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(-)-Epigallocatechin-3-gallate, reduces corneal damage secondary from experimental grade II alkali burns in mice

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ABSTRACT

Background: Since recent reports have shown that (-)-Epigallocatechin-3-gallate (EGCG) could be used for treating proliferative and inflammatory disorders, we explored its use for the management of corneal chemical burns.

Materials and methods: Initially, EGCG was assayed on the rabbit corneal epithelial cell line RCE1(5T5) to establish the best testing conditions, and to avoid unwanted outcomes in the experimental animals. Then, we studied its effects on cell proliferation, cell cycle progression and cell differentiation. Afterwards, we instilled EGCG in experimental grade II corneal alkali burns in mice, three times a day up to 21 days, and evaluated by slit lamp examination and histological sections of corneal epithelial, corneal endothelial and stromal edema, as well as the presence of inflammatory cells and neovascularization.

Results: EGCG reduced cell growth and led to a decline in the proportion of proliferative cells in a concentration dependent manner. At 10 μM, EGCG promoted cell differentiation, an effect not related with apoptosis or cytotoxicity. When 10 μM EGCG was instilled in corneal alkali burns in mice three times a day up to 21 days, EGCG significantly reduced corneal opacity and neovascularization. The improved clinical appearance of the cornea was associated to a controlled epithelial growth; epithelial morphology was similar to that observed in normal epithelium and contrasted with the hyperproliferative, desquamating epithelium observed in control burn wounds. EGCG reduced corneal, stromal and endothelial edema, and wound inflammation.

Conclusion: This work constitutes the first evidence for the use of EGCG in the acute phase of a corneal alkali burn, representing a possible novel alternative to improve patient outcomes as an add-on therapy.

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1. Introduction

Chemical injuries to the eye represent between 7–22% of ocular traumas in the human population [1,2], and account for about 3-4% of total occupational injuries, which mostly occur among young men [2,3]. Frequently, they result in limbal stem cell deficiency and conjunctival damage, leading to permanent unilateral or bilateral visual impairment. Therefore, patients benefit from an immediate and intensive intervention to suppress inflammation, promote ocular surface re-epithelialization and minimize severe complications [4,5].

In general, ocular burns may implicate a severe trauma of the anterior segment including the development of ocular pannus [6–8]; besides an additional chemical and blunt trauma to the retina, and elevated intraocular pressure [9–11]. In most cases, chemical injuries are caused by alkali [2], which lead to the release of different mediators that promote tissue damage and proliferation. Thus, the alteration of the ocular structures can be observed at different levels, ranging from corneal epithelial damage to ischemia and necrosis [12].

According to its severity, the management of corneal burns involves the use of an anti-inflammatory therapy to reduce damage; the prevention of stromal breakdown to avoid corneal ulceration and melting, and the promotion of eye surface re-epithelialization [13]. In the acute setting, ocular surface reconstruction comprises surgical techniques such as transplantation of conjunctival and limbal tissue [14], the grafting of cultured limbal stem cells [14–18], and the use of amniotic membranes or cultured oral epithelia [19]. In spite of these different therapies aimed to restore vision, treatment of chemical burns is a challenge and many patients undergo a bad outcome that progresses into a permanent damage [20]. Therefore, there is an unmet need to develop complementary treatments that could reduce the use of surgery for visual rehabilitation and the incidence of visual disabilities.

(-)-Epigallocatechin-3-gallate (EGCG) is a polyphenolic compound that belongs to the family of catechins, which are formed as secondary metabolites in many of the plants used in traditional Chinese medicine. Several studies showed that EGCG inhibits normal [21] and transformed cell proliferation [22,23]; and due to its effects as an antimutagenic and antioxidant agent, it is also used in chemoprevention [24]. Importantly, EGCG shows anti-inflammatory activity by suppressing the response to inflammatory cytokines [25,26], and interfering with the associated signal transduction pathways [27–29].

Partial and extensive corneal damage is accompanied by alterations which include epithelial hyperplasia, severe inflammation, vascular tissue proliferation, conjunctivalization and active fibrosis [30–33], which are all hallmarks of corneal alkali burns. Since EGCG inhibits some of these processes, it is plausible its use to prevent sequelae. In this paper we first assayed the biological activity of EGCG on the rabbit corneal epithelial cell line RCE1(5T5) [34,35], which in vitro mimics corneal epithelial differentiation [34], and based on our in vitro results, we considered imperative to evaluate its effects on the evolution of experimental grade II corneal alkali burns in mice. Our results show that burned eyes treated with EGCG showed a statistically significant reduction in opacity, edema, neovascularization, and epithelial hyperproliferation following the alkali burn, possibly by regulation of corneal re-epithelialization and wound healing.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT). (-)Epigallocatechin gallate (≥95%) was from Sigma-Aldrich (St. Louis, MO, USA). The AE5 mouse monoclonal antibody (AE5 mAb), specific for the differentiation-linked K3 cytokeratin, was a generous gift from Dr. Tung-Tien Sun (New York University School of Medicine). All other reagents were analytical grade.

2.2. Cell culture

We used the RCE1(5T5) corneal epithelial cell line [34], since it accurately mimics the differentiation process of rabbit corneal epithelial cells. Cells were seeded at 2.7×10^3 cells/cm^2 together with 2.2×10^4 cells/cm^2 mitomycin C-treated 3T3 cells [36], and maintained as previously [35]. The 3T3 fibroblasts [37] were cultured as described [38]. Media were changed every 3 days; the cultures were maintained in a 10% CO_2 and 90% air humidified atmosphere.

To determine the effect of EGCG on the amount of proliferative cells, we determined the number of colony forming units in indicator cultures [39]. For this purpose, RCE1(5T5) cells were grown first in medium containing or not 10 μM EGCG. Four days after seeding, cells were disaggregated and transferred to four indicator dishes at 500 cells/60-mm culture dish, together with 2.2×10^4 cells/cm^2 mitomycin C-treated 3T3 cells. These cultures were grown in culture medium without the catechin, and 14 days after plating, they were fixed, and stained with rhodamine-B [38] to determine the number and size of RCE1(5T5) colonies.

2.3. EGCG preparation

EGCG was prepared before use as an aqueous 4×10^{-2} M stock solution, sterilized by filtration through a 0.22-μm pore size MF-Millipore membrane (Millipore Corporation, Bedford, MA.), and added to the culture medium to the indicated final concentration.

2.4. Transmission electron microscopy

For electron microscopy, epithelia were grown up to 5-days after confluence. Then, cultures were fixed for 60 min with 2.5%(v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Afterwards, samples were processed for electron microscopy as previously [40]. Thin sections (60 nm) were examined in a JEOL-JEM-1011 electron microscope. Semi-thin sections stained with toluidine blue were used to analyze the structure of the cultured epithelia.

2.5. Cell growth assessment

In some experiments EGCǴs effect on cell number was determined after staining fixed cultures with Rhodamine B,

which preferentially stains epithelial cells [38]. Alternatively, cells were quantified after disaggregation, using a Neubauer chamber under the microscope stage.

2.6. Keratin extraction and western blot

Total keratin extracts were obtained, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% gels, transferred to a nitrocellulose paper and stained for K3 cytokeratin [41]. K3 levels were calculated with the Quantity One v. 4.5 image analysis software (Bio-Rad Laboratories, Hercules, CA), using total keratins from a Coomasie Blue stained PAGE-SDS gel as a standard.

2.7. Flow cytometry

One day after seeding, RCE1(5T5) cells were supplemented with medium containing 10 ng/ml EGF, with or w/o 10 μM EGCG. At fourth day, after removal of 3T3 feeders, cultures were disaggregated and cell suspensions were prepared for flow cytometry, using 3×10^5 cells/assay [35]. For cell cycle analysis, cells were stained with 5 mg/ml propidium iodide in the dark. To reveal K3 cytokeratin positive cells, fixation and permeabilization were done using FACS Perm2 solution (BD Biosciences, San Jose, CA, USA) for 10 min at room temperature before incubation with the AE5 mAb [41], for 20 min on ice; and stained with a secondary anti-mouse IgG (FITC-labeled). Isotype-control matched antibodies (Santa Cruz Biotechnology, Inc.; Dallas, TX) were used as negative control. The amount of proliferative cells was also determined using the Click-iT® Plus EdU Pacific Blue™ Flow Cytometry Assay (Cat No. C10636, Thermo Fisher Scientific, Waltham, MA), according to manufacturer's instructions. All experiments were analyzed with a CyAn ADP cytometer (Beckman Coulter, Brea, CA).

2.8. Alkali-burn injury

Experiments in male BALB/c mice, aged 6–8 weeks (15–20 grams weight) were carried out in compliance with institutional regulations, according to the NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23 (revised 1985) and according to the ARVO statement for the use of animals in Ophthalmic and Vision Research. This study was approved by the research committee at the Hospital "Dr. Luis Sanchez Bulnes" from the Asociación para Evitar la Ceguera en México I. A.P. (Protocol number CO-16-09).

Animals were quarantined and acclimatized for 10 days before the experiment. On Day 0, mice received 5 mg/kg xylazine intraperitoneally as pre-anesthetic, followed by induction with 3–4% (v/v) sevoflurane and maintenance with 2% (v/v) sevoflurane. During the procedure, 0.01 mg/kg intramuscular buprenorphine was used as analgesic; and 2 mg/kg meloxicam was used for post-procedure analgesia during 5 days.

A controlled grade II alkali burn injury was done according to Anderson et al. [42]. Briefly, 2-mm diameter filter paper soaked in 1M NaOH were applied on the right eyes of mice to generate an acute alkali-burn of about 2 × 2 mm. Afterwards, mice were divided in two experimental groups. Group 1 received isotonic saline eye drops containing 10 μM EGCG three times a day. Group 2 received, with the same schedule, topical instillation of saline solution alone. At the indicated times, mice were euthanized with sodium pentobarbital (90 mg/kg). Ocular surface changes were examined using a slit-lamp on Days 1, 7 and 21 and evaluated using the Draize-derived ocular irritation scoring system devised by Pauly et al. [43] to grade corneal opacity and neovascularization.

2.9. Histological analysis

Following euthanization, eyes were proptosed and photographed. Then, they were carefully removed, dehydrated, paraffin-embedded; and 8-μm serial sections were cut and stained either with hematoxylin-eosin to observe the histological appearance of the cornea; with Masson trichrome to evaluate neovascularization and presence of scarring tissue; or with periodic acid-Schiff reaction (PAS) to evaluate corneal conjunctivalization and basement membrane integrity [44]. Slides were then photographed under a Leica DM2000 microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Corneal layers were evaluated regarding epithelial and endothelial edema, stromal edema and the presence of inflammatory cells and neovascularization, according to a 0-3 scale, where 0 denotes absence, 1: mild, 2: moderate, 3: severe. Epithelial thickness was determined using the ImageJ 1.46r analysis software [45]. Histologic parameters were evaluated by a pathologist blinded to group assignment.

2.10. Apoptosis assay

Apoptosis was determined by flow cytometry of 4-days RCE1 (5T5) cell cultures grown in medium with or without 10 μM EGCG, using the CellEvent™ Caspase-3/7 Green Detection Reagent (Cat. No. C10740, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

2.11. Data management and statistical analysis

Results correspond to the average of at least four duplicated experiments. Data are presented as the mean ± standard deviation. Data were analyzed by the two-tailed Student's T-test or, when appropriate, with the Mann-Whitney Rank Sum Test. Statistical significance was accepted if the P value was lower than 0.05. Data were analyzed using GraphPad Prism v 6.0 statistical software (GraphPad Software, Inc.; La Jolla, CA)

3. Results

3.1. EGCG inhibits cell proliferation by promoting cell differentiation

Considering previous results which showed that EGCG inhibits the proliferation of normal and transformed cells [21–23], we studied the effects of this polyphenolic compound in corneal epithelial cell growth, using as an experimental model the immortalized rabbit corneal epithelial cell line RCE1(5T5) [34]. As shown in Fig. 1(A,B), EGCG inhibited cell proliferation when 2.5 μM or higher concentrations were added to culture medium. The ED50 for EGCG was about 6.5 μM, and the

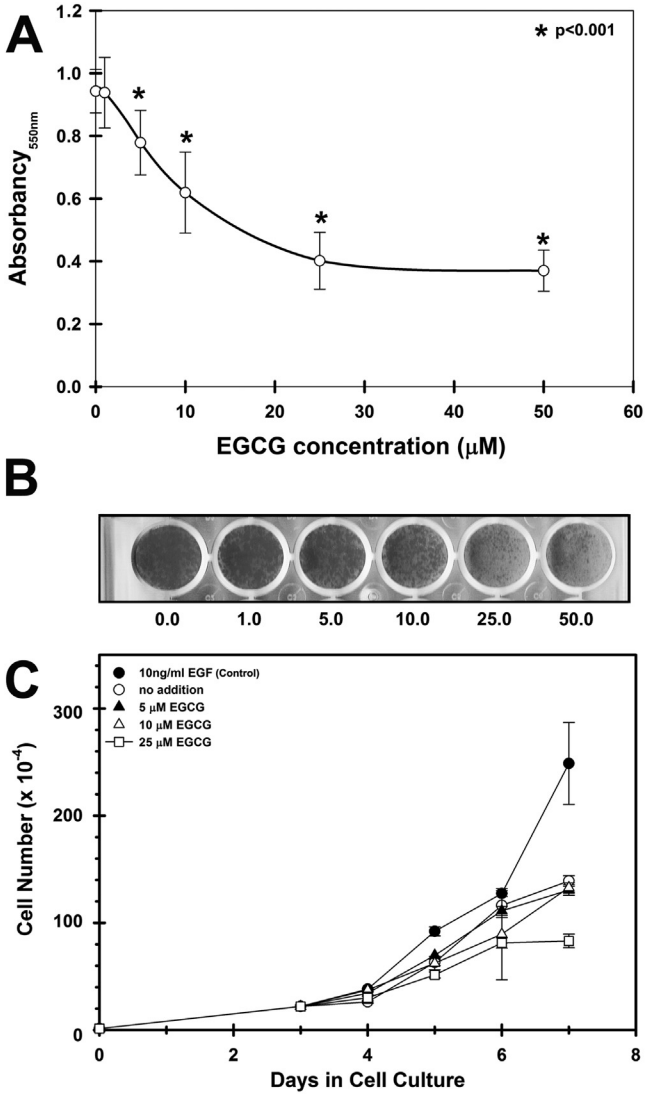

Fig. 1 – EGCG reduces growth rate of corneal epithelial RCE1(5T5) cell cultures in a concentration-dependent manner. (A, B) One day after seeding, cells were supplemented with culture medium containing 0-50 μM EGCG and 10 ng/ml EGF; control cultures consisted in cells supplemented with medium containing 10 ng/ml EGF. At 5th day, cultures were fixed and stained with rhodamine B as described (see Cell growth assessment). Then, they were photographed (B) and extracted to determine the size of cell population (A). In other experiments, cultures were trypsinized and counted every day to determine cell number and proliferation rate (C). Note that cell growth was not completely inhibited by EGCG. In (C), controls consisted in cells supplemented with medium containing of 10 ng/ml EGF, which as reported, should show a maximal proliferative rate, and cells cultured in medium without EGF to obtain the lowest proliferative rate.

maximal inhibition was obtained with 25 μM or higher concentrations (Fig. 1A,B), as determined by a Rhodamine B colorimetric assay.

These results were confirmed when proliferation in the presence or absence of 5–25 μM EGCG was quantified by an everyday direct cell counting after trypsinization of cultures (Fig. 1B). As expected, cells supplemented with medium containing 10 ng/ml EGF alone, displayed the highest proliferation rate (Fig. 1C). In contrast, cells which received increasing EGCG concentrations together with 10 ng/ml EGF, showed a reduction in their proliferative ability proportional to the added EGCG concentration (Fig. 1C). Since cultures grown in the presence of 5–10 μM EGCG had similar cell numbers to those maintained in medium with no additives (Fig. 1C), the results suggest that EGCG did not completely block cell proliferation. Moreover, we found that EGCG caused a significant augment on cell doubling time, with a higher increase and variability in those cultures grown in the presence of 25 μM EGCG (Supplementary Table S1). Based on the above results, further analyses used 10 μM EGCG as the conventional experimental condition.

To extend our examination of EGCG effects on the proliferative potential, we assayed the colony forming efficiency (CFE) of cells grown in the presence of 10 μM EGCG. For

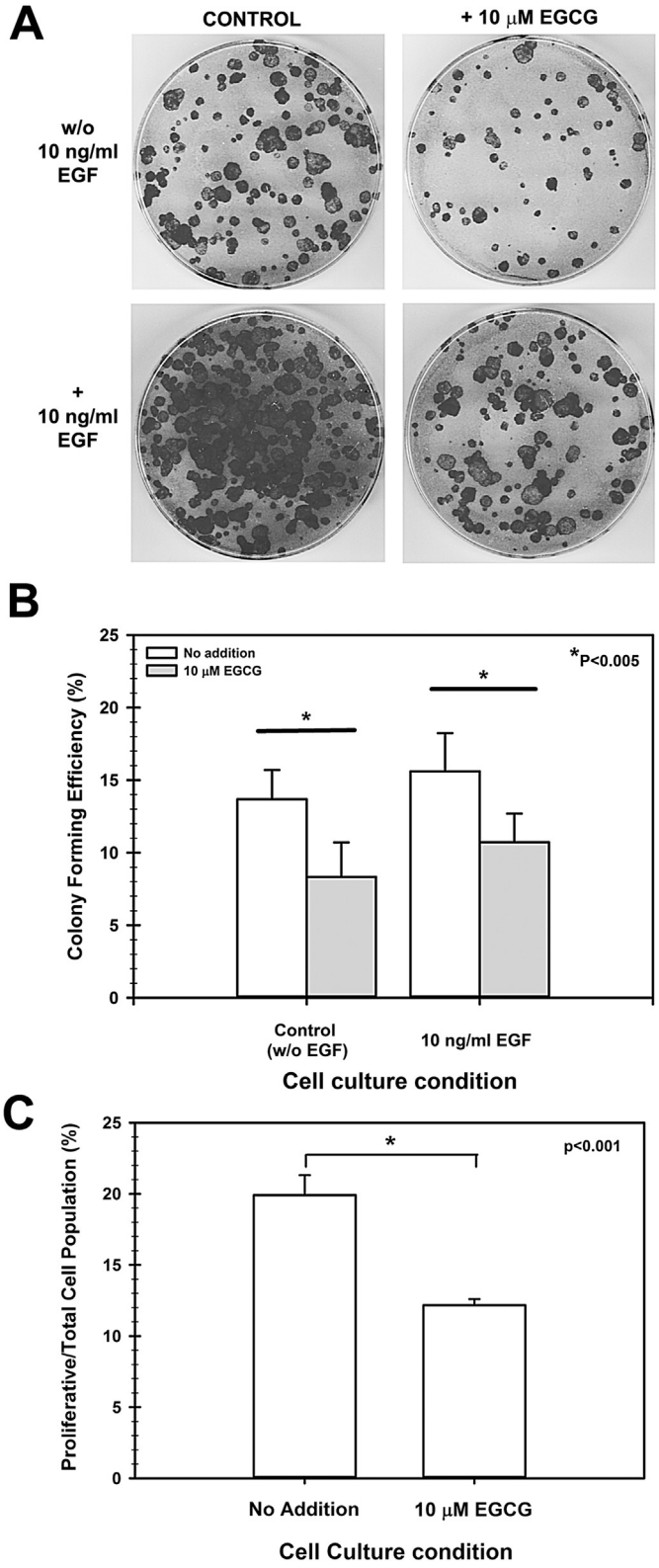

Fig. 2 – EGCG reduces the number of proliferative cells. To determine the number of proliferative cells, we followed two different approaches: RCE1 cells were plated in 35-mm culture dishes and supplemented either with medium containing or not 10 ng/ml EGF and 10 μM EGCG. Four days after seeding, cells were harvested and plated in indicator dishes at 500 cells/60-mm under standard culture conditions. After 14 days, cultures were fixed and stained with Rhodamine B (A) and the number cells with colony forming ability was determined (B). Alternatively, (C) the number of proliferative cells was determined by flow cytometry after S-phase cells staining with Pacific Blue™ dye (see Flow cytometry section).

these experiments, cells were plated in 35-mm culture dishes and supplemented with medium containing or not 10 ng/ml EGF and 10 μM EGCG. Control cultures were fed with medium without the catechin. Four days after seeding, cells were harvested and plated on indicator dishes (see cell culture section) and maintained under standard conditions. Under such cultivation scheme, if EGCG exerted a long-term inhibitory effect on cell proliferation it would be detected in the indicator cultures. Fig. 2 shows that EGCG promoted a significant 40% decrease in CFE both in cultures grown in the presence or the absence of 10 ng/ml EGF (Fig. 2A, B), indicating that EGCG reduced the number of proliferative cells. We confirmed these effects by measuring the size of the proliferative cell compartment in flow cytometry experiments which showed a 40% reduction of proliferative cells in EGCG treated cultures (Fig. 2C).

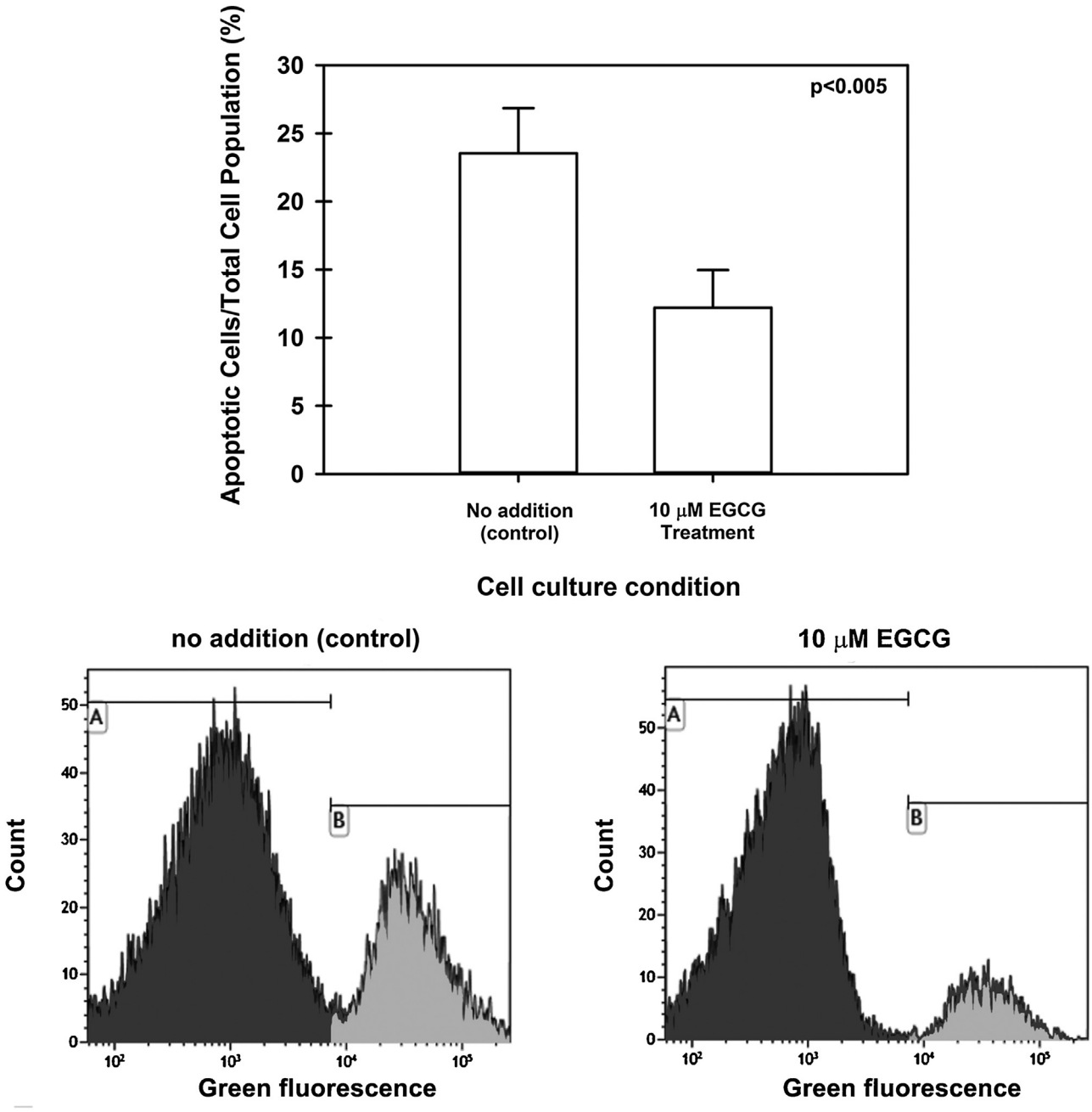

Fig. 3 – EGCG does not promote apoptosis of RCE1(5T5) cells. Cells were grown in the presence of 10 μM EGCG. After 4 days, cell cultures were disaggregated and apoptosis was determined by determination of the number of cells with active Caspase 3/7. Control cultures were supplemented with medium without the catechin. (Dark grey corresponds to caspase 3/7 negative cells; Light gray corresponds to cells with activated caspase 3/7).

Table 1 – Cell Cycle distribution of RCE1(5T5) cells treated with EGCG.

Cell cycle phase	Cell culture condition		
	No addition	10 mM EGCG	P-value
G_1	47.3±1.0	52.4±1.8	0.003
S	42.1±0.7	39.2±2.1	0.017
G_2/M	10.8±1.6	7.4±0.8	0.006

Distribution of cell population along the cell cycle was determined, after removal of 3T3-feeder cells, by flow cytometry of propidium iodide stained cultures. The RCE1(5T5) cells were cultured as described, and supplemented with medium containing or not 10 μM EGCG. Results are the average of six duplicated experiments (±SD). Statistical significance was accepted when the P value was lower than 0.05.

The results were further validated when we determined the distribution of cells along cell cycle. Cells treated with EGCG underwent a G_0/G_1 cell cycle arrest, as demonstrated by the increase in the number of cells in this phase, and the reduction of cell populations in S and G2 (Table 1). Together, the above results led us to propose that EGCG inhibits corneal epithelial cell growth through a reduction in the size of the proliferative compartment, induced either by promotion of cell differentiation, or via the induction of apoptosis.

To distinguish between these alternatives, we analyzed whether EGCG promoted apoptosis. After growing during 4 days in the presence of the catechin, cells showed a 50% decrease in the amount of apoptotic cells compared to non-treated cultures (Fig. 3). Similar results were obtained when cell viability was assayed after treatment with EGCG (not shown).

Afterwards, we determined whether EGCG promoted cell differentiation assessing the amount of K3-cytokeratin positive cells. First, we found that proliferative cultures treated during 4-days with 10 μM EGCG underwent an increase in the amount of differentiated cells: while cultures grown under standard cell conditions contained their characteristic low number of K3-cytokeratin positive cells (3.3% of total cell population), the growing cultures treated with EGCG showed a 10-fold increase in the number of differentiated cells (Fig. 4A).

Similar results were obtained when we evaluated K3-cytokeratin levels by densitometric analysis of western blots. In such experiments, when added to proliferative cells EGCG led to a 40% increase in K3 keratin content compared with control cultures grown without the catechin (Fig. 4B, C). Furthermore, in confluent cultured epithelia (8 days after inoculation), characterized by their high content of K3-cytokeratin levels, treatment with 10 μM EGCG augmented 30% the levels of this differentiation-linked keratin (Fig. 4B, C).

In conclusion, our results show that EGCG inhibits cell proliferation by induction of a cell cycle arrest and promotion of corneal epithelial cell differentiation. As a consequence, cells grown in the presence of 10 μM EGCG formed epithelia with a lower number of cell layers than control cultures (Supplemental Fig. S1 A, B), although there was not an evident effect in the ultrastructure of the epithelial cells, as demonstrated by electron microscope analysis (Supplemental Fig. S1 C, D).

3.2. *Instillation of (-)-Epigallocatechin-3-gallate onto corneal alkali burn injuries reduces corneal opacity, epithelial hyperplasia, edema and neovascularization*

The first 20 days after a chemical eye injury constitute the acute and early phases of wound recovery [4], and the management determines either the completion of healing with good visual prognosis, or the development of late complications that include poor vision, corneal scarring, xerophthalmia, symblepharon and corneal pannus among others [46]. Depending on the depth and severity of corneal damage, these phases are associated with epithelial hyperplasia, conjunctivalization, squamous metaplasia, active fibrosis, severe inflammation, and active neovascularization [6,30]. Since it has been reported that burned patients with epithelial hyperplasia show an unfavorable outcome compared to patients without hyperplasia [6,47] and in view of our results obtained from the treatment of cell cultures with the catechin, we explored the effects of EGCG's instillation on experimentally induced alkali-burn injuries in mice.

The instillation of saline solution containing 10 μM EGCG on corneal alkali-burn injuries led to a reduction in corneal opacity as evaluated by slit-lamp examination on Days 1, 7 and 21 after the injury, compared to control eyes treated with saline solution only. As shown in Fig. 5 and Fig. 7(A), the effect of EGCG on corneal opacity was observed along the treatment, being more evident 21 days after the injury when mice had an almost clear cornea (see Fig. 5D,E), while in control mice corneal tissue remained opaque and with neovascularization (Fig. 5B,C). Evaluation of corneal opacity demonstrated that differences between treated and untreated corneas were evident from 7 days after injury (Fig. 7A; $p<0.05$), and markedly significant at 21 days (see Fig. 7A; $p<0.001$).

To obtain a better understanding of our clinical observations, we carried out the histological examination of alkali-burned corneas from both groups. As observed after clinical examination of the burned corneas, we did not find differences in corneal re-epithelialization between both groups. One of the most evident findings was a 2-fold increase of epithelial thickness 7 days after the injury, observed only in control corneas (Fig. 6B, G and K; Fig. 7B). Such augment in epithelial thickness is partly explained by the severe epithelial edema observed in this group (Fig. 6B, G and K; Fig. 7C), and by an epithelial hyperproliferative response that led to a slight increase in the number of cell layers and epithelial desquamation (see Fig. 6B, G and K, arrowheads). In contrast, corneas treated with 10 μM EGCG showed mild epithelial edema and a reduced number of epithelial cell layers (Fig. 6E, I and M; Fig. 7C) that may be explained as a result of the slower growth rate caused by the catechin (see above). This effect was also supported by an epithelial thickness similar to that found in normal, intact corneas (Fig. 6A, 7 B). Subsequently, 21 days after the alkali burn injury, corneal epithelial thickness returned to normal values in both groups (Figs. 6A, C, F; and 7 B). However, edema and corneal opacity persisted in control corneas, while EGCG-treated corneas recovered an almost normal transparency without evidence of edema (Fig. 7A and C).

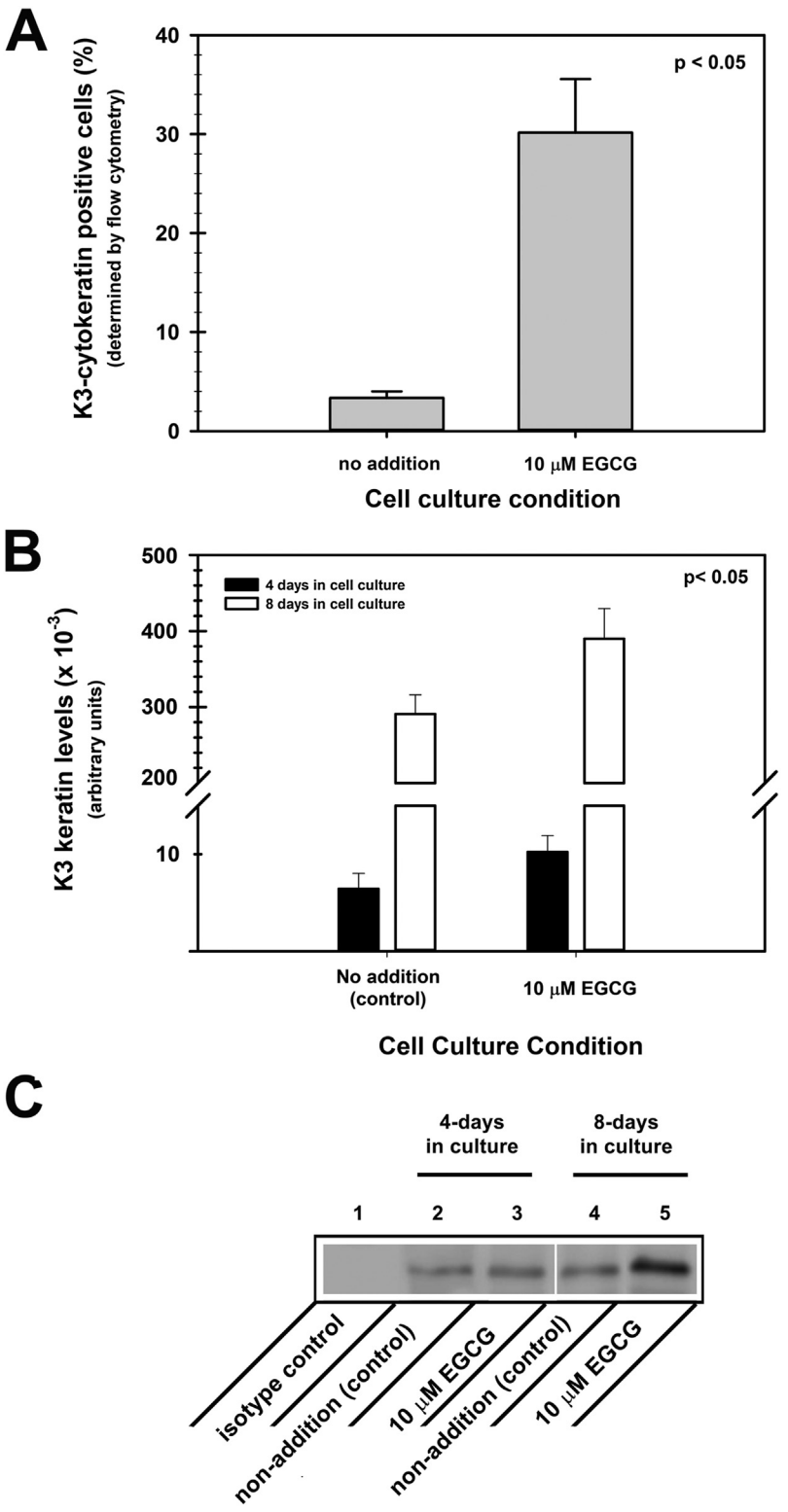

Fig. 4 – EGCG stimulates corneal epithelial cell differentiation. Effect of EGCG on cell differentiation was determined by growing cells in the presence of 10 μM EGCG and (A) quantification of cells immunostained with the monoclonal antibody AE5 which is specific for the differentiation-linked K3 cytokeratin; or (B, C) extracting intermediate filaments from cell cultures grown in the presence of EGCG during 4 or 8 days after seeding. (B) Densitometric analysis of western blots immunodetected with the AE5 antibody, similar to that shown in (C). Note that in (A), the number of K3-positive cells was determined in proliferative cultures (5 days in cell culture), which contain a low number of differentiated cells.

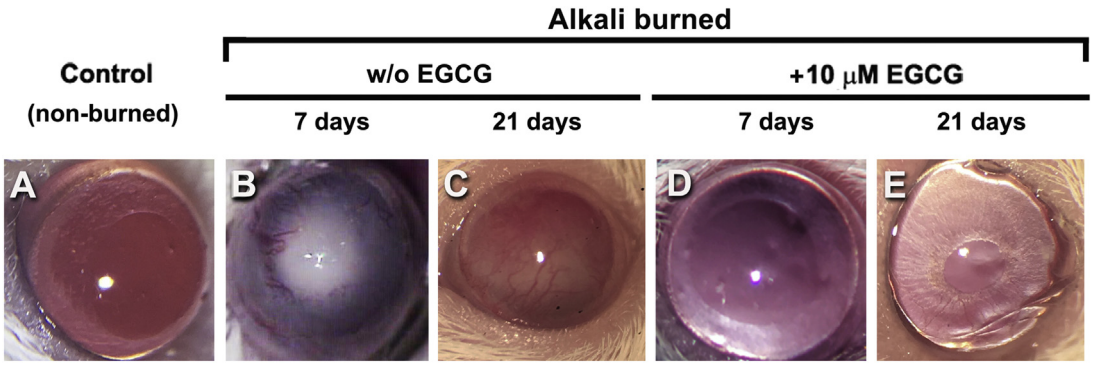

Fig. 5 – Treatment of corneal alkali burns with EGCG improves corneal transparency and reduces neovascularization. Representative photographs showing the evolution of (D, E) EGCG-treated and (B, C) control corneal alkali burn wounds at 7 and 21 days after injury. (A) A non-injured eye is shown for comparison with treated eyes. Note that in control corneas, opacity and superficial neovascularization were evident as early as the 7th day after wounding (B) and persisted along the experiment (C). In contrast, opacity in EGCG-treated eyes was lower or absent at 7th day (D) and receded at 21st day (E), when corneas were completely clear. In EGCG treated wounds we observed a minimal neovascularization.

Additionally, we also evaluated the differences between groups for stromal edema, stromal inflammatory cells, endothelial edema and neovascularization 7 and 21 days after the corneal alkali-burn. As shown in Fig. 6 and Fig. 8, re-establishment of basement membrane (Fig. 6G–J) and infiltration of inflammatory cells within corneal stroma (Fig. 8B) did not show significant differences between control and treated eyes at any time. In contrast, stromal and endothelial edema were moderate to severe in control eyes, and mild in those eyes treated with the catechin (Fig. 8A, C); these differences were more significant 21 days after the injury, when control eyes (see Fig. 6C) were compared to those treated with EGCG (Fig. 6F, I and N), the latter showing an almost normal appearance, while in control eyes stromal and endothelial edema persisted. Finally, when corneal neovascularization was examined, we observed that control animals developed neovessels starting 7 days after the burn (see Fig. 5B), that continued growing until 21st day, when we observed completely neovascularized corneas (see Fig. 5C). Growth of new blood vessels in the control group (Fig. 6H, L), and absence of neovascularization in eyes treated with 10 μM EGCG (Fig. 6J, N) was also demonstrated by histological evaluation. Quantitation of clinical neovascularization showed that those animals treated with EGCG had a slight development of new blood vessels at day 7; however, there was not further growth of neovessels, and 21 days after injury, corneas were non-vascularized and almost transparent (Fig. 5E, 8 D).

Together, the results suggest that EGCG may improve the outcome of corneal burn injuries through its effects on the acute phase of wound repair. Such effects could be related, at least in part, with its activity as a regulator of epithelial growth and differentiation.

4. Discussion

Chemical burns of the cornea are a potentially sight-threatening condition whose outcome depends on the management of the wound. Treatment of alkali burns during the acute phase is of major importance to decrease inflammation, avoid further epithelial and stromal breakdown, and foster re-epithelialization [2]; if the response to standard therapy during the acute phase is not adequate, patients undergo unsatisfactory outcomes and severe visual disability.

Under normal conditions, corneal epithelium undergoes a rapid renewal with a turnover rate of 4 to 6 days [48]; among its multiple biological activities, it regulates metabolic activity and collagenase production by stromal keratocytes [33]. It has been shown that the recovery of an intact and phenotypically normal corneal epithelium is the most important determinant for a favorable outcome following chemical injuries, to prevent the invasion by fibrotic tissue, reduce the inflammatory process that impairs normal repair, and improve corneal transparency [33,49–50].

In view of recent results that showed that EGCG might be a promising agent for treating proliferative and inflammatory systemic disorders [22–24,26], and considering that partial and extensive corneal burn damage is accompanied by such alterations, we explored the possible use of EGCG as a therapeutic agent in the management of corneal chemical burn injuries.

To this purpose, we first evaluated the effects of EGCG on the rabbit corneal epithelial cell line RCE1(5T5), which was demonstrated as an excellent in vitro model to study the mechanisms that regulate proliferation and differentiation of corneal epithelial cells [34,51–53], and the role of growth factors, cytokines and other molecules on epithelial cell growth [35,38,40,52–54]. Since EGCG shows differential effects depending on cell type, we used this cell line to look for possible unsought outcomes on corneal epithelial growth, differentiation and viability that could discard it as a potential therapeutic agent, and in order to support its further utilization in the experimental treatment of corneal alkali burns.

As demonstrated, the addition of increasing amounts of EGCG to culture medium led to a reduction in cell growth rate,

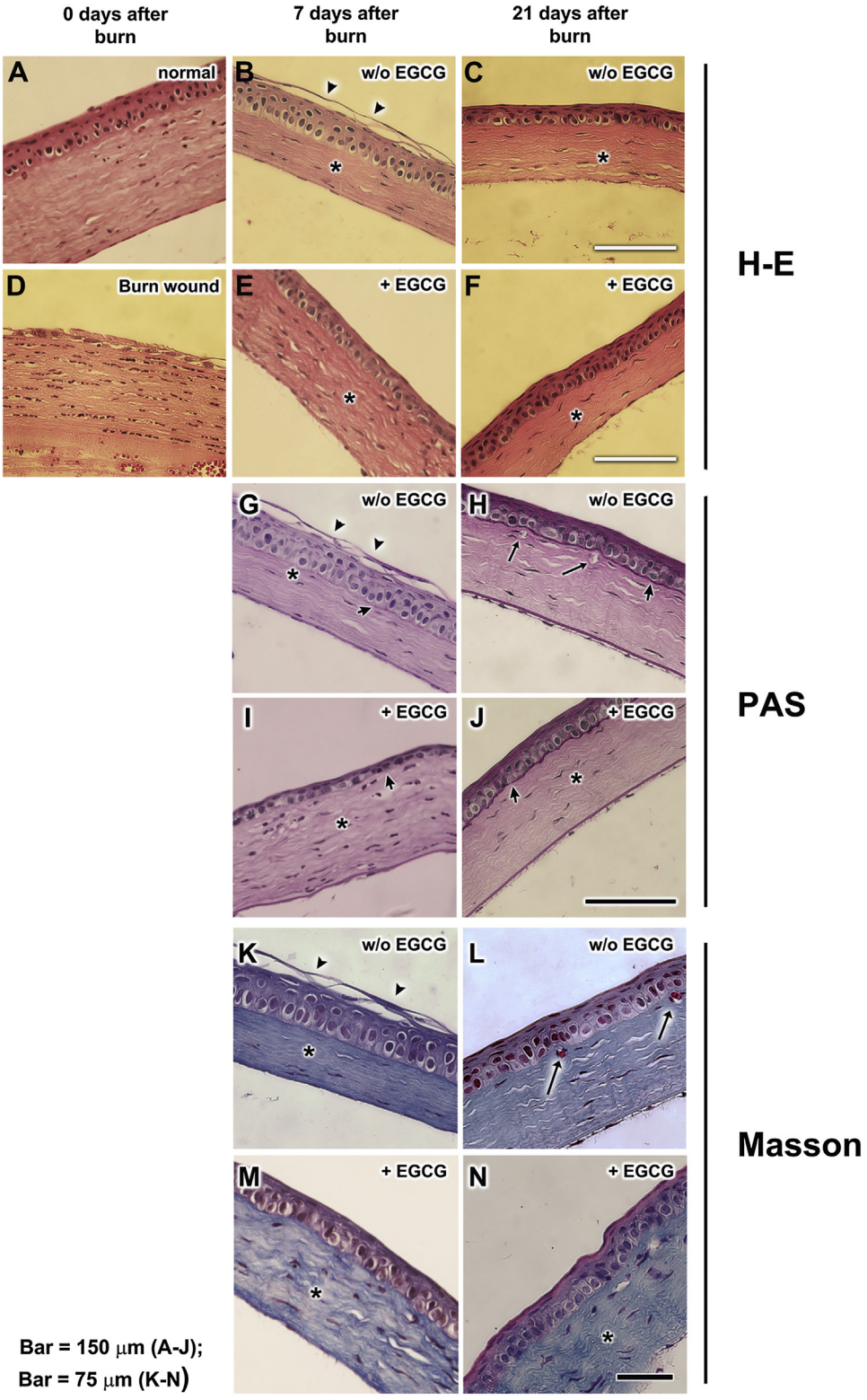

Fig. 6 – Histological changes promoted by EGCG treatment in corneal alkali burn wounds. The differences between control and EGCG-treated burn wounds at 7 days (B, E, G, I, K, M) and 21 days (C, F, H, J, L, N) after injury, are shown in histological sections stained either with hematoxylin-eosin (H-E), PAS or Masson trichrome techniques. (A) Normal unwounded cornea; (D) corneal wound bed. Note that at 7 days of treatment with EGCG, wounds were covered with a thinner epithelium (E, I, M) than non-treated burn wounds (B, G, K). At 21 days after wounding, epithelial differences disappeared, but superficial neovascularization was evident in non-treated eyes (H, L; thin arrows). Asterisks show keratocyte and inflammatory cells in stroma; Arrowheads correspond to epithelial desquamation and small arrows point to basement membrane. Scale bar = 150 μm in (A–J), and 75 μm in (K–N).

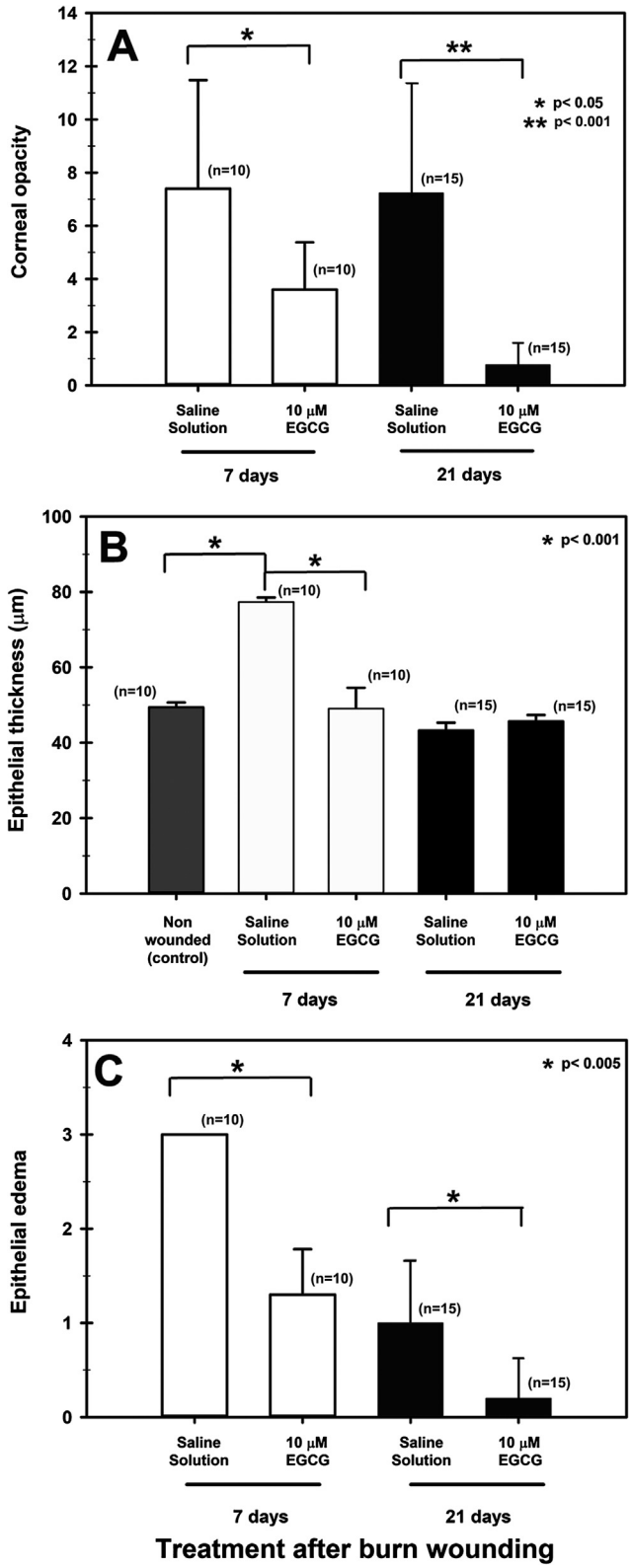

Fig. 7 – EGCG reduces corneal opacity, epithelial edema and epithelial hyperproliferation after alkali burn injury. Quantitative analysis of corneal opacity, epithelial thickness and epithelial edema in EGCG-treated and control wounded corneas 7 and 21 days after injury. (A) Evaluation of opacity was made according to the Pauly scoring system (2007) and (C) epithelial edema with a 0-3 scale as described (see Histological Analysis section). (B) Epithelial thickness was evaluated by the use of a micrometric scale. Results correspond to the average and standard deviation from 10–15 mice per experiment, in which 10 fields each from at least 3 slides/animal were evaluated.

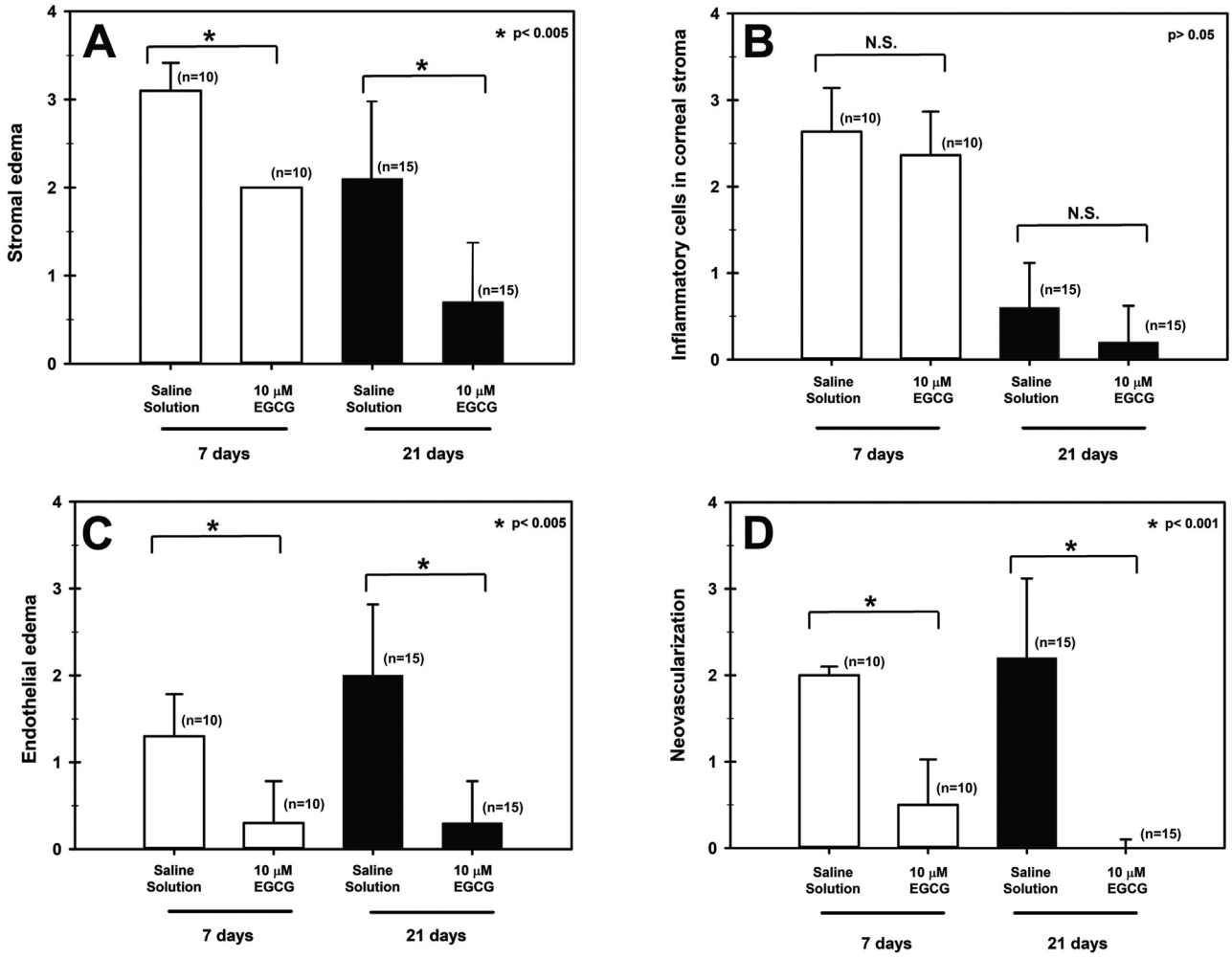

Fig. 8 – Treatment of corneal alkali burn wounds with EGCG reduces stromal and endothelial edema, and corneal neovascularization. (A) Stromal edema, (B) presence of inflammatory cells in corneal stroma, (C) endothelial edema and (D) neovascularization were evaluated in tissue sections by a blinded pathologist to avoid the bias of results (see Histological Analysis). The results did not show a significant difference on inflammatory cells between treated and non-treated wounds. Results correspond to the average and standard deviation from 10-15 mice per experiment, in which at least 10 fields each from 3 slides/animal were evaluated.

associated with a decline in the number of colony forming cells and a decrease in the proportion of proliferative cells. This reduction in cell growth was not due to a blockage of cell proliferation given that cells treated with the higher EGCG concentrations still proliferated but with a slower growth rate. These effects are explained as result of the augment in the proportion of cells that remain in the G1 phase of cell cycle, and as shown, they are not related with the promotion of apoptosis. Interestingly, EGCG reduced apoptosis as observed in other non-transformed, normal cell types [55,56]; but contrary to our expectations, this effect did not increase cell proliferation neither augmented the size of cell colonies.

Instead, the results strongly suggest that the catechin induced cell differentiation as indicated by the increase in the expression of the differentiation-linked K3 cytokeratin [41], and by a significant reduction in the number of cell layers observed in the cultured epithelia. These findings are consistent with previous observations in normal human epidermal keratinocytes, in which the catechin induced differentiation as evaluated by the expression of molecular markers such as K1 cytokeratin, involucrin, TGase1 and filaggrin [57–59]; but it did not promote apoptosis [57,58]; suggesting that EGCG stimulates the expression of the terminal differentiated phenotype in normal stratified epithelia.

Therefore, we proceeded to examine the in vivo effects of EGCG on a murine model, which is a common and reliable method to evaluate corneal alkali burns [42,60–61]. For the experiments, we chose the 10 μM EGCG concentration because

it led to more consistent results without knocking down the proliferative abilities of the corneal epithelial cells.

As shown, treatment of corneal alkali burns by instillation of an ophthalmic solution containing 10 μM EGCG led to a reduction of corneal opacity and neovascularization as clinically evaluated by slit lamp examination. In addition, histological comparison between treated and non-treated eyes indicated that EGCG exerted a significant role by inducing a controlled epithelial proliferative response and promoting cell differentiation, producing a reduction in the number of epithelial cell layers and epithelial thickness mainly at the 7th day of treatment, the time point when the hyperproliferative response reached its maximum. These effects were similar to those observed in cell culture experiments, and anticipated by previous results in human epidermal keratinocytes [57–59]. Afterwards, as observed at 21 days of treatment, epithelial thickness returned to normal values comparable to those found in unwounded corneas. Moreover, we did not observe any effect of EGCG on the re-epithelialization of the wound bed, which suggests that the catechin did not affect epithelial cell migration nor wound closure.

The improved clinical appearance of the cornea was also associated with a reduction in corneal, stromal and endothelial edema, and to a decrease in neovascularization as demonstrated both clinically and by H-E and Masson Trichrome stained tissue. We did not observe changes on basement membrane formation nor conjunctivalization of the corneal surface as shown by PAS staining. The effect on neovascularization can be explained by the reported effects of EGCG on the proliferation, migration and tube formation ability of endothelial cells [62–64], hampering the development of neovessels.

On the other hand, we did not find a significant difference in the infiltration of corneal stroma by inflammatory cells, although our clinical findings, the inhibition of corneal neovascularization (see above), and results from other laboratories suggest that treatment with the catechin should decrease the inflammatory response subsequent to corneal injury [65,66]. Our ongoing experiments should enable us to clarify the mechanisms that lead to the improvement of corneal repair due to the treatment with this polyphenolic compound.

5. Conclusion

These results demonstrate a better corneal repair response after alkali burn injuries by the use of EGCG. This compound promoted a regulated re-epithelialization, reduced corneal edema and neovascularization. Although topical EGCG has been used as a therapeutic agent in the experimental treatment of dry eye in mice [67], as well as to inhibit neovascularization and vascular permeability [63–65,68], to the best of our knowledge, this work constitutes the first evidence of the use of EGCG in the acute phase of corneal alkali burns. This work opens the possibility of the use of EGCG as a novel alternative to improve patient outcomes as an add-on therapy, potentially diminishing or eliminating the occurrence of severe visual sequelae. Its effectiveness in humans remains to be evaluated in further clinical studies.

Conflict of interest

Authors declare no potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.burns.2018.08.021.

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