-grooming behavior in this species, for which they use the forelegs.

Rabbits have been chosen because they are easier to operate, manipulate, and work with in a laboratory setting, less expensive to maintain in large numbers, and inexpensive (compared to larger species), inbred strains are readily available, and only a previous unsuccessful study had been described using contact lenses. The smaller globe size of rats, mice, or hamsters is more difficult to examine in vivo and experimentally handle. Furthermore, some of them have demonstrated a self-limiting development of AK, resulting in a clinical ocular disease dissimilar to that observed in humans.

In conclusion, the use of infected contact lenses after epithelial debridement results in a reliable infection rate in rabbits, being an alternative model to induce AK and reproduces its course. To our knowledge, this is the first rabbit model successful in the development of experimentally AK using contact lenses, being also not self-limiting. Thus, the rabbit is not only a model of infection, but it also is a model of disease clinically and histopathologically similar to its human counterpart.

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