The Ocular Surface 18 (2020) 64–73

Contents lists available at ScienceDirect

The Ocular Surface

journal homepage: www.elsevier.com/locate/jtos

Original Research

Repetitive magnetic stimulation protects corneal epithelium in a rabbit model of short-term exposure keratopathy

Ifat Sher[a,b], Adi Tzameret[a,b], Zehavit Goldberg[a,b], Ettel Bubis[a,b], Noa Avni-Zauberman[a], Ofra Kalter-Leibovici[b,c], Arie L. Marcovich[d,e], Guy Ben Simon[a,b], Ygal Rotenstreich[a,b,*]

[a] Goldschleger Eye Institute, Sheba Medical Center, Tel Hashomer, Israel
[b] Sackler Faculty of Medicine, Tel Aviv University, Israel
[c] Unit of Cardiovascular Epidemiology, Gertner Institute for Epidemiology and Health Policy Research, Ramat Gan, Israel
[d] Department of Plant Sciences and Environmental Health, The Weizmann Institute of Science, Rehovot, Israel
[e] Department of Ophthalmology Kaplan Medical Center, Rehovot, Israel

ARTICLE INFO

Keywords:
SD-OCT
Exposure keratopathy
Corneal defects
Epithelial erosions
Fluorescein
Magnetic stimulation
RMS
TMS

ABSTRACT

Purpose: To investigate the effect of repetitive magnetic stimulation (RMS) on corneal epithelial permeability in a rabbit model of exposure keratopathy.

Methods: 61 female New Zealand White (NZW) rabbits were treated on one eye with repetitive magnetic stimulation (RMS) at a frequency of 20 Hz for 15 min. The other eye was untreated. Rabbit eyes were kept open for 2 h to induce acute corneal desiccation. The extent of fluorescein corneal staining was evaluated using *EpiView* software and the concentration of fluorescein in the anterior chamber was determined by a fluorometer. Safety was evaluated by electroretinogram, spectral domain optical coherence tomography (SD-OCT) and histopathology. Expression pattern of corneal cell markers was determined by immunofluorescence.

Results: A significant decrease in fluorescein concentration in the anterior chamber (54 ± 8.4 ng/ml vs. 146.5 ± 18.6 ng/ml, $p = 0.000001$) and in corneal surface fluorescein staining score (1.7 ± 0.2 vs. 4.6 ± 0.6, $p = 0.00001$) was obtained in RMS-treated eyes compared with control eyes, respectively. RMS treatment reduced by nearly 4 fold the percentage of corneal area with epithelial erosions by anterior segment SD-OCT. The therapeutic effect was maintained for at least 3 months. Increased expression of epithelial tight junction protein Zo-1 was observed in treated eyes. SD-OCT and histopathology analysis revealed no pathological changes in the treated or non-treated eyes.

Conclusions: RMS treatment decreases epithelial corneal erosions in a rabbit model of exposure keratopathy, with no indication of pathological changes. RMS may present a novel treatment for protection of corneal epithelium from desiccation.

1. Introduction

The maintenance of ocular surface lubrication is critical for proper corneal function, as it protects the non-keratinized corneal epithelium from dehydration, provides nourishment to the avascular cornea and plays a significant role in the refractive power of the cornea [1]. Tear-film production and secretion is tightly controlled by a neural feedback loop which involves the corneal nerves and corneal epithelial cells [2].

Exposure keratopathy is a clinical syndrome characterized by damage to the cornea from dryness due to incomplete eyelid closure (lagophthalmos) or low blink frequency that result in evaporative tear loss and tear film insufficiency. Patients with exposure keratopathy may suffer from epithelial erosions, pain, foreign body or burning sensation, blurring of vision, watering, redness and photophobia. If left untreated, and in severe cases, exposure keratopathy might cause corneal edema, microbial keratitis, corneal perforation, endophthalmitis and vision loss [3–5]. Risk factors for exposure keratopathy include lid malposition, exophthalmos, iatrogenic events such as anesthesia and ocular surgery, trigeminal (V) and facial (VII) cranial nerve palsy, multiple sclerosis and tumors [5–7].

The corneal epithelium is innervated by a dense network of neuronal endings originating from the trigeminal nerve that mediate sensory inputs of mechanical, chemical, and thermal stimuli (reviewed in Ref. [8]). These neurons secrete trophic factors that mediate cell-cell communication in corneal epithelial cells and contribute to the maintenance of corneal epithelium homeostasis and barrier functions

* Corresponding author. Goldschleger Eye Research Institute, Sheba Medical Center, 52621, Tel-Hashomer, Israel.
E-mail address: Ygal.rotenstreich@sheba.health.gov.il (Y. Rotenstreich).

https://doi.org/10.1016/j.jtos.2019.09.009
Received 13 March 2019; Received in revised form 16 September 2019; Accepted 25 September 2019

[9–11]. The critical role of corneal neuronal function was demonstrated in studies in which lesions in the trigeminal nerve caused corneal dystrophy, and sensory denervation reduced corneal wound healing [12,13]. In vitro studies demonstrated that the corneal neurons and epithelial cells communicate via purinergic and glutamatergic receptors that mediate epithelial cell wound response [14].

Transcranial Magnetic Stimulation (TMS) is a neurostimulation and neuromodulation technique, based on the principle of electromagnetic induction of an electric field in the brain [15]. The basic principle of TMS is that most neuronal axons that fall within the volume of magnetic stimulation become electrically excited, trigger action potentials and release neurotransmitter into the postsynaptic neurons. When TMS pulses are applied repetitively (rTMS) they can modulate cortical excitability, decreasing or increasing it, depending on the parameters of stimulation, even beyond the stimulation duration [16]. Repetitive TMS allows for a more sustained neurological intervention than TMS, generating lasting cortical effects that remained after the cessation of stimulation [17]. Repetitive TMS has been studied in clinical trials for non-invasive mapping of human brain physiology and for treatment of numerous neurological and psychiatric illnesses, such as depression (FDA approved since 2008), schizophrenia, Parkinson's disease, and pain syndromes [18–20].

Repetitive TMS modulates the turnover of neurotransmitters and ion (Na^+, K^+, and calcium) channels and the ion balance in neuronal systems [21,22]. In addition, rTMS has long-term effects by induction of intra-cortical inhibitory circuits and long-lasting potentiation that lead to cortical remodeling [16,23–25]. Animal studies demonstrated that some of these long term effects are mediated by the induction of transcription factors such as c-fos and CREB, activation of signaling pathways such as ERK1/2 and expression of growth factors such as BDNF [26–28].

Recently, rTMS was reported to modulate the glutamate-mediated blood brain barrier by neuronal activation [29] and glutamate was shown to play a key role in the corneal epithelium wound response [14]. Since, as indicated above, corneal epithelium health depends on the function of corneal neurons and since repetitive magnetic stimulation (RMS) was shown to affect neuronal activity, glutamate-mediated barrier function and the expression of growth factors in the brain, we hypothesized that RMS treatment may potentially improve the health and barrier functions of the corneal epithelium in exposure keratopathy. Here we demonstrate that RMS treatment protects corneal epithelial barrier function under acute dryness conditions in rabbit eyes.

2. Materials and methods

2.1. Animals

Sixty one female New Zealand White (NZW) Rabbits age 12–14 weeks were purchased from Envigo (previously, Harlan) Laboratories, Israel, Rehovot, and were housed at the Goldschleger Eye Research Institute animal facility at the Sheba Medical Center. All animal procedures and experiments were conducted with approval and under the supervision of the Institutional Animal Care Committee at the Sheba Medical Center, Tel-Hashomer (IRB numbers 754/12, 1009/16) and conformed to the recommendations of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Study groups are detailed in Supplementary Table 1.

2.2. RMS treatment

Repetitive magnetic stimulation (RMS) was applied to one eye pre-evaluated for the absence of epithelial damage. The eye was exposed to a single session of RMS at 20 Hz for a total duration of about 15 min, according to Epitech Mag Ltd. protocol (Supplementary Fig. 1).

2.3. Rabbit exposure keratopathy model

Corneal desiccation was performed following the protocol we previously described [30]. Briefly, rabbits were anesthetized with an intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg) and eyes were kept open using 15 mm wire speculum for 140 min under controlled temperature and humidity conditions (24 °C, 55% relative humidity). Then, the speculums were removed and fluorescein corneal staining was performed, followed by AS-SD-OCT imaging. Five microliters of 5 mg/ml sodium fluorescein (Fluorescein® Novartis) were instilled on the anesthetized rabbit corneas. After 30 s, corneas were extensively washed with 0.9% w/v Sodium Chloride (B. Braun, Melsungen AG Germany). Five minutes after fluorescein instillation, the corneas were photographed with a SONY digital HD video camera (HDR-SE12E) with the lens covered with a Rosco yellow (E-COLOR+). The eyes were illuminated with a fiber optic light source illuminator (Volpi AG intralux 6000, Schlieren, Switzerland) covered with a blue filter (Rosco E-COLOR+). The fluorescein corneal staining was graded using EpiView® software, as we previously described [30].

2.4. Fluorometry measurement

0.1 ml taps of the anterior chamber were evaluated for fluorescein concertation using a fluorometer (FLx800 Fluorescence Microplate Reader, BioTek) at wavelengths of 485 nm (excitation) and 528 nm (emission).

In preliminary studies the fluorescein concertation in the anterior chamber differed by up to 20% between desiccated non-treated eyes (data not shown). Hence, a positive therapeutic effect was defined as a 30% lower fluorescein concentration in anterior chamber in RMS-treated eye vs. the contralateral non-treated eye for the therapeutic effect duration and dose response experiments.

2.5. Spectral Domain Optical Coherence Tomography (SD-OCT)

All rabbits were tested by multicolor fundus imaging and SD-OCT imaging of the visual streak 1 week before and 5 h after RMS treatment and then every 2-week following treatment. Three rabbits completed a follow-up of 1 year. Rabbits were anesthetized as indicated above. Eye drops of 0.5% tropicamide (Fisher Pharmaceuticals Labs) were applied before image acquisition. Eyes were subjected to OCT imaging with Spectralis® Multicolor OCT (Heidelberg Engineering, Heidelberg, Germany), as we previously described [31,32]. Rabbits were placed on a platform with the visual streaks (3 mm ventral to the optic nerve head, ONH) located at the center of the image. Duration of the procedure was approximately 15 min per eye. Rectangular scans were performed with 768 A-scan per B-scan, with a total of 25 B-scan per frame.

2.6. Anterior Segment Spectral Domain Optical Coherence Tomography (AS-SD-OCT)

Spectralis Multi Color Blue Peak OCT plus (Heidelberg) equipped with anterior segment module was used to assess the anatomy of the cornea, as we previously described [30]. AS-SD-OCT imaging was performed following fluorescein staining. Rectangular scans of the central cornea were performed with 1024 A-scans per B-scan, with a total of 21 B scans per frame. To evaluate percentage of corneal area with an epithelial defect, the length of the corneal section (LCS) and the total length of epithelial defects (LED) in each B-scan were measured using *Image J* (v, 1.48, National Institutes of Health, Bethesda, MD, USA). The LCS and LED from all B-scans were summed up and the percentage of corneal area with an epithelial defect was calculated by the formula: AS-SD-OCT Score = 100X Sum [LED1-21]/Sum [LCS1-21], as we previously described [30]. The LEDs and LCSs were measured by three observers and only measurements that were agreed upon by all three observers were included.

2.7. Intraocular pressure (IOP) measurement

IOP measurements were performed using a TONO-PEN XL Tonometer (Reichert, Inc., USA) under anesthesia. The measurement included covering of the instrument tip with a purpose-made cup, calibration of the instrument per manufacturer instructions and gently touching the cornea with the tip of the tonometer. Five readings were taken and the average value was recorded.

2.8. Electroretinogram (ERG) recording

Rabbits were anesthetized as indicated above. ERGs were recorded from both eyes simultaneously using corneal contact lens ERG-jet electrodes (Micro Components, Universo Pastique, Switzerland) as we previously described [32]. Dark-adapted ERG was performed following 2 h dark adaptation. Light-adapted ERG was tested after 10 min light adaptation. Light intensities were 0.023, 0.25, 2.4, 4.4 and 23.5 cd-s/m^2.

2.9. Histology

Rabbits were sacrificed 5 h, 1 day, 1 week, 1 month, 2 months and 6 months following treatment, and their eyes were fixed in 3.7% formaldehyde for 24 h, followed by embedding in paraffin [31,32]. Sections were stained with H&E and carefully examined for any changes in eye structure.

2.10. Immunofluorescence of eye sections

Paraffin sections were incubated following citrate buffer antigen retrieval with antibodies detailed in Supplementary Table 2 followed by extensive washes in PBS and incubation with fluorescently labeled secondary antibodies detailed Supplementary Table 2.

TUNEL staining was performed following manufacturer instructions (In Situ Cell Death Detection Kit, TMR red, Roche). Briefly, following rehydration, sections were incubated in permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice, followed by incubation with the TUNEL reaction mixture for 1 h at 37 °C.

All sections were counterstained with 4′,6-Diamidino-2-phenylindole (Vector, Laboratories, Burlingame CA) and examined and photographed by a fluorescent microscope (Olympus BX51).

Corneal epithelial cell apoptosis and proliferation were determined by counting the number of TUNEL-positive and Ki67-positive corneal epithelial cells, respectively, in the entire cornea section, divided by the length of the cornea, using *Image J*.

2.11. Immunofluorescence of whole-mount cornea tissues

Under a dissecting microscope (Leica Wild M690; Wild Herring, Herring, Switzerland), the retina, lens, and iris were discarded and six radial incisions were made in each cornea. Each cornea was fixed in situ in PBS with 3.7% formaldehyde for 5 min, and then was permeabilized with acetone for 3 min at −20 °C. Subsequently, the corneas were washed in PBS with 1% Triton X-100 and 1% dimethyl sulfoxide (DMSO; PTD buffer). Corneas were incubated in 1% BSA diluted in PTD buffer for 1 h followed by incubation with a solution containing rat anti-rabbit ZO-1 antibody (dilution 1:100, clone R40.76, Millipore) for 16 h with agitation at 4 °C. The tissues were extensively washed with PTD buffer, incubated with FITC-conjugated secondary antibody (Sigma) for 1 h at 4 °C. The whole-mount cornea tissues were mounted epithelial side up on a slide, counter-stained with DAPI and examined with a confocal microscope (Leica TCS SP5 II.)

2.12. Statistical analysis

Normality of data was tested by the Shapiro-Wilk test. Wilcoxon signed ranks test was used to assess the effect of RMS treatment on fluorescein concertation in anterior chamber, corneal surface fluorescein staining score, the percentage of corneal area with epithelial erosions by AS-OCT. The difference in the ratio between treated vs. non treated eyes between RMS treatments at output 40% and 50% was assessed by Mann-Whitney. Kaplan - Meier estimate was used to evaluate the duration of RMS therapeutic effect. To test the significance of RMS effect on Schrimer's test and IOP, Wilcoxon signed-ranks test was used for pairwise comparisons at each time point. Friedman Test was used for testing the significance of change in Schrimer's test between time points. One way ANOVA analysis was performed to evaluate the effect of RMS treatment on ERG, with Bonferroni correction for multiple comparisons. All analyses were performed using SPSS for windows version 20.0. Differences were considered significant if $p < 0.05$.

3. Results

3.1. Evaluation of RMS treatment efficacy

3.1.1. Determination of fluorescein penetration through the corneal epithelial barrier in rabbit eyes

To test the effect of RMS treatment on corneal epithelium barrier integrity we selected sodium fluorescein as the model-penetrating agent. We chose to use fluorescein due to its common use in clinical setting for diagnosis of corneal epithelial damage, high ocular safety profile (used daily in clinical practice), its hydrophilic nature, and the fact that its concentration in the anterior chamber can be measured with good precision and reproducibility using a fluorometer. In healthy eyes, the baseline corneal permeability of this hydrophilic substance is low, due to the corneal barrier function. As shown in Supplementary Fig. 2, fluorescein concentration in the anterior chamber (AC) increased with time, with a peak at 60 min following dye application. Fluorescein concentration in the anterior chamber was significantly higher, by up to 70 fold, in desiccated eyes as compared with healthy-non treated eyes.

The concentration of fluorescein found in the AC of the rabbits presented similar dynamics to those found in a study on human eyes in which permeation of healthy eyes was compared with that of eyes with compromised corneal epithelium [33].

3.1.2. RMS treatment protects corneal epithelium under desiccation conditions

Next, we examined the effects of RMS treatment on corneal epithelium health. For this aim, 36 rabbits were treated in one eye with RMS 1hr prior to desiccation and fluorescein application. The contralateral eye was not treated with RMS and was used as control. Both eyes were left open for 140 min to induce eye desiccation as detailed in "Materials and Methods" section. Rabbits treated with RMS at 40% output intensity presented with significantly reduced fluorescein penetration into the anterior chamber following desiccation, by nearly 3-fold (mean ± standard error (SE): 176.0 ± 26.8 ng/ml vs. 56.7 ± 13.0 ng/ml for control vs. RMS treatment, respectively, Wilcoxon signed ranks test $p = 0.0004$, $n = 16$). Similarly, rabbits treated with RMS treatment at 50% presented with significantly lower fluorescein penetration into the anterior chamber following desiccation (121.3 ± 25.0 ng/ml vs. 51.9 ± 11.3 ng/ml for control and treated eyes, respectively, Wilcoxon signed ranks test $p = 0.001$, $n = 20$). There was no significant difference in the ratio between treated vs. non treated eyes between the two treatment groups (Mann-Whitney $p = 0.132$). Taken together, RMS treatment significantly reduced fluorescein penetration into the AC following desiccation, by nearly 3-fold (146.5 ± 18.6 ng/ml vs. 54 ± 8.4 ng/ml for control and treated eyes, respectively, Wilcoxon signed ranks test p value = 0.000001, Fig. 1A).

In addition, rabbit eyes were examined by a fluorescent slit lamp microscope. Following 140 min of desiccation, significant corneal fluorescein staining was evident in control eyes, reflecting

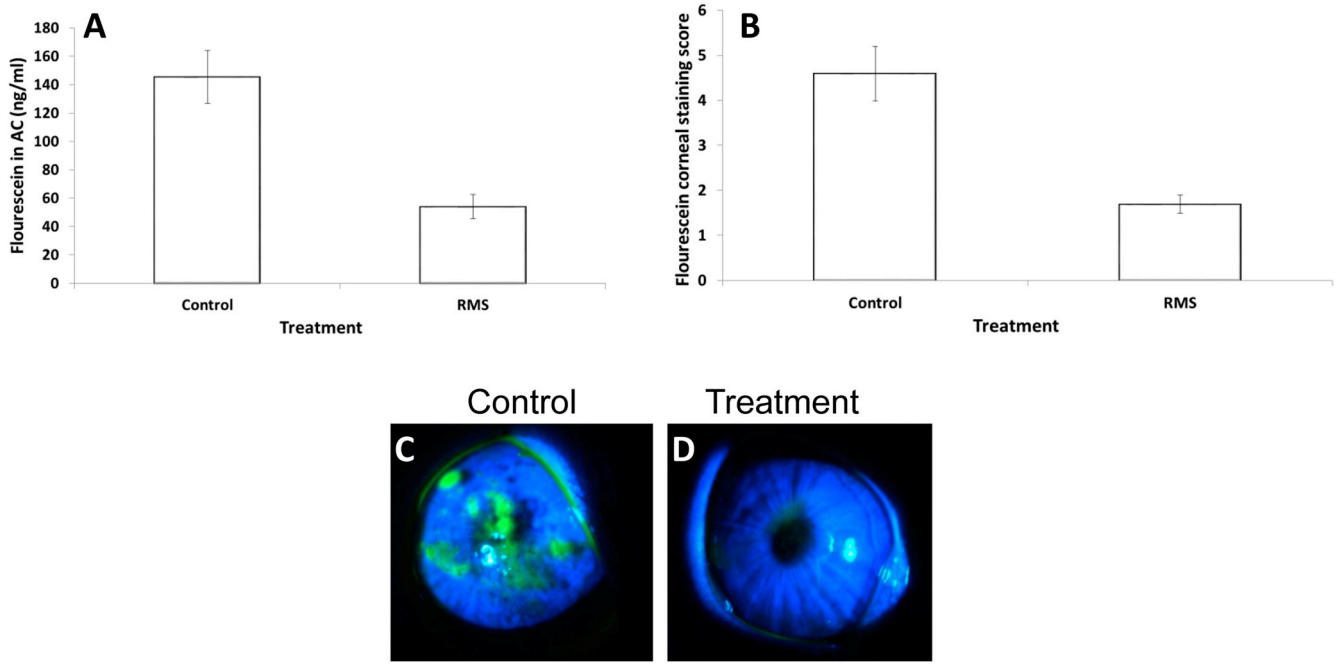

Fig. 1. RMS treatment significantly reduced fluorescein penetration into the AC and corneal staining in desiccated eyes.
In each rabbit, one eye was non-treated (control) and the second eye was treated with RMS prior to desiccation of both eyes. **A-** Fluorescein concentration in the anterior chamber was measured 60 min after application of fluorescein (n = 36; Mean ± SE). **B-** Fluorescein corneal staining score was determined using EpiView® software [30] (n = 30; Mean ± SE). **C**- Representative images of fluorescein corneal staining of both eyes of a rabbit, one eye was not treated (Control) and the second eye was treated with RMS (Treatment) prior to desiccation of both eyes.

discontinuities in the tear film and damage to the corneal epithelium (Fig. 1C). Fluorescein corneal staining was quantified using the *Epiview*® software as describe in the "Material and Methods" section. In rabbits treated with RMS at 40% and 50% output intensity, the fluorescein staining score was significantly lower in treated eyes compared with the contralateral non-treated eyes (mean ± SE: 1.8 ± 0.5 for RMS 40% vs. 5.5 ± 0.94 for control, Wilcoxon signed ranks test p value p = 0.007, n = 10; 1.6 ± 0.2 for RMS 50% vs. 4.1 ± 0.7 for control, Wilcoxon signed ranks test p value p = 0.001, n = 20). There was no significant difference in the ratio between treated vs. non treated eyes between the two treatment groups (Mann-Whitney p = 0.53). Taken together, the fluorescein staining score in control eyes ranged between 0.5 and 11.9 [mean ± SE, 4.6 ± 0.6, Fig. 1B]. By contrast, RMS treated eyes presented significantly reduced fluorescein corneal staining with fluorescein staining score ranging between 0.1 and 5.4 (mean ± SE, 1.7 ± 0.2, Wilcoxon signed ranks test p value p = 0.00001, Fig. 1B).

3.1.3. RMS treatment prevents corneal epithelium erosions as determined by AS-SD-OCT

We have previously demonstrated that AS-SD-OCT may be used to evaluate corneal lesions following desiccation in rabbit eyes in vivo [30]. As shown in Fig. 2, RMS treatment reduced by nearly 4-fold the percentage of corneal area with an epithelial defect as determined by AS-SD-OCT imaging analysis. RMS treated eyes had significantly smaller epithelial defects compared to control non-treated eyes (2.1% [SE = 0.87] vs 7.8% [SE = 2.4], Wilcoxon Signed-Rank Test p = 0.012).

3.1.4. RMS treatment protects corneal epithelium tight junction structures under desiccation conditions

Next, we evaluated the effect of RMS treatment on corneal epithelium tight junctions (TJs). Staining for Zonula occledens-1 (Zo-1) demonstrated that the RMS treatment protected Zo-1 positive TJ structures in corneal epithelium under desiccation conditions. In control eyes (that underwent desiccation with no RMS treatment), the pattern of Zo-1 staining was discontinuous and patchy, and areas lacking Zo-1 expression were observed (Fig. 3A, highlighted with a white asterisk). By contrast, in RMS-treated desiccated corneas, a continuous pattern of ZO-1 immunoreactivity around the perimeter of corneal epithelial cells was demonstrated (Fig. 3B).

3.1.5. The therapeutic effect of a single RMS treatment lasts for at least 3 months

To examine the long-term effect of RMS treatment, seven rabbits were treated with RMS on one eye at the baseline time point, followed by exposure to desiccation conditions of both eyes every two weeks. The proportion of rabbits demonstrating a positive therapeutic effect (as defined in the "Materials and Methods" section 2.4) was determined at each time point. The therapeutic effects of a single RMS treatment were maintained for at least 3 months in all rabbits (Fig. 4). Two rabbits demonstrated significantly longer (11 month) duration of therapeutic effect.

3.1.6. Dose response of RMS treatment

Next, we determined the minimal effective magnetic stimulation intensity required for protecting corneal epithelium from desiccation. To this aim, rabbits were treated with RMS at lower intensities (10 and 1% output intensity). As shown in Table 1, the minimal magnetic stimulation intensity resulting in > 80% of the rabbits presenting with successful treatment is 40% output intensity.

3.2. Evaluation of RMS treatment safety

3.2.1. Bio-microscopy analysis demonstrates no pathological changes following RMS treatment

Five rabbits underwent a comprehensive complete ophthalmic slit-lamp biomicroscopy clinical-grade examination by a senior ophthalmologist (a retina specialist) before and following RMS treatment on one eye at 40% (2 rabbits) and 50% (3 rabbits) RMS output intensity at

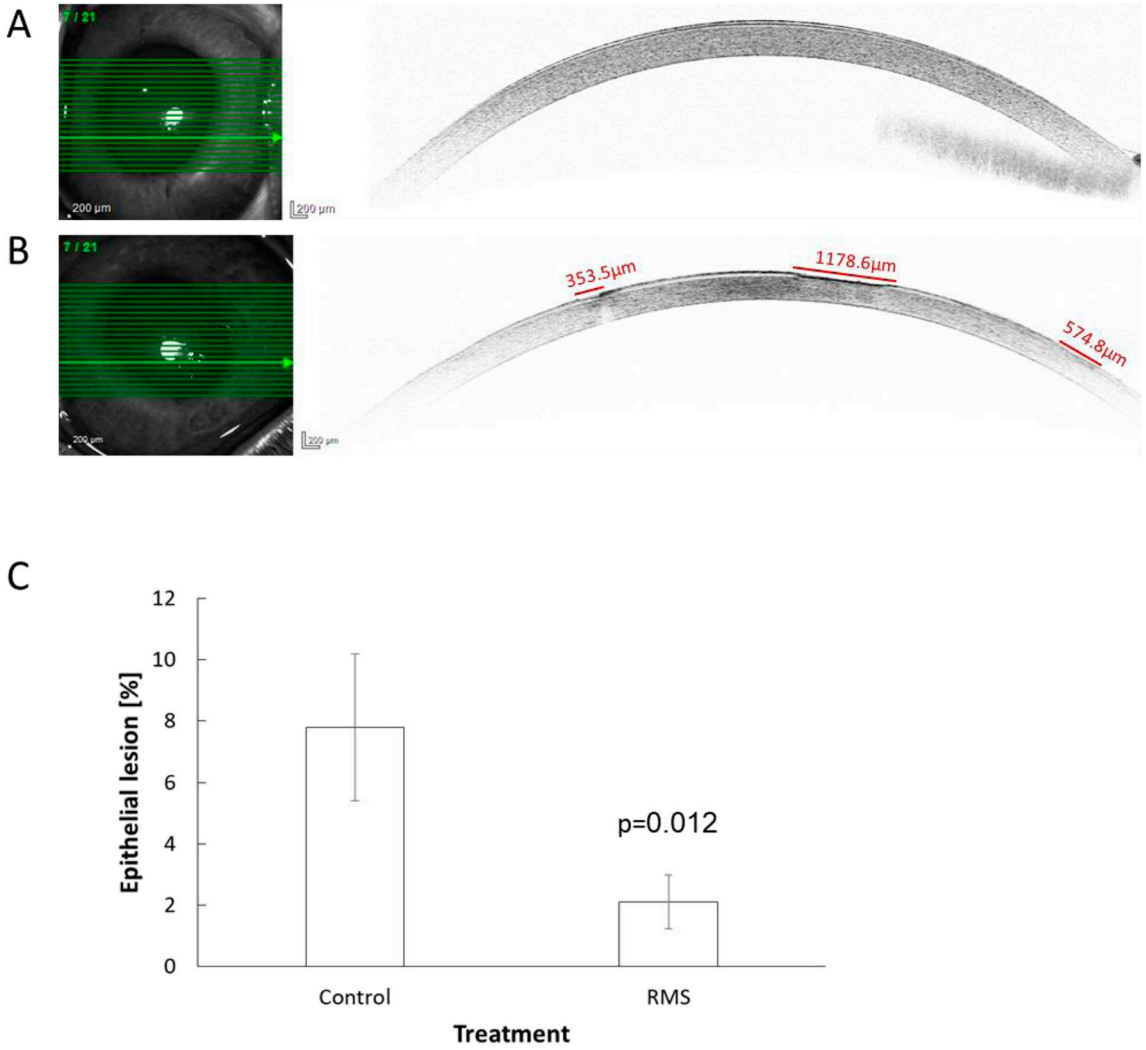

Fig. 2. RMS treatment reduced the percentages of corneal area with an epithelial defect as determined by AS-SD-OCT.
Representative AS-SD-OCT images of RMS treated (A) and contralateral non treated eye (B) following desiccation. AS-SD-OCT was performed and the percentages of corneal area with an epithelial defect was determined as described in the "Materials and Methods" section (C). Data are presented as mean ± SE (n = 8).

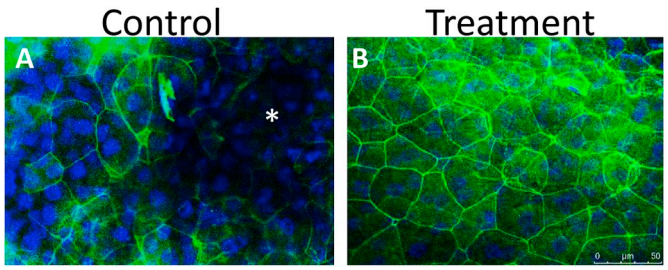

Fig. 3. RMS treatment protect TJ structure under desiccation conditions.
Whole-mounted rabbit corneas removed immediately following acute desiccation conditions from control non-treated (A) and RMS-treated eyes (B) were stained with an antibody directed against Zo-1 (green). Cell nuclei were counterstained with DAPI (blue). An area with patchy Zo-1 staining is highlighted with a white asterisk in the representative image of a control eye (A). N = 4 each group.

1 day, 1, 2, 5 and 9 weeks. In all eyes (treated and contralateral controls eyes) of all rabbits, no pathological findings were observed in any of the ocular tissues: eyelids, conjunctiva, cornea, anterior and posterior chambers, iris, pupil, lens, angle, vitreous body, optic nerve, optic disc (data not shown).

In addition, SD-OCT analysis of the posterior segment demonstrated no pathological findings in any of the rabbits tested at the time points indicated in the "Materials and methods" section. No retinal detachment or choroidal hemorrhages or any other pathologies were demonstrated in any of the treated or contralateral control eyes following 40% or 50% RMS treatments. Retinal thickness was measured in 10 rabbits. There were no significant differences in retinal thickness between the different time points following RMS treatment (all $p > 0.05$, Supplementary Tables 3 and 4).

3.2.2. Histopathology analysis of the posterior segment demonstrates no adverse effects of RMS treatment

To further evaluate the safety of RMS treatment, twenty five rabbits were sacrificed at various time points following RMS treatment as indicated in the "Materials and methods" section. No gross or microscopic changes or inflammatory reactions were observed in any of the treated eyes, in either 40% (n = 11) or 50% (n = 14) output intensity or in the contralateral non-treated control eyes. As shown in Supplementary Tables 5 and 6, there were no significant differences in the thickness of retinal photoreceptor outer nuclear layer (ONL), inner nuclear layer (INL) or ganglion cell density. Furthermore, no significant changes were detected in central corneal thickness or endothelial cell count in RMS treated vs. non-treated eyes.

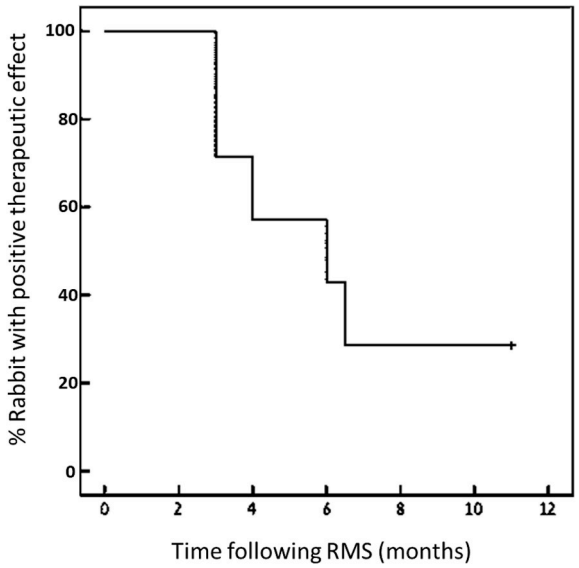

Fig. 4. A single RMS treatment protects corneal epithelium for at least 3 months.
The percentage of rabbits with positive therapeutic effect is plotted against months following a single RMS treatment in a Kaplan–Meier estimator (n = 7).

Table 1
Dose response of RMS treatment efficacy.

Output intensity	Successful treatment (No. of rabbits)	% Successful therapy
50%	17/20	85
40%	15/16	94
10%	3/5	60
1%	1/3	33

Rabbits were treated with a single RMS treatment at indicated output intensity, followed by desiccation for 140 minutes and fluorescein application. Treatment was considered successful if the fluorescein concentration in the anterior chamber was at least 30% lower in RMS treated eye compared with the contralateral non-treated eye, as described in "Materials and methods" section 2.4.

3.2.3. RMS does not significantly affect tear secretion

To determine the effect of RMS treatment on tear secretion, Schrimer's test was performed in three rabbits. As shown in Fig. 5, there were no significant changes in tear secretion following 50% RMS treatment (all P-values > 0.11). There were no significant differences in the Schrimer's test scores between time points in each group (control: p = 0.711; RMS: p = 0.07).

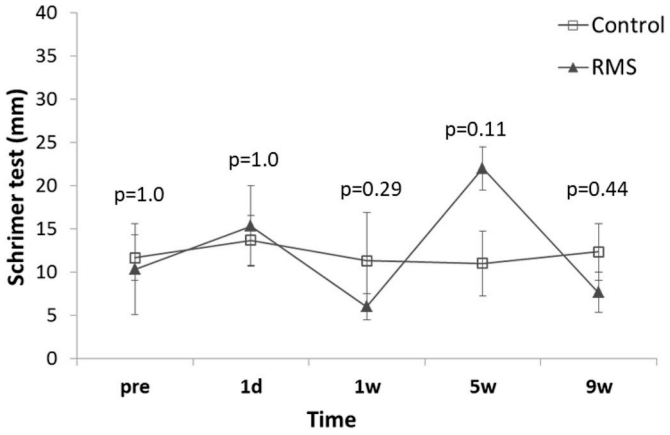

Fig. 5. RMS treatment has no significant effect on tear secretion.
Three rabbits were tested by Schrimer's test before (pre) and at indicated time points [1 day (1d); 1 week (1w); 5 weeks (5w) and 9 weeks (9w)] following RMS treatment at 50% output intensity on one eye in 3 rabbits. The contralateral eyes were not treated and used as control. Wilcoxon signed-ranks test p-Value for each time point is indicated in the graph.

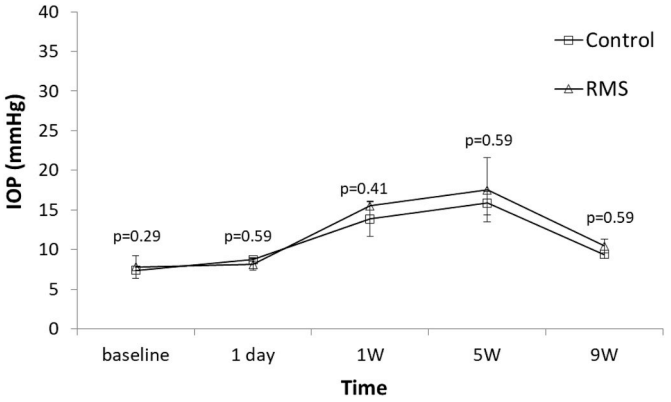

Fig. 6. RMS treatment at 50% output intensity has no significant effect on IOP.
IOP was measured in both eyes of 3 rabbits before (baseline) and at indicated time points following RMS treatment on one eye in the treated (RMS, triangles) and in the non-treated contralateral eyes (control, squares). Measurements were performed at indicated time points (x-axis). Data are presented as mean ± SE. IOP measurements were performed using a TONO-PEN XL Tonometer (Reichert, Inc., USA). Five readings were taken and the average value was recorded. W-weeks. Wilcoxon signed-ranks test p-Value for each time point is indicated in the graph.

3.2.4. RMS has no significant effect on retinal function

To determine the effect of RMS treatment on retinal function, both eyes of three rabbits were tested by electroretinogram (ERG) 1 week before and at various time points following 50% RMS treatment on one eye. ERG testing assesses photoreceptor (a-wave component) and second-order neuron (b-wave component) function [34–36]. The function of cones and rods can be assessed by performing the test under light or dark adaptation, respectively [36,37]. As shown in Supplementary Figs. 3 and 4, RMS treatment had no significant effect on a-wave (p = 0.667, p = 0.612) or b-wave (p = 0.817, p = 0.977) recordings under dark or light adaptations, respectively.

3.2.5. RMS has no significant effect on intraocular pressure (IOP)

IOP was measured in control and RMS treated eyes before RMS treatment and at various time points following RMS treatment in 3 rabbits (Fig. 6). IOP was below 21 mmHg throughout the follow-up period and no significant changes in IOP were recorded between control and RMS treated eyes (all p > 0.29).

3.2.6. RMS treatment has no effect on rabbit general health and weight

Rabbits were monitored weekly for general health and weight by a veterinarian. All rabbits presented with good general health. As shown in Fig. 7, no weight loss was recorded following RMS treatment.

3.2.7. Effect of RMS treatment on corneal epithelial apoptosis

Corneal cell apoptosis rate was determined by TUNEL staining. In all groups very few cells were TUNAL-positive, with a mean of less than 11 cells/10 mm corneal length (Fig. 8). Although the mean TUNEL-positive cells/10 mm cornea was lower in the RMS group at 1 day and 1 week following treatment compared with the contralateral non-treated eyes, this difference was not statistically significant (Fig. 8G, all p > 0.11).

3.2.8. Effect of RMS on corneal epithelial cell proliferation and differentiation

Corneal cell proliferation was determined by staining with a Ki67

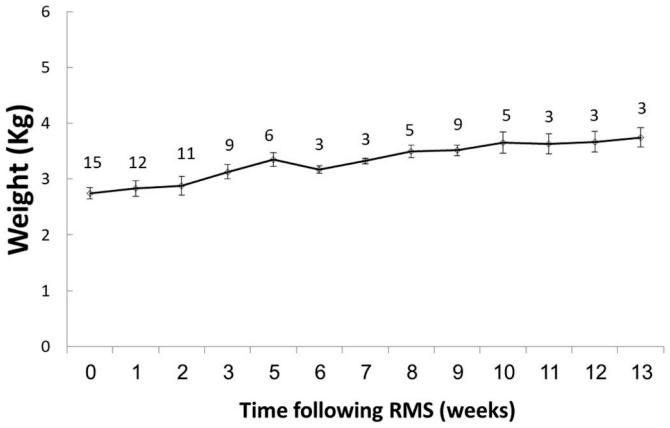

Fig. 7. RMS treatment has no significant effect on rabbit eight gain.
Rabbits were weighed weekly at indicated weeks following RMS treatment. Data are presented as mean ± SE. Number of rabbits weighed at each time point is indicated above the graph.

antibody. In all groups, very few cells were Ki67-positive, with a mean of less than 10 cells/10 mm corneal length (Fig. 9). There were no significant differences between the groups at 4 h, one day and one week following RMS treatment (all $p > 0.1$, Fig. 9G).

In addition, there were no substantial differences in the expression patterns of epithelial cell markers between groups in those time points, including: p63 that is specifically expressed in corneal stem cells in the basal and suprabasal layers in the limbus and mostly in the basal layer in the cornea; cytokeratin-3 which was expressed throughout the corneal epithelium and in the upper epithelium layers in the limbus; Cytokeratin 14 that was confined to the limbal cells in the basal and supra-basal layers in all groups, suggesting the RMS treatment did not significantly affect the differentiation pattern of cornel epithelium cells (data not shown).

3.2.9. Effect of RMS on corneal epithelial gap junction and adherens junction

No substantial differences were observed in the expression patterns of the gap junction marker connexin 43 and adherens junction marker integrin β1 between treatment and control groups at 4 h, 1 day and 1 week following RMS treatment. Connexin 43 staining was observed throughout the corneal and limbal epithelium, whereas integrin β1 was more strongly expressed in the basal and supra-basal epithelial cell layers in the cornea and was confined to the basal cells in the limbus (Fig. 10 and data not shown).

4. Discussion

In this study we report that RMS treatment protects corneal epithelial barrier function under repeated, severe and acute desiccation in rabbit eyes. Corneal fluorescein staining, the widely used clinical testing for corneal surface health was significantly lower in RMS treated eyes and significantly less fluorescein penetrated through the cornea into the anterior chamber, strongly suggesting that RMS treatment protected corneal epithelial cells from the desiccation stress. AS-OCT imaging demonstrated significantly less erosions under desiccation conditions in treated eyes compared with control eyes, further supporting the ability of RMS to protect corneal epithelial cells from desiccation.

The formation of TJs between adjacent epithelial cells at the apical plasma membrane forms the physical and functional corneal epithelial barrier [38]. Zonula occludens-1 (Zo-1) is one of the major protein components of TJs and plays a key role in TJ formation and maintenance and in maintaining corneal epithelial barrier function [38–40]. The major role that Zo-1 plays in corneal TJ formation prompted us to examine the effect of RMS treatment on Zo-1 expression pattern in whole-mounted corneas. Our results suggest that RMS treatment ameliorating the loss of these inter-cellular structures under desiccation conditions. To the best of our knowledge this study demonstrates for the first time such effect of RMS on corneal cells.

As epithelial cell health and barrier function depends on a cross talk with the trigeminal nerve endings [12,14], it is plausible that RMS treatment activated the trigeminal nerve that innervates the cornea [41], leading to secretion of neurotransmitters and neurotrophic factors that mediated the epithelial resistance to desiccation stress. Even though no significant changes were observed in tear secretion by Schrimer's test between RMS and control eyes, we cannot exclude the possibility that RMS therapeutic effects may be mediated by the activation of the pterygopalatine ganglion cells that mediate the parasympathetic innervation of the lacrimal gland and conjunctival goblet cells [42]. Recent translational studies by the Palanker group in rabbits

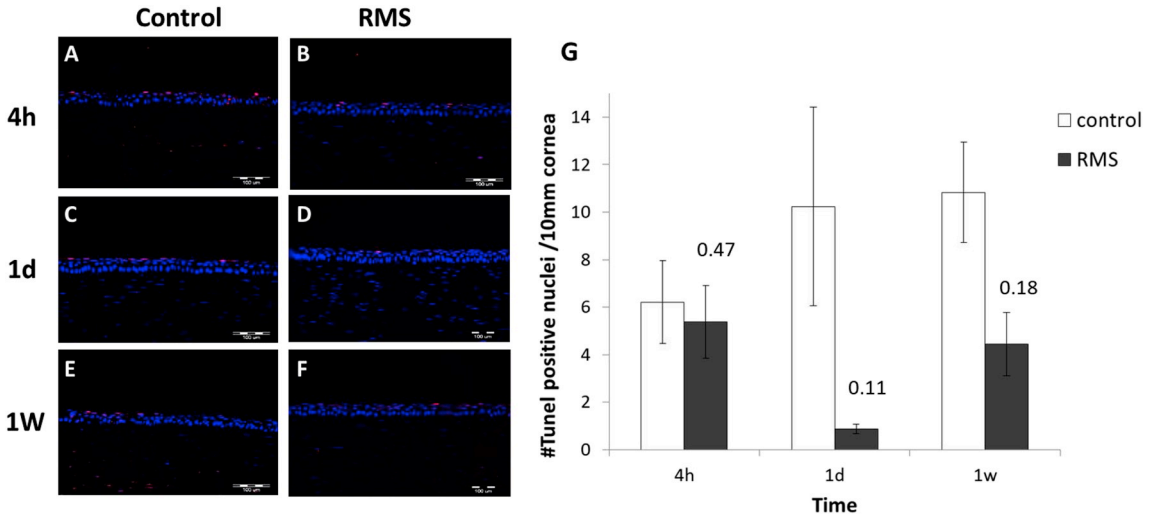

Fig. 8. RMS treatment has no significant effect on apoptosis rate in desiccated eyes.
Rabbit corneal sections were analyzed for apoptosis using TUNEL staining at 4 h (A & B, 4 h, n = 4), 1 day (C & D, 1d, n = 3) or 1 week (E & F, 1w, n = 3) following desiccation. Representative images of non-treated control eyes (A, C, E) and contralateral RMS treated eyes (B, D, F) are shown. Scale – 100 μm. G- Quantification of TUNEL-positive cells. Data are presented as mean number of TUNEL positive cells/10 mm cornea ± SE. Wilcoxon signed-ranks p-values for comparison of RMS treated and contralateral non-treated eyes at each time point are presented.

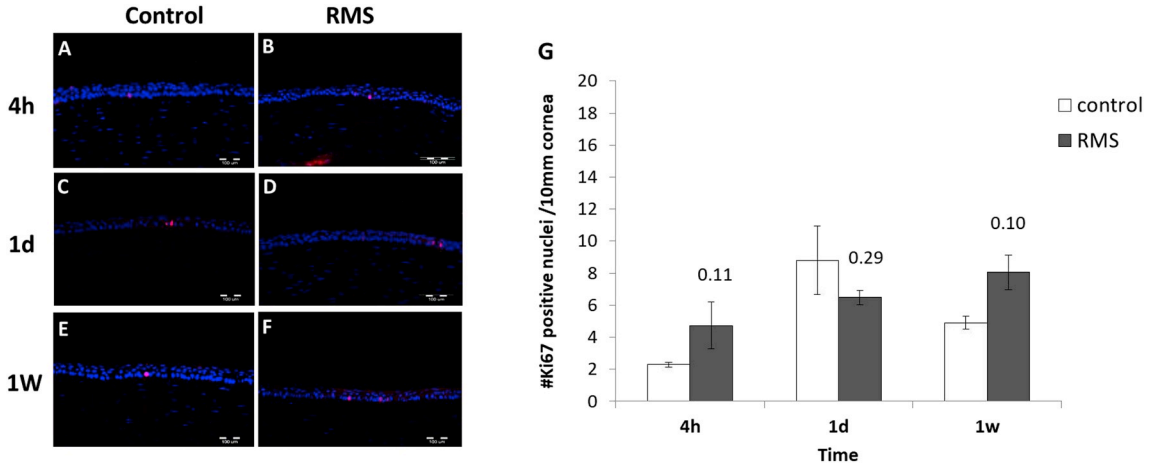

Fig. 9. RMS treatment has no significant effect on cell proliferation in desiccated eyes.
Rabbit corneal sections were analyzed for number of cells in the cell cycle by staining for Ki67 at 4 h (A & B, 4 h, n = 4), 1 day (C & D, 1d, n = 3) or 1 week (E & F, 1w, n = 3) following desiccation. Representative images of non-treated control eyes (A, C, E) and contralateral RMS treated eyes (B, D, F) are shown. Scale – 100 μm. G – Quantification of Ki67-positive cells. Data are presented as mean number of Ki67 positive cells/10 mm cornea ± SE. Wilcoxon signed-ranks test was used for comparison of RMS treated and contralateral non-treated eyes at each time point.

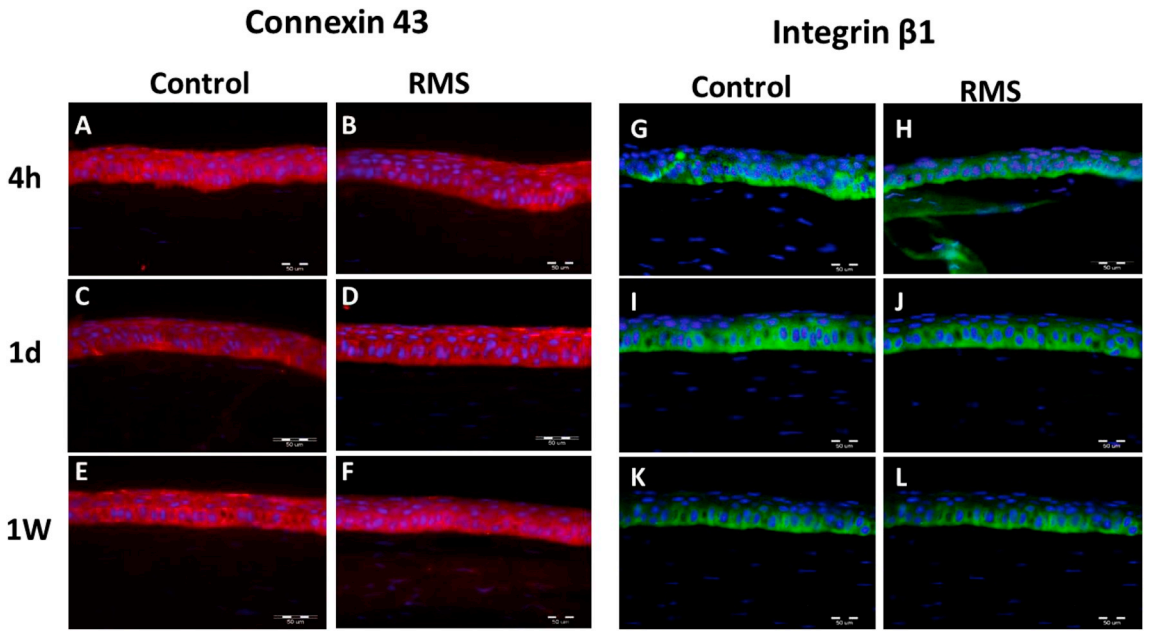

Fig. 10. RMS treatment has no significant effect on connexin43 or Integrin β1 expression pattern.
Rabbit corneal sections were analyzed at 4 h (A & B, G& H, 4 h, n = 4), 1 day (C & D, I & J, 1d, n = 3) or 1 week (E & F, K & L, 1w, n = 3) following desiccation for connexin 43 (red, A-F) or Integrin β1 (green, G - L) expression pattern. Blue- DAPI counter staining. Scale – 50 μm.

followed by clinical trials suggest that electric stimulation of the lacrimal gland and afferent nerves may alleviates dry eye symptoms [43–45]. Another possibility to be explored in future studies is a direct effect of RMS on the corneal epithelial cells. Studies in rabbits demonstrated that application of continuous electrical stimulation (100 mV/mm) on the eye lid increased the rate of epithelial healing following epithelium removal [46].

Our findings demonstrated that a single RMS treatment protected the cornea for at least three months of repeated desiccation, suggesting that the treatment may have a significant long-term therapeutic effect. Repetitive *trans*-cranial Magnetic stimulation (rTMS) treatments have also been demonstrated to have short and long term effects on the central nerve system. Long term effects of rTMS treatment were suggested to be mediated by induction of changes in gene and protein expression [23,26,28,47–49]. The effects of RMS corneal treatment may be mediated by secretion of neurotrophic factors from the activated trigeminal nerve that may induce short and long-term changes in the corneal epithelium [14]. One possible candidate is the trigeminal nerve neuropeptide substance P that was shown to enhance corneal epithelial cell attachment in vitro by inducing E-cadherin expression [50]. Substance P also induces DNA synthesis and corneal epithelial cell growth in-vitro [51,52]. A second candidate may be calcitonin gene-related peptide (CGRP) that is expressed in the trigeminal ganglion cells in animals and in humans [53] and was shown to induce prolonged proliferative effects in corneal epithelial cells in vitro [52]. The rabbit model established here will enable future studies to decipher the molecular mechanisms underlying the short- and long-term RMS therapeutic effects in the cornea.

RMS treatment was performed in awake animals without inducing any apparent pain, retinal or corneal side effects. Comprehensive safety testing performed in 60 rabbits showed no adverse effects and no indication of any pathological findings in the structure or function of the

rabbits' retina or cornea, including OCT imaging, electrophysiology measurement of retinal function, bio-microscopy and histology analyses.

Our findings suggest that RMS treatment may protect the cornea from severe desiccation stress with no adverse effects. Hence, RMS may present an efficient and safe treatment for patients with exposure keratopathy. The RMS treatment may ameliorate the discomfort, pain and reduced vision in these patients. The translation of our findings to dry eye disease associated with tear film instability remains to be determined. Furthermore, RMS may be applicable for treatment of other conditions where epithelium surface integrity is impaired such as diabetic neuropathy [54]. Future studies will be aimed at deciphering the molecular mechanism underlying RMS therapeutic effects and its application in corneal neuropathies.

Disclosure

The study was supported by a research grant from Epitech Mag Ltd, Israel. Epitech Mag Ltd. staff participated in the review of the manuscript but had no role in the design or conduct of this research. IS and YR were consultants to Epitech Mag Ltd. during data collection and analysis (2015–2017). IS and YR are consultants to Everads Therapy LTD (not related to the subject matter). IS and YR have a patent application (Sheba Medical Center).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtos.2019.09.009.

References

[1] Clayton JA. Dry eye. N Engl J Med 2018;378:2212–23.
[2] Meng ID, Kurose M. The role of corneal afferent neurons in regulating tears under normal and dry eye conditions. Exp Eye Res 2013;117:79–87.
[3] Jammal H, Khader Y, Shihadeh W, Ababneh L, AlJizawi G, AlQasem A. Exposure keratopathy in sedated and ventilated patients. J Crit Care 2012;27:537–41.
[4] Holland EJ, Mannis MJ, Lee WB. Ocular surface disease: cornea, conjunctiva and tear film: expert consult-online and print. Elsevier Health Sciences; 2013.
[5] Hartford JB, Bian Y, Mathews PM, De Rojas J, Garg A, Rasool N, et al. Prevalence and risk factors of exposure keratopathy across different intensive care units. Cornea 2019;38:1124–30.
[6] Bonini S, Rama P, Olzi D, Lambiase A. Neurotrophic keratitis. Eye 2003;17:989.
[7] Elner VM, Mauffray RO, Fante RG, Harris M, Morton AD, Hassan AS. Comprehensive midfacial elevation for ocular complications of facial nerve palsy. Arch Facial Plast Surg 2003;5:427–33.
[8] Muller LJ, Marfurt CF, Kruse F, Tervo TM. Corneal nerves: structure, contents and function. Exp Eye Res 2003;76:521–42.
[9] Okada Y, Reinach PS, Kitano A, Shirai K, Kao WW, Saika S. Neurotrophic keratopathy; its pathophysiology and treatment. Histol Histopathol 2010;25:771.
[10] Wang F, Gao N, Yin J, Fu-Shin XY. Reduced innervation and delayed re-innervation after epithelial wounding in type 2 diabetic Goto-Kakizaki rats. Am J Pathol 2012;181:2058–66.
[11] Yang L, Di G, Qi X, Qu M, Wang Y, Duan H, et al. Substance P promotes diabetic corneal epithelial wound healing through molecular mechanisms mediated via the neurokinin-1 receptor. Diabetes 2014;63:4262–74.
[12] Beuerman RW, Schimmelpfennig B. Sensory denervation of the rabbit cornea affects epithelial properties. Exp Neurol 1980;69:196–201.
[13] Nagano T, Nakamura M, Nakata K, Yamaguchi T, Takase K, Okahara A, et al. Effects of substance P and IGF-1 in corneal epithelial barrier function and wound healing in a rat model of neurotrophic keratopathy. Investig. Ophthalmol. Vis. Sci. 2003;44:3810–5.
[14] Oswald DJ, Lee A, Trinidad M, Chi C, Ren R, Rich CB, et al. Communication between corneal epithelial cells and trigeminal neurons is facilitated by purinergic (P2) and glutamatergic receptors. PLoS One 2012;7:e44574.
[15] Barker AT, Jalinous R, Freeston IL. Non-invasive magnetic stimulation of human motor cortex. The Lancet 1985;325:1106–7.
[16] Huerta PT, Volpe BT. Transcranial magnetic stimulation, synaptic plasticity and network oscillations. J NeuroEng Rehabil 2009;6:1.
[17] Pascual-Leone A, Valls-Sole J, Wassermann EM, Hallett M. Responses to rapid-rate transcranial magnetic stimulation of the human motor cortex. Brain 1994;117(Pt 4):847–58.
[18] George MS, Nahas Z, Borckardt JJ, Anderson B, Foust MJ, Burns C, et al. Brain stimulation for the treatment of psychiatric disorders. Curr Opin Psychiatr 2007;20:250–4. discussion 247-9.
[19] Rossi S, Hallett M, Rossini PM, Pascual-Leone A. Safety, ethical considerations, and application guidelines for the use of transcranial magnetic stimulation in clinical practice and research. Clin Neurophysiol 2009;120:2008–39.
[20] Slotema CW, Blom JD, Hoek HW, Sommer IE. Should we expand the toolbox of psychiatric treatment methods to include Repetitive Transcranial Magnetic Stimulation (rTMS)? A meta-analysis of the efficacy of rTMS in psychiatric disorders. J Clin Psychiatry 2010;71:873–84.
[21] Lenz M, Galanis C, Müller-Dahlhaus F, Opitz A, Wierenga CJ, Szabó G, et al. Repetitive magnetic stimulation induces plasticity of inhibitory synapses. Nat Commun 2016;7.
[22] Tan T, Xie J, Tong Z, Liu T, Chen X, Tian X. Repetitive transcranial magnetic stimulation increases excitability of hippocampal CA1 pyramidal neurons. Brain Res 2013;1520:23–35.
[23] Feng S, Shi T, Wang W, Chen Y, Tan Q. Long-lasting effects of chronic rTMS to treat chronic rodent model of depression. Behav Brain Res 2012;232:245–51.
[24] Dayan E, Censor N, Buch ER, Sandrini M, Cohen LG. Noninvasive brain stimulation: from physiology to network dynamics and back. Nat Neurosci 2013;16:838.
[25] Zhang L, Xing G, Fan Y, Guo Z, Chen H, Mu Q. Short-and long-term effects of repetitive transcranial magnetic stimulation on upper limb motor function after stroke: a systematic review and meta-analysis. Clin Rehabil 2017;31:1137–53.
[26] Hausmann A, Weis C, Marksteiner J, Hinterhuber H, Humpel C. Chronic repetitive transcranial magnetic stimulation enhances c-fos in the parietal cortex and hippocampus. Mol Brain Res 2000;76:355–62.
[27] Gersner R, Kravetz E, Feil J, Pell G, Zangen A. Long-term effects of repetitive transcranial magnetic stimulation on markers for neuroplasticity: differential outcomes in anesthetized and awake animals. J Neurosci 2011;31:7521–6.
[28] Hellmann J, Jüttner R, Roth C, Bajbouj M, Kirste I, Heuser I, et al. Repetitive magnetic stimulation of human-derived neuron-like cells activates cAMP-CREB pathway. Eur Arch Psychiatry Clin Neurosci 2012;262:87–91.
[29] Vazana U, Veksler R, Pell GS, Prager O, Fassler M, Chassidim Y, et al. Glutamate-mediated blood-brain barrier opening: implications for neuroprotection and drug delivery. J Neurosci 2016;36:7727–39.
[30] Sher I, Tzameret A, Szalapak AM, Carmeli T, Derazne E, Avni-Zauberman N, et al. Multimodal assessment of corneal erosions using optical coherence tomography and automated grading of fluorescein staining in a rabbit dry eye model. Transl Vis Sci Technol 2019;8:27.
[31] Rotenstreich Y, Tzameret A, Kalish SE, Bubis E, Belkin M, Moroz I, et al. A minimally invasive adjustable-depth blunt injector for delivery of pharmaceuticals into the posterior pole. Acta Ophthalmol 2017;95(3):e197–205. https://doi.org/10.1111/aos.13238.
[32] Tzameret A, Kalish SE, Sher I, Twito L, Meir A, Levy I, et al. Long-term safety of transplanting human bone marrow stromal cells into the extravascular spaces of the choroid of rabbits. Stem Cells Int 2017;2017.
[33] Matsuo H, Tomidokoro A, Araie M. Penetration of topically applied fluorescein into eyes with avascular filtering bleb after trabeculectomy. Am J Ophthalmol 2005;140:742–4.
[34] Kofuji P, Ceelen P, Zahs KR, Surbeck LW, Lester HA, Newman EA. Genetic inactivation of an inwardly rectifying potassium channel (Kir4.1 subunit) in mice: phenotypic impact in retina. J Neurosci 2000;20:5733–40.
[35] Penn RD, Hagins WA. Signal transmission along retinal rods and the origin of the electroretinographic a-wave. Nature 1969;223:201–4.
[36] Young B, Eggenberger E, Kaufman D. Current electrophysiology in ophthalmology: a review. Curr Opin Ophthalmol 2012;23:497–505.
[37] Wurziger K, Lichtenberger T, Hanitzsch R. On-bipolar cells and depolarising third-order neurons as the origin of the ERG-b-wave in the RCS rat. Vis Res 2001;41:1091–101.
[38] Harhaj NS, Antonetti DA. Regulation of tight junctions and loss of barrier function in pathophysiology. Int J Biochem Cell Biol 2004;36:1206–37.
[39] Stevenson BR, Anderson JM, Bullivant S. The epithelial tight junction: structure, function and preliminary biochemical characterization. Mol Cell Biochem 1988;83:129–45.
[40] Anderson JM, Fanning AS, Lapierre L, Van Itallie CM. Zonula occludens (ZO)-1 and ZO-2: membrane-associated guanylate kinase homologues (MAGuKs) of the tight junction. Biochem Soc Trans 1995;23:470–5.
[41] Kubilus JK, Linsenmayer TF. Developmental guidance of embryonic corneal innervation: roles of Semaphorin3A and Slit2. Dev Biol 2010;344:172–84.
[42] Toshida H, Nguyen DH, Beuerman RW, Murakami A. Evaluation of novel dry eye model: preganglionic parasympathetic denervation in rabbit. Investig Ophthalmol Vis Sci 2007;48:4468–75.
[43] Brinton M, Chung JL, Kossler A, Kook KH, Loudin J, Franke M, et al. Electronic enhancement of tear secretion. J Neural Eng 2015;13:016006.
[44] Gumus K, Schuetzle K, Loudin JD, Pflugfelder SC. Randomized, controlled, crossover trial comparing the impact of sham or intranasal neurostimulation on conjunctival goblet cell degranulation. Investig. Ophthalmol. Vis. Sci. 2016;57:2864.
[45] Friedman NJ, Butron K, Robledo N, Loudin J, Baba SN, Chayet A. A nonrandomized, open-label study to evaluate the effect of nasal stimulation on tear production in subjects with dry eye disease. Clin Ophthalmol 2016;10:795–804.
[46] Ghaffarieh A, Ghaffarpasand F, Dehghankhalili M, Honarpisheh N, Nirumandi S, Tanideh N. Effect of transcutaneous electrical stimulation on rabbit corneal epithelial cell migration. Cornea 2012;31:559–63.
[47] Yamada M, Higuchi T. Functional genomics and depression research: beyond the monoamine hypothesis. Eur Neuropsychopharmacol 2002;12:235–44.
[48] Kudo K, Yamada M, Takahashi K, Nishioka G, Tanaka S, Hashiguchi T, et al. Repetitive transcranial magnetic stimulation induces kf-1 expression in the rat brain. Life Sci 2005;76:2421–9.
[49] Wang HY, Crupi D, Liu J, Stucky A, Cruciata G, Di Rocco A, et al. Repetitive transcranial magnetic stimulation enhances BDNF-TrkB signaling in both brain and

lymphocyte. J Neurosci 2011;31:11044–54.
[50] Araki-Sasaki K, Aizawa S, Hiramoto M, Nakamura M, Iwase O, Nakata K, et al. Substance P-induced cadherin expression and its signal transduction in a cloned human corneal epithelial cell line. J Cell Physiol 2000;182:189–95.
[51] Reid TW, Murphy CJ, Iwahashi CK, Foster BA, Mannis MJ. Stimulation of epithelial cell growth by the neuropeptide substance P. J Cell Biochem 1993;52:476–85.
[52] Garcia-Hirschfeld J, Lopez-Briones LG, Belmonte C. Neurotrophic influences on corneal epithelial cells. Exp Eye Res 1994;59:597–605.
[53] Uusitalo H, Krootila K, Palkama A. Calcitonin gene-related peptide (CGRP) immunoreactive sensory nerves in the human and Guinea pig uvea and cornea. Exp Eye Res 1989;48:467–75.
[54] Markoulli M, Flanagan J, Tummanapalli SS, Wu J, Willcox M. The impact of diabetes on corneal nerve morphology and ocular surface integrity. Ocul Surf 2018;16:45–57.