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The healing of alkali-injured cornea is stimulated by a novel matrix regenerating agent (RGTA, CACICOL20) - a biopolymer mimicking heparan sulfates reducing proteolytic, oxidative and nitrosative damage

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Summary. The efficacy of a chemically modified dextran - heparan sulfate mimicking regenerating agent (RGTA) on the healing of the rabbit cornea injured with alkali was examined. The eyes were injured with 0.15 N NaOH applied on the cornea or with 1.0 N NaOH using a 8 mm diameter filter paper disk. Then RGTA or placebo was applied on the cornea. In the last group of rabbits, corneas injured with the high alkali concentration were left without any treatment for four weeks; subsequently, the corneas were treated with RGTA or placebo. The central corneal thickness was measured using a pachymeter. The corneas were examined morphologically, immunohistochemically and for real time-PCR. Compared to control (unaffected) corneas, following the application of low alkali concentration the expression of urokinase-type plasminogen activator, metalloproteinase 9, nitric oxide synthase and xanthine oxidase was increased in the injured corneal epithelium of placebo-treated eyes, whereas the expression of antioxidant enzymes was reduced. Nitrotyrosine and malondialdehyde stainings appeared in the corneal epithelium. RGTA application suppressed the antioxidant/prooxidant imbalance and reduced the expression of the above-mentioned immunohistochemical markers. The corneal thickness increased after alkali injury, decreased during corneal healing after RGTA treatment faster than after placebo application. Following the injury with the high alkali concentration, corneal inflammation and neovascularization were highly pronounced in placebo-treated corneas, whereas in RGTA-treated corneas they were significantly supressed. When RGTA or placebo application was started later after alkali injury and corneas were ulcerated, subsequent RGTA treatment healed the majority of them. In conclusion, RGTA facilitates the healing of injured corneas via a reduction of proteolytic, oxidative and nitrosative damage.

Key words: Alkali-injured cornea, RGTA treatment

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Introduction

Severe alkali injury of the cornea evokes acute corneal inflammation, leading to rapid infiltration of the corneal stroma with neutrophils, followed by chronic inflammation, during which inflammatory cells migrate into the cornea for a long period of time, resulting in profound corneal destruction. Within three to four weeks after a severe large alkali injury, the majority of corneas are ulcerated or perforated, leading to the loss of the eye or reduced vision due to scar formation.

However, not only injuries with highly concentrated alkalis are dangerous to the eye, but less concentrated alkalis also threaten vision. Kubota et al. (2011) described that soon after the injury of the cornea with 0.15 N NaOH, oxidative stress appeared in the cornea, preceding intracorneal inflammation.

To save vision after an alkali injury, a number of therapies have been investigated, including epidermal growth factor (Gonul et al., 1995), fibronectin (Phan et al., 1991), ascorbate (Levinson et al., 1976; Pfister and Paterson, 1980; Pfister et al., 1982), citrate (Pfister et al., 1981; Haddox et al., 1989; Pfister et al., 1991), metalloproteinase inhibitors (Schultz et al., 1992; Wentworth et al., 1992; Paterson et al., 1994; Pfister et al., 1997; Sotozono et al., 1999), bovine lactoferrin (Pattamatta et al., 2009), bevacizumab (Mello et al., 2011) and hydrogen (H_2) irrigation solution (Kubota et al., 2011). Although these therapeutic approaches lead to the decrease of incidences of corneal ulcerative and scarring processes, the new methods of therapy are being devoloped with the aim of optimizing the course of healing with the restoration of corneal transparency and vision restitution.

With the aim of contributing to the effective therapy of corneal alkali injury, we employed in this study the regenerating agent (RGTA, CACICOL20) for the treatment of alkali-injured corneas in a rabbit experimental model. RGTA is an engineered biopolymer mimicking heparan sulfates as a protector and stabilizer of the actions of heparin-binding growth factors, and is known to stimulate wound healing under various conditions in different in vivo systems (Garcia-Filipe et al., 2007; Chebbi et al., 2008; Groah et al., 2011; Zakine et al., 2011). Recently, Aifa et al. (2012) described the effective healing of chronic ulcers in humans using RGTA drops. However, until now, there have existed only preliminary findings dealing with alkali-injured corneas treated with RGTA (Brignole et al., 2005). Therefore, in this study we decided to examine the efficacy of RGTA treatment on the alkali-injured rabbit cornea in detail.

In the healthy cornea a physiological balance between antioxidant and prooxidant mechanisms exists, which becomes disturbed under various corneal injuries or diseases, particularly those in which oxidative stress plays an important role (Buddi et al., 2002; Cejkova et al., 2005; Arnal et al., 2011; Zhou et al., 2012). The amount of antioxidants decreases in the cornea, leading to the antioxidant/prooxidant imbalance. Reactive oxygen species are insufficiently cleaved contributing to the induction and activation of various enzymes, such as metalloproteinases, serine proteases and nitric oxide synthases, enzymes that generate nitric oxide (Cejkova et al., 2005; Arnal et al., 2011). Toxic oxygen and nitrogen products with proteolytic enzymes degrade the cornea (e.g. Buddi et al., 2002; Zhou et al., 2012). To examine corneal destructive processes evoked by oxidative stress to the cornea after alkali injury and their possible suppression by RGTA, the following immunohistochemical markers were employed; the expression of superoxide dismutase (an enzyme, cleaving superoxide and belonging to antioxidant enzymes) and xanthine oxidase (an enzyme that generate reactive oxygen species and belongs to prooxidant enzymes). After the oxidative injury, the activity as well as expression of xanthine oxidase remain at normal levels or are even increased in the corneal epithelium, whereas the activity as well as expression of antioxidant enzymes are decreased. This contributes to the prooxidant/antioxidant imbalance in the corneal epithelium (Cejkova et al., 2004; Tessem et al., 2006). Of a group of metalloproteinases, matrix metalloproteinase 9 belonging to gelatinases/type IV collagenases was detected in this study. Matrix metalloproteinases, secreted as proenzymes, are modulated by activating proteases (Woessner, 1991), e.g. by serine proteases. Of the serine proteases, plasminogen activator of urokinase type, investigated in this study, is suggested as a potent activator of various destructive proteases. This enzyme is highly involved in corneal neovascularization, inflammation and chemotaxis (e.g. Martell-Pelletier et al., 1991). Of the nitric oxide synthases, the expressions of endothelial nitric oxide synthase and inducible nitric oxide synthase were examined in alkali injured corneas. To follow these enzymes is important because nitric oxide present in high amounts reacts with superoxide to form very toxic peroxynitrite. Peroxynitrite formation serves as a marker of oxidative stress (Ceriello 2002; Chirinio et al., 2006). Immunohistochemically, the formation of peroxynitrite is investigated by the staining of nitrotyrosine residues. Moreover, we detected malondialdehyde, a marker of lipid peroxidation (Buddi et al., 2002; Del Rio et al., 2005). Lipid peroxidation may result from oxidative destruction of cell membranes and formation of cytotoxic aldehydes. These aldehydes may induce activities of destructive enzymes and inhibition of DNA/RNA protein synthesis (Esterbauer et al., 1991). Because the expressions of the above mentioned immunohistochemical markers are low in the normal cornea (metalloproteinase 9, plasminogen activator of urokinase type, endothelial nitric oxide synthase) or they are completely absent (inducible nitric oxide synthase, nitrotyrosine, malondialdehyde), their positive staining in injured or diseased cornea clearly points to corneal destruction or degeneration. Finally, we investigated the corneal inflammatory response and corneal neovascularization in alkali-injured cornea after RGTA or placebo treatment using anti-VEGF and anti-macrophage antibody. Quantitative evaluation of our experiments was examined by real time-PCR.

Material and methods

Alkali injuries in experimental animals

Adult female New Zealand white rabbits (2.5-3.0 kg) were used in our experiments. The investigation was conducted according to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Rabbits were anesthetized by an intramuscular injection of Rometar (Xylazinum hydrochloricum, Spofa, Prague, CR, 2%, 0.2 ml/kg body weight) and Narkamon (Ketaminum hydrochloricum, Spofa, 5%, 1 ml/kg body

weight).

In the first experiment the corneas of anesthetized rabbits were injured by applying 0.15 N NaOH on the corneal surface for 1 min, then the eyes were rinsed with tap water. One hour after the injury, RGTA was applied on the right eye in the first group of animals and dextran or saline on the right eyes of the second and third groups of rabbits (six rabbits in each group). Subsequently, RGTA or placebo was dropped every day until sacrificing the animals. For immunohistochemical examination, rabbits were sacrificed on day four after the injury. For measurement of central corneal thickness (using a pachymeter as described below) and for real-time PCR, the animals were sacrificed after six days following the injury (on day 7). For pachymetry and real-time PCR dextrane was used as placebo.

In the second experiment a filter paper disk 8 mm in diameter, soaked with alkali (1.0 N NaOH), was placed on the center of each right cornea for 1 min; afterwards the corneas were rinsed with tap water. The rabbits were divided into three groups (six rabbits in each group). In the first group the regenerating agent (RGTA) was applied on the right eye one hour after the injury. In the second group dextran and in the third group saline were applied on the right eye as a placebo. Treatment with RGTA, dextran or saline continued every second day until sacrificing of the animals, which took place on day four after burning (for morphological examination) or following three or six weeks of continued treatment. At these latter time intervals the corneas were examined morphologically as well as immunohistochemically.

In the third experiment the corneas were injured with alkali similarly as in the second experiment and then the burned corneas were left without any treatment for one month. Subsequently, the corneas were treated with RGTA or placebo (every second day) for four weeks. (Six rabbits in each group was employed). The rabbits were sacrificed and the corneas examined morphologically and immunohistochemically.

After the alkali injury and awakening from anesthesia, the rabbits were treated with analgesia (ketoprofen, 1.0 mg/kg i.m.) two times daily for three days in the case of the lower concentration of alkali and for ten days in the case of the higher concentration of alkali.

During the whole experiment, photographs of corneas were performed.

The animals were sacrificed following an i.v. injection of thiopental anesthesia (Thiopental, Spofa, 30 mg/kg) following premedication with an intramuscular injection of Rometar/Narkamon.

In all experiments with alkali injury, the corneas of healthy rabbit eyes served as controls.

Matrix regenerating agent (RGTA)

RGTA is a sterile solution of CACICOL20 (dextran and poly/carboxymethylglucose suplphate/). The placebos employed were dextran or saline. The drugs employed were supplied by Laboratoires Thea, Clermont-Ferrand, France.

The measurement of central corneal thickness (as an index of corneal hydration)

The central corneal thickness of anesthetized animals was measured using an Ultrasonic Pachymeter SP-100 (Tomey Corporation, Noritake-shinmachi, Nishi-ku, Nagoya, Japan) in the corneal center. The corneal thickness was measured in the same corneas before alkali injury (normal, undamaged corneas), one day after alkali injury and then on day six. Corneas were treated with RGTA or placebo (dextran) one hour after the injury and then every second day. Every cornea was measured four times with the Pachymeter, and the mean value of the thickness and the standard deviation were computed.

Preparation of cryostat sections, immunohistochemical and morphological examinations

After sacrificing the animals, the eyes were enucleated and the anterior eye segments dissected out and quenched in light petroleum chilled with an acetone-dry ice mixture. Sections were cut on a cryostat and transferred to glass slides.

Subsequently, the cryostat sections were fixed in acetone at 4°C for 5 min. For the immunohistochemical localization of peroxynitrite (by nitrotyrosine residues), malondialdehyde, urokinase-type plasminogen activator, endothelial nitric oxide synthase, inducible nitric oxide synthase, xanthine oxidase, superoxide dismutase, vascular endothelial growth factor and macrophages, the following primary antibodies were used: monoclonal mouse anti-nitrotyrosine (Abcam, Cambridge, UK), polyclonal goat anti-malondialdehyde (US Biological, Swampscott, MA, USA), monoclonal mouse anti-urokinase-type plasminogen activator Ab-1 (Neomarkers, Fremont, CA, USA), monoclonal mouse anti-human endothelial nitric oxide synthase (Biosciences, Sant Jose, CA, USA), monoclonal mouse anti-human inducible nitric oxide synthase (Biosciences, Sant Jose, CA, USA), monoclonal mouse anti-xanthine oxidase AB-2 (NeoMarkers, Fremont, CA, USA), monoclonal mouse anti-superoxide dismutase (Biogenesis, Poole, UK), monoclonal mouse anti-vascular endothelial growth factor (VEGF) (Abcam, Cambridge, UK) and monoclonal mouse anti-macrophage (Abcam, Cambridge, UK). The binding of the primary antibodies was demonstrated using the HRP/DAB Ultra Vision detection system (Thermo Scientific, Fremont, CA, USA) following the instructions of the manufacturer: hydrogen peroxide block (15 min), ultra V block (5 min), primary antibody incubation (60 min), biotinylated goat anti-mouse or donkey anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA, USA) secondary antibody incubation (10 min) and peroxidase-labeled streptavidin incubation

were used (10 min). Visualization was performed using a freshly prepared DAB substrate-chromogen solution. Cryostat sections in which the primary antibodies were omitted from the incubation media served as negative controls. Some sections were counterstained with Mayer's hematoxylin.

For morphological examination, Harris haematoxylin-eosin staining was employed.

Real-time PCR

The expression of genes for the interleukin-1ß (IL-1ß), inducible nitric oxide synthase (iNOS) and interferon-γ (IFN-γ) in corneas from alkali-injured and placebo- or RGTA-treated eyes was determined by quantitative real-time polymerase chain reaction (PCR) (Trosan et al., 2012). For this investigation, corneas were injured with 0.15 N NaOH and treated with RGTA or placebo (dextran) for 7 days or injured with 1.0 N NaOH and treated with RGTA or placebo (dextran) for 21 days. The injuries with alkali as well as the treatment with RGTA or placebo were performed similarly as for immunohistochemical examination. After sacrificing the animals corneas were excised using Vannas scissors and transferred into Eppendorf tubes containing 500 μl of TRI Reagent (Molecular Research Center, Cincinnatti, OH). The details of RNA isolation, transcription and the PCR parameters have been described previously (Trosan et al., 2012). In brief, total RNA was extracted using TRI Reagent according to the manufacturer's instructions. One μg of total RNA was treated using deoxyribonuclease I (Promega) and subsequently used for reverse transcription. The first-strand cDNA was synthesized using random primers (Promega, Madison, WI) in a total reaction volume of 25 μl using M-MLV Reverse Transcriptase (Promega).

Quantitative real-time PCR was performed in an StepOnePlus real-time PCR system (Applied Biosystems). The relative quantification model with efficiency correction was applied to calculate the expression of the target gene in comparison with GAPDH used as the housekeeping gene. The following primers were used for amplification: GAPDH - 5'-CCCAACGTGTCTGTCGTG (sense), 5'- CCGACCCA GACGTACAGC (antisense), IL-1ß - 5'- CTGCGGCA GAAAGCAGTT (sense), 5'- GAAAGTTCTCAGG CCGTCAT (antisense), iNOS - 5'- AGGGAGTGTTG TTCCAGGTG (sense), 5'- TCCTCAACCTGCT CCTCACT (antisence), and IFN-γ - 5'-GGGTAACTGT GAATGTTCAATGG (sense), 5'- GCTCAGAAAC CCAGTTGCAT (antisense). The PCR parameters included denaturation at 95°C for 3 min, then 40 cycles at 95°C for 20 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s. Fluorescence data were collected at each cycle after an elongation step at 80°C for 5 s and were analyzed on the StepOne Software, version 2.2.2 (Applied Biosystems). Each individual experiment was done in triplicate. The results are summarized in Fig. 13.

Statistics

For statistical evaluation of the central corneal thickness measured by an ultrasonic Pachymeter, a paired t-test was employed to investigate the differences between alkali-injured corneas treated with RGTA vs. the same corneas before injury or injured corneas treated with placebo (dextran) vs. the same corneas before alkali injury (Table 1).

For immunohistochemistry, the Mann-Whitney U test (and unpaired t-test) were used to analyze the differences between the control (normal corneas of animals which were not engaged in experiments) and individual experimental groups or differences between RGTA treated and placebo treated corneas were analyzed. A p value <0.05 was considered to indicate statistical significance. Using the immunohistochemical markers, the number of positively stained cells (and inflammatory cells) was counted (central as well as limbal corneal regions) over an area 100 μm long and 100 μm wide in sagittal cryostat sections. The

Table 1. The central thickness of corneas (considered as an indicator of corneal hydration) one day after the injury with 0.15 N NaOH was significantly increased compared to the central thickness of the same corneas before alkali injury (unaffected corneas). Differences between the thickness of corneas before alkali injury and the same corneas after alkali injury and treatment with RGTA for six days were not significant, whereas differences between the thickness of corneas before alkali injury and the central thickness of same corneas after alkali injury and placebo treatment for six days were significant.

Corneas before alkali injury N=6	Corneas one day after alkali injury N=6	Paired t-test P value (significance) corneas one day after alkali injury vs. corneas before alkali injury	Corneas six days after alkali injury treated with RGTA N=6	Paired t-test P value (significance) corneas six days after alkali injury and treatment with RGTA vs. corneas before alkali injury
353.17±3.43	655.00±3.16	P value is less than 0.0001 (h.s.)	355.67±4.46	P value equals 0.1614 (n.s.)
Corneas before alkali injury N=6	Corneas one day after alkali injury N=6	Paired t-test P value (significance) corneas one day after alkali injury vs. corneas before alkali injury	Corneas six days after alkali injury treated with placebo N=6	Paired t-test P value (significance) corneas six days after alkali injury and treatment with placebo vs. corneas before alkali injury
347.00±3.58	646.50±4.76	P value is less than 0.0001 (h.s.)	401.17±40.15	P value equals 0.0177 (s.)

h.s., higly significant; s., significant; n.s., not significant.

representative data are presented (Table 2).

Results

Morphological examination of corneas injured with high alkali concentration (1.0 N NaOH)

On day four after the injury with the high alkali concentration (1.0 N NaOH), the corneal epithelium was lost and the corneal stroma was swollen without any living cells (Fig. 1a). Following three weeks of RGTA treatment started one hour after the injury, the cornea was reepithelialized (Fig. 1b), whereas after the same time interval of placebo treatment, re-epithelialization proceeded very poorly (Fig. 1c,d). Following six weeks of RGTA treatment of corneas injured with the high alkali concentration, the majority of corneas were healed (Fig. 1e, the arrow points to some inflammatory cells present in the limbal region). Following six weeks of placebo treatment, corneal ulcers developed (Fig. 1f). When the treatment of corneas injured with the high alkali concentration (1.0 N NaOH) was begun one month after alkali injury, when the corneas were ulcerated (Fig. 1g), subsequent RGTA treatment for four weeks healed the majority of corneas (Fig. 1h), whereas the ulcers generally persisted after subsequent placebo treatment for four weeks (Fig. 1i, the rest of the corneal stroma was vascularized, arrows).

Immunohistochemical examination of corneas injured with low alkali concentration (0.15 N NaOH). Treatment with RGTA or placebo started one hour after injury

On day four after the alkali injury, the expression of superoxide dismutase was low in the epithelium of placebo-treated corneas (Fig. 2c), whereas in the epithelium of alkali-injured corneas treated with RGTA drops, the expression of superoxide dismutase was higher (Fig. 2b) - very similar to the epithelium of control cornea (Fig. 2a). In contrast, the expression of xanthine oxidase was increased in placebo-treated corneas (Fig. 2g), whereas in injured corneas treated with RGTA the expression of xanthine oxidase was lower in the corneal epithelium (Fig. 2f), again very similar to the epithelium of control cornea (Fig. 2e). Also, the expression of matrix metalloproteinase 9 was high in the epithelium of alkali-injured, placebo-treated corneas (Fig. 3c), whereas after RGTA treatment its expression was much less in the corneal epithelium (Fig. 3b), similar to the epithelium of control cornea (Fig. 3a). The expression of malondialdehyde was high in the epithelium of placebo-treated corneas (Fig. 4c), whereas in alkali-injured corneas on which RGTA was applied, the staining for malondialdehyde was greatly reduced (Fig. 4b).

Immunohistochemistry of corneas injured with high alkali concentration (1.0 N NaOH) and treated with RGTA or placebo

After three weeks of RGTA treatment of the injured corneas, the expression of urokinase-type plasminogen activator was low in the regenerated epithelium (Fig. 5a), whereas after three weeks of placebo treatment it was strongly expressed in the poorly regenerated epithelium (Fig. 5c). At later time intervals of RGTA treatment (6 weeks), the expression of urokinase-type plasminogen activator (Fig. 5b) was similar to the epithelium of control cornea (Fig. 5e). In contrast, after six weeks of placebo treatment urokinase-type plasminogen activator was strongly expressed in the irregular epithelium and the cornea was vascularized (Fig. 5d, arrows). The immunohistochemical detection of inducible nitric oxide synthase showed that treatment with RGTA gradually decreased its expression in the cornea during the healing process (Fig. 6a - after three weeks of RGTA treatment, Fig. 6b - after 6 weeks of RGTA treatment), whereas in corneas on which placebo was applied after alkali-injury, staining for inducible nitric oxide synthase was strong in inflammatory cells in the corneal stroma (Fig. 6c - following 3 weeks of placebo treatment, arrows point to enzyme positive inflammatory cells, keratocytes and the epithelium). After six weeks of placebo drops, inducible nitric oxide

Table 2. The effect of RGTA vs. placebo in alkali-injured cornea.

anti-oxidative 0.01 N NaOH, after 4 days	anti-inflamamtory 1.0 N NaOH, after 3 and 6 weeks	anti-angiogenic 1.0 N NaOH, after 3 and 6 weeks
SOD s.	e-NOS s.	VEGF s.
XOX s.	i-NOS s.	
NT s.	u-PA s.	
MDA s.	macrophage s.	
MMP9 s.		

Differences between RGTA treated and placebo treated alkali-injured corneas (0.01 N NaOH or 1.0 N NaOH) were analyzed using unpaired t-test and Mann-Whitney U-test. RGTA vs. placebo treatments of alkali injured corneas in various time intervals after the injury (Mann-Whitney U test). A p value <0.05 was considered to indicate statistical significance. Expression of following immunohistochemical markers: SOD, superoxide dismutase; XOX, xanthine oxidase; NT, nitrotyrosine; MDA, malondialdehyde; e-NOS, endothelial nitric oxide synthase; i-NOS, inducible nitric oxide synthase; MMPS, matrix metalloproteinase; VEGF, vascular endothelial growth factor; u-PA, plasminogen activator of urokinase type. s., significant increase; s., significant decrease.

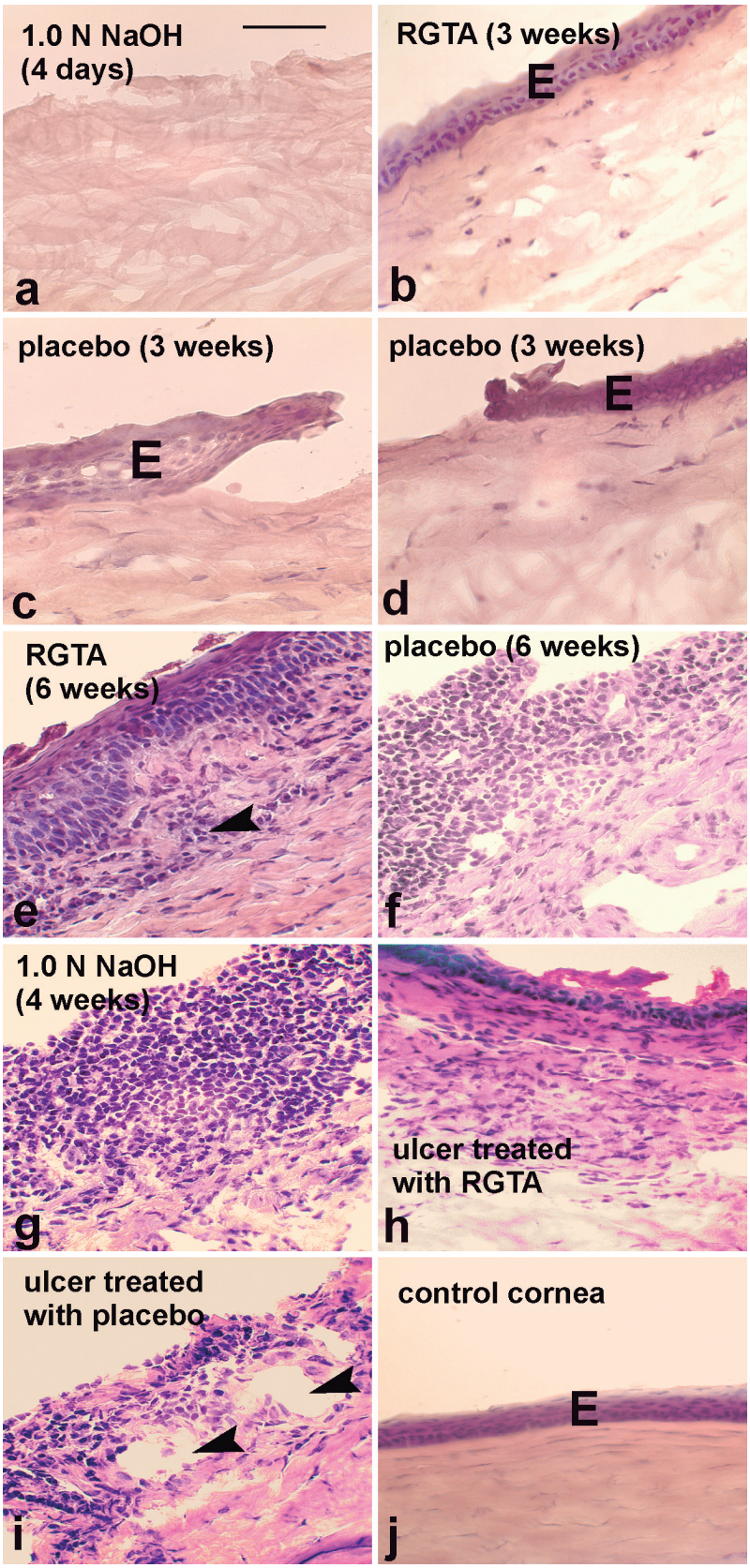

Fig. 1. Morphological examination of corneas injured with NaOH (haematoxylin-eosin staining. HE). **a.** Cornea four days after the injury with 1.0 N NaOH. The corneal epithelium is lost and the swollen corneal stroma contains no living keratocytes. **b.** Cornea injured with 1.0 N NaOH and subsequently treated with RGTA for three weeks. The cornea is re-epithelialized (E - epithelium). **c, d.** Cornea injured with 1.0 N NaOH and treated with placebo for three weeks. The re-epithelialization proceeds very poorly (E - epithelium). **e.** Cornea injured with 1.0 N NaOH and then treated with RGTA for six weeks. The cornea is healed. The arrow point points to some inflammatory cells in the corneal stroma (limbal region). **f.** Cornea injured with 1.0 N NaOH and then treated with placebo for six weeks. The cornea is ulcerated. **g.** Cornea injured with 1.0 N NaOH and left without any treatment for four weeks. The cornea is ulcerated. **h.** Cornea injured with 1.0 N NaOH, left without any treatment for four weeks and subsequently treated with RGTA for four weeks. The cornea is healed. **i.** Cornea injured with 1.0 N NaOH, left without any treatment for four weeks and subsequently treated with placebo for four weeks. The cornea is ulcerated and the rest of the corneal stroma vascularized (arrows). **j.** Control cornea. Scale bar: 10 μm.

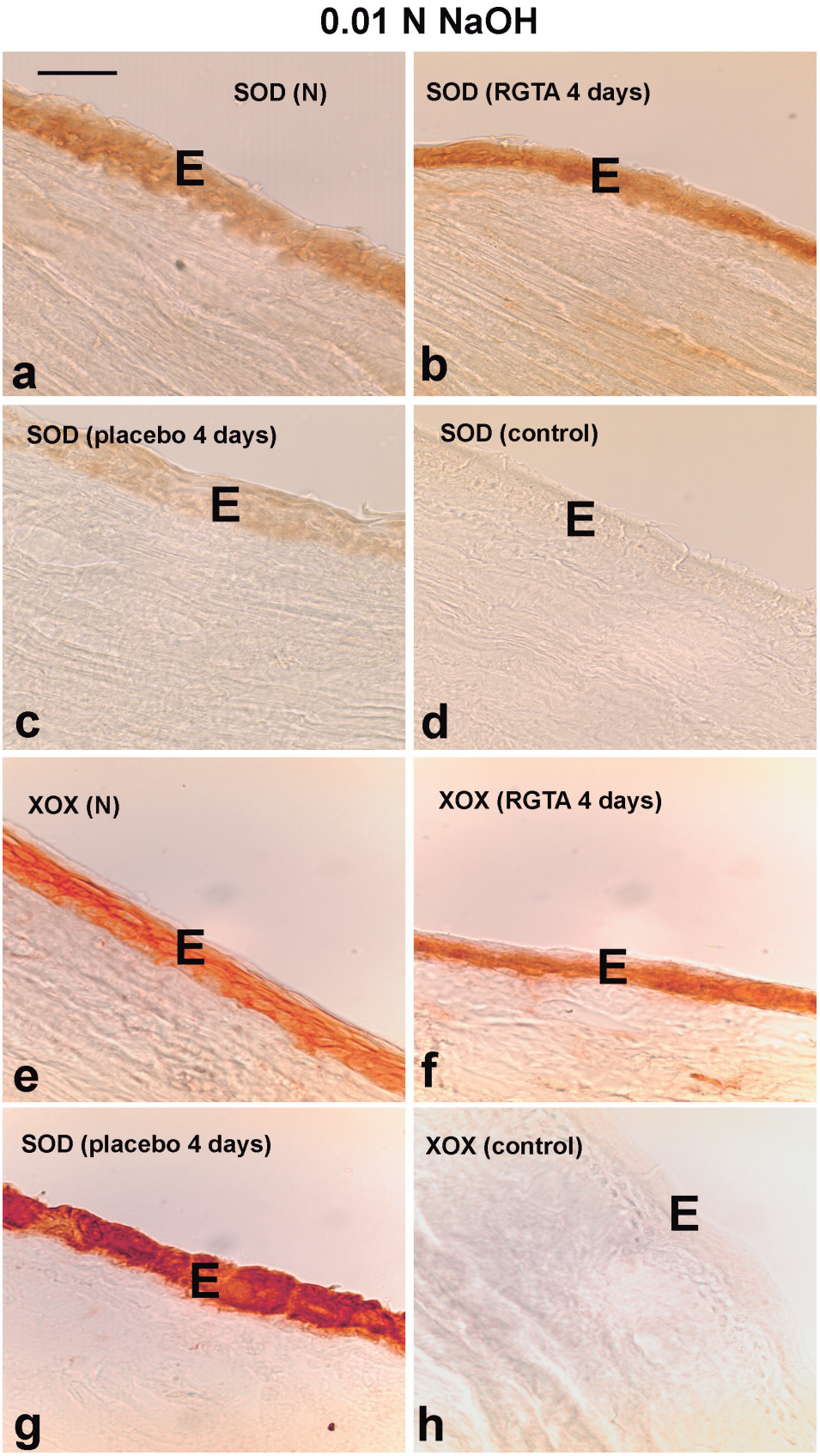

Fig. 2. Immunohistochemical demonstration of superoxide dismutase (SOD) and xanthine oxidase (XOX). **a.** Control cornea. The expression of SOD is high in the epithelium (E). **b.** Cornea four days after injury with 0.15 N NaOH and treatment with RGTA. The staining for SOD is strong, very similar to the epithelium of control cornea (a). **c.** Cornea four days after injury with 0.15 N NaOH and treatment with placebo. The staining for SOD is weak in the epithelium (E). **d.** Negative control. The primary antibody was omitted from the incubation medium. No staining appears. **e.** Control cornea. The staining for XOX is strong in the epithelium (E). **f.** Cornea four days after injury with 0.15 N NaOH and treatment with RGTA. The enzyme expression is very similar to the epithelium of control cornea **(e)**. **g.** Cornea four days after injury with 0.15 N NaOH and placebo application. The expression of XOX is higher in the epithelium (E); compare with the staining of the enzyme in the epithelium of control cornea **(e)**. **h.** Negative control. No staining appears in the epithelium (E). The primary antibody was omitted from the incubation medium. Scale bar: 10 μm.

synthase staining in the corneal stroma was strong in inflammatory cells as well as in keratocytes (Fig. 6d, arrows). The expression of endothelial nitric oxide synthase was low in the regenerated corneal epithelium after three weeks of RGTA treatment (Fig. 7a) compared to corneas treated with placebo, where endothelial nitric oxide synthase was more pronounced in the epithelium and the corneas were highly vascularized (arrows, Fig. 7c). Following 6 weeks of RGTA treatment the cornea was re-epithelialized, and the expression of endothelial nitric oxide synthase (Fig. 7b) was similar to the epithelium of control cornea (Fig. 7e), whereas

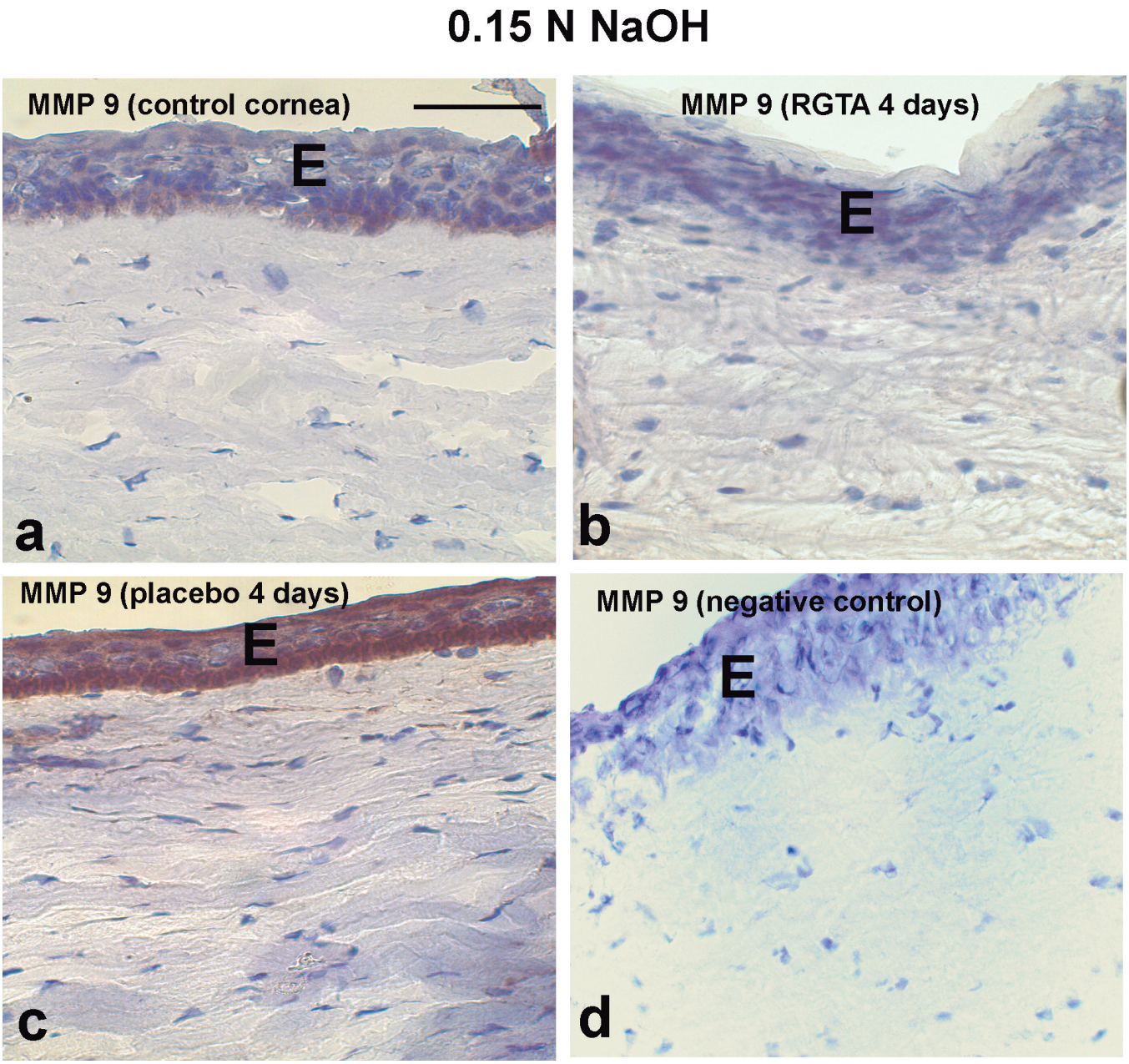

Fig. 3. Immunohistochemical staining for matrix metalloproteinase 9 (MMP9). **a.** Control cornea. The enzyme expression is low in the epithelium (E). **b.** Cornea four days after the injury with 0.15 N NaOH and RGTA application. The expression of MMP9 is low in the epithelium (E), similar to the epithelium of control cornea **(a)**. **c.** Cornea four days after injury with 0.15 N NaOH and treatment with placebo. MMP9 is high in the epithelium (E). **d.** Negative control (the primary antibody was omitted from the incubation medium). The section is stained by counterstaining only. The sections are counterstained with haematoxylin. Scale bar: 10 μM

following six weeks of placebo treatment the corneal stroma was highly vascularized (arrows) and the expression of endothelial nitric oxide synthase was increased in the poorly regenerated epithelium (E) and in the vascularized corneal stroma (Fig. 7d, arrows point to the vessels). The staining of macrophages (monocytes) and vascular endothelial growth factor in alkali-injured corneas treated with RGTA is much less compared to placebo-treated alkali-injured corneas - in both time intervals of treatment (three or six weeks) (Fig. 8). Corneas left without any treatment for four weeks after alkali injury were ulcerated. In Fig. 9 (immunohistochemical demonstration of nitrotyrosine) the arrow points to the strong expression of nitrotyrosine in a corneal ulcer (Fig. 9a, E - residual corneal epithelium). Treatment with RGTA healed the ulcer (Fig. 9b);

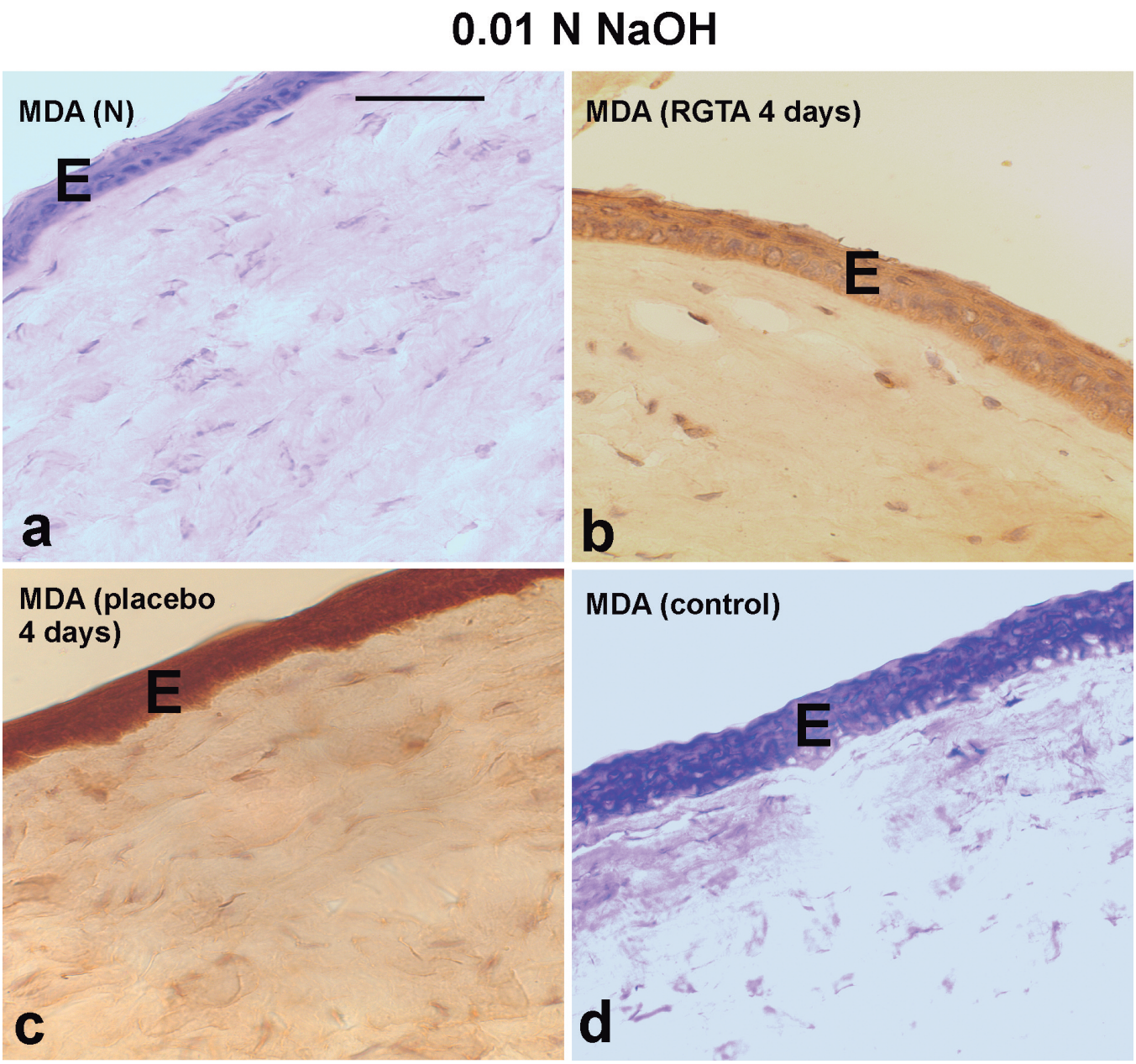

Fig. 4. Immunohistochemistry of malondialdehyde (MDA). **a.** Control cornea. MDA staining is nearly absent (E, epithelium). **b.** Cornea four days after injury with 0.15 N NaOH and RGTA treatment. The staining for MDA is weak in the epithelium (E). **c.** Cornea four days after injury with 0.15 N NaOH and placebo treatment. The staining for MDA is strong in the epithelium (E). **d.** Negative control (the primary antibody was omitted from the incubation medium). No positive staining appears. The section is stained by counterstaining (E, epithelium). Scale bar: 10 μM.

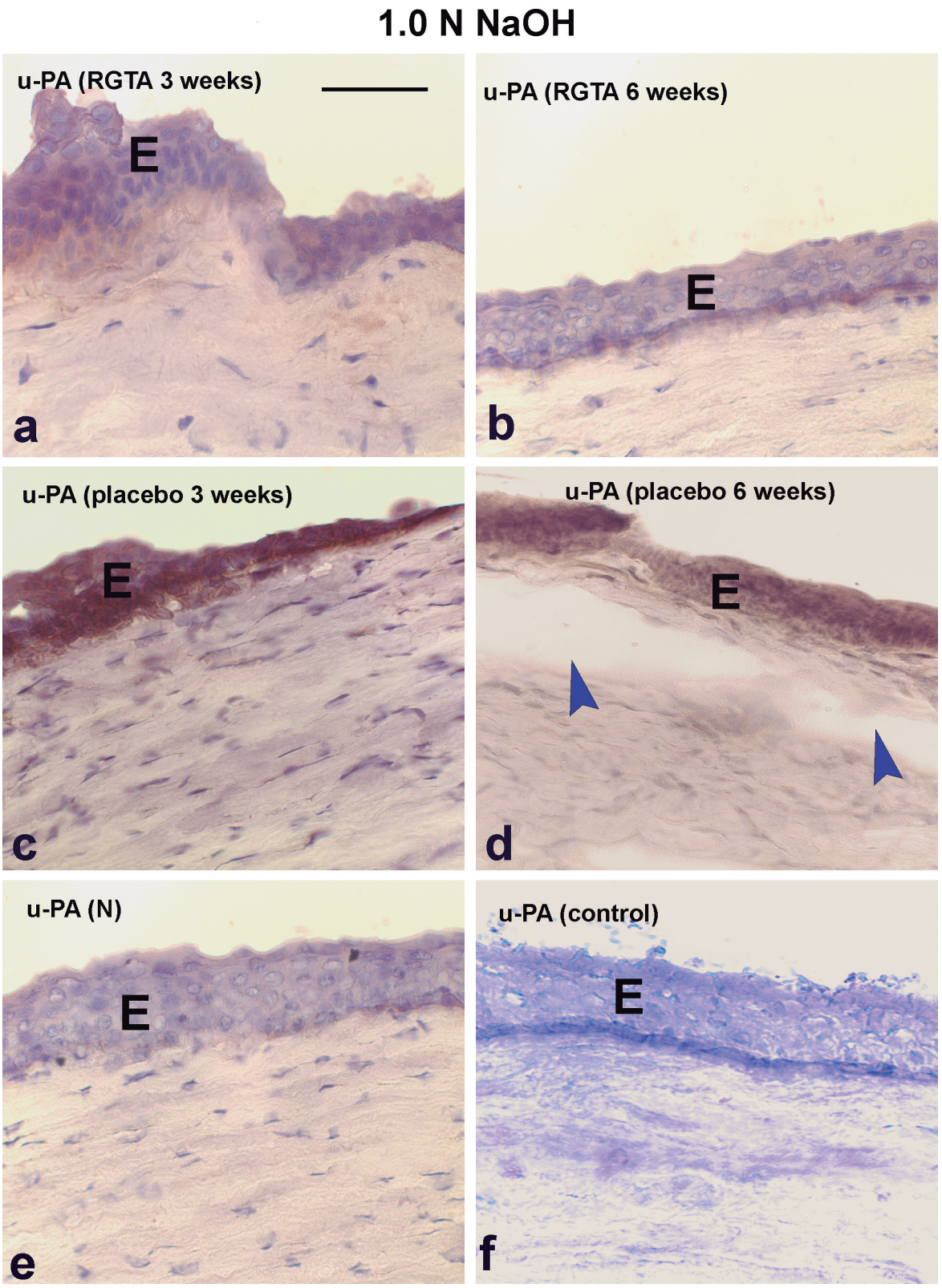

Fig. 5. Immunohistochemistry of urokinase-type plasminogen activator (u-PA). **a.** Cornea injured with 1.0 N NaOH and treated for three weeks with RGTA. The cornea is re-epithelialzed (E, epithelium), and the expression of U-PA is similar to the epithelium (E) of control cornea. **b.** Cornea injured with 1.0 N NaOH, treated with RGTA for six weeks. The expression of U-PA is low (compare with the epithelium (E) of control cornea, **(e)**. **c.** Cornea injured with 1.0 N NaOH and treated with placebo for three weeks. The expression of U-PA is high in the irregularly re-epithelialized cornea (E, epithelium). **d.** Cornea injured with 1.0 N NaOH, treated with placebo for six weeks. The expression of U-PA is high in the corneal stroma. Compare with the corneal stroma after RGTA treatment **(b)**. **e.** Control cornea. The staining for U-PA is weak in the epithelium (E). **f.** Negative control. The primary antibody was omitted from the incubation medium. The section is stained only with counterstaining. In Figure 5 the sections are lightly counterstained (Haematoxylin). Scale bar: 10 μm.

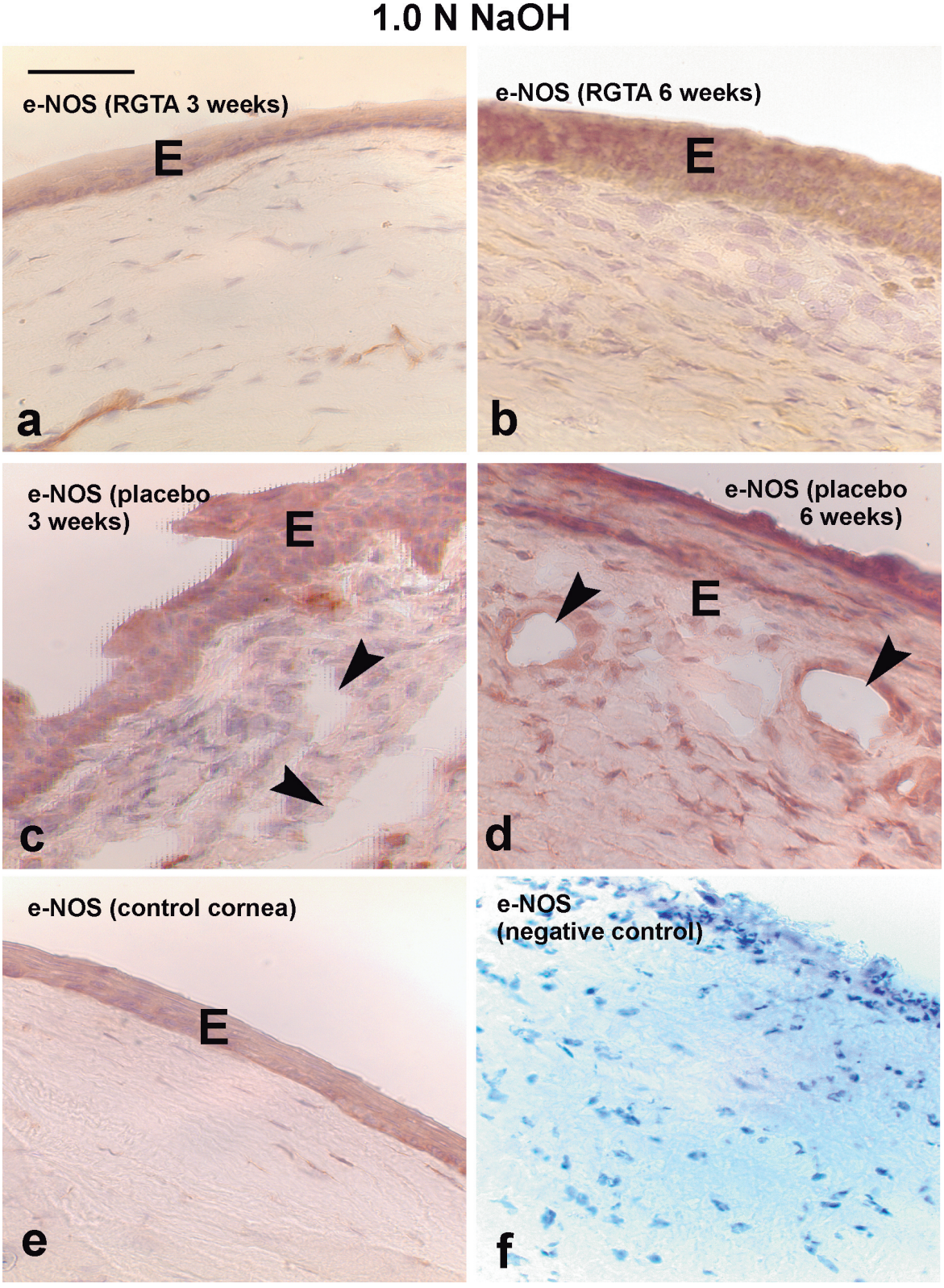

Fig. 6. Immunohistochemical staining of endothelial nitric oxide synthase (e-NOS). **a.** An alkali-injured cornea treated with RGTA for three weeks. The expression of e-NOS is low in the regenerating epithelium (E). **b.** A cornea injured with 1.0 N NaOH and treated with RGTA for six weeks. The regenerated epithelium (E) reveals the slight expression of e-NOS. **c.** Cornea injured with 1.0 N NaOH and treated with placebo for three weeks. The enzyme is expressed in the irregularly regenerated corneal epithelium (E). The corneal stroma is vascularized (arrows). **d.** Cornea injured with 1.0 N NaOH and treated with placebo for six weeks. The expression of e-NOS is high in the irregular thin epithelium (E) and in the vascularized corneal stroma (arrows). **e.** Control cornea. The expression of e-NOS is low in the epithelium (E). **f.** Negative control. The primary antibody was omitted from the incubation medium. The section is stained with counterstaining only. The sections are not counterstained with Haematoxylin. Scale bar: 10 μM.

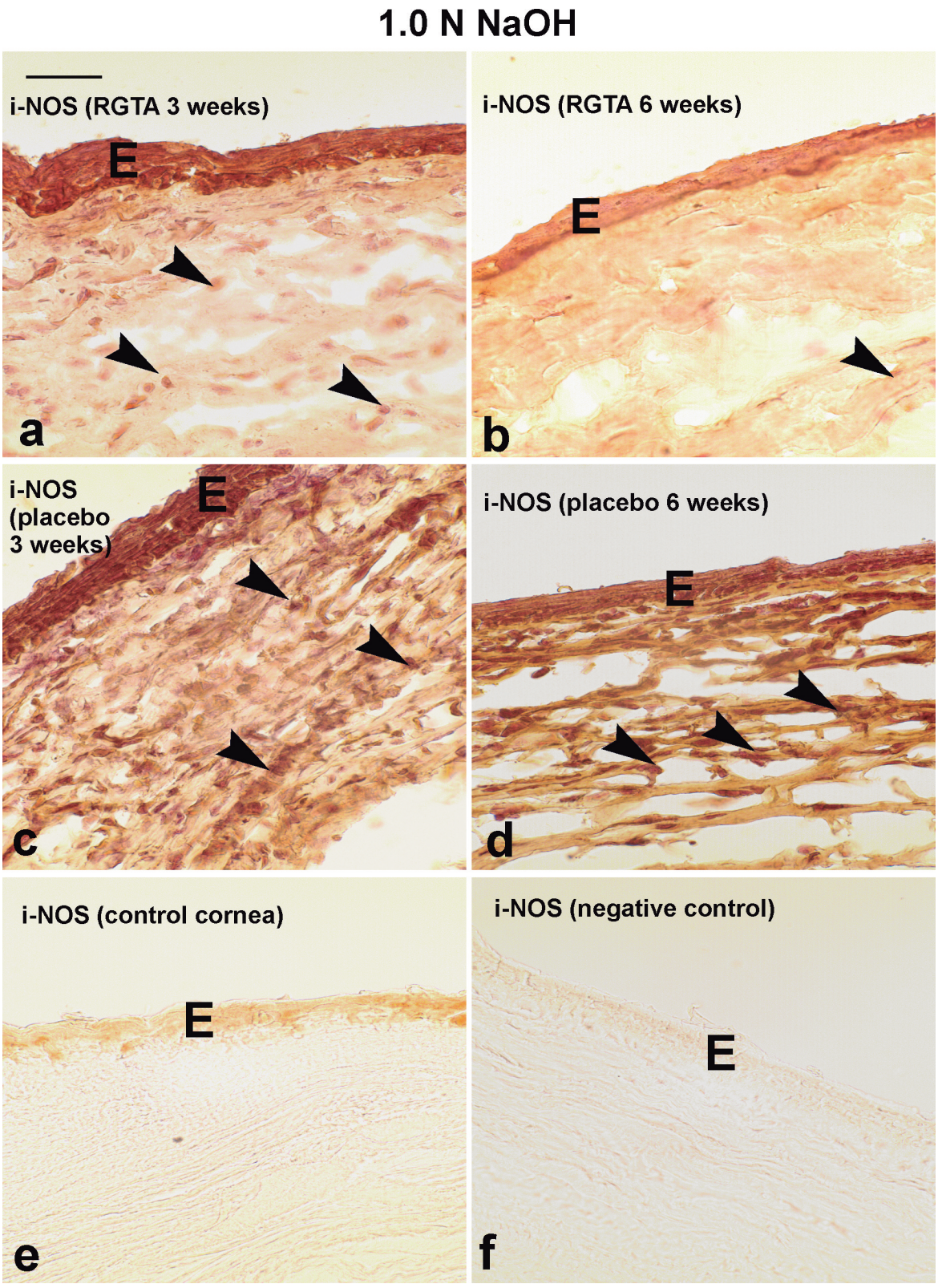

Fig. 7. Immunohistochemical demonstration of inducible nitric oxide synthase (i-NOS). **a.** Cornea injured with 1.0 N NaOH and treated for three weeks with RGTA. The enzyme is expressed in some polymorphonuclear leukocytes in the corneal stroma (arrows), keratocytes and in the re-epithelialized corneal epithelium (E). **b.** Cornea injured with 1.0 N NaOH and treated with RGTA for six weeks. Only slight i-NOS staining is present in the corneal epithelium (E) and rare inflammatory cells (arrow) in the corneal stroma. **c.** Cornea injured with 1.0 N NaOH and treated with placebo for three weeks. The expression of i-NOS is strong in numerous inflammatory cells (arrows) in the corneal stroma, in keratocytes and in the irregular epithelium (E). **d.** Cornea injured with 1.0 N NaOH and treated with placebo for six weeks. The staining of i-NOS is high in inflammatory cells and keratocytes (arrows) in the corneal stroma. **e.** Control cornea. The expression of i-NOS is nearly absent. **f.** Negative control. The primary antibody was omitted from the incubation medium. No staining appears. Scale bar: 10 μM.

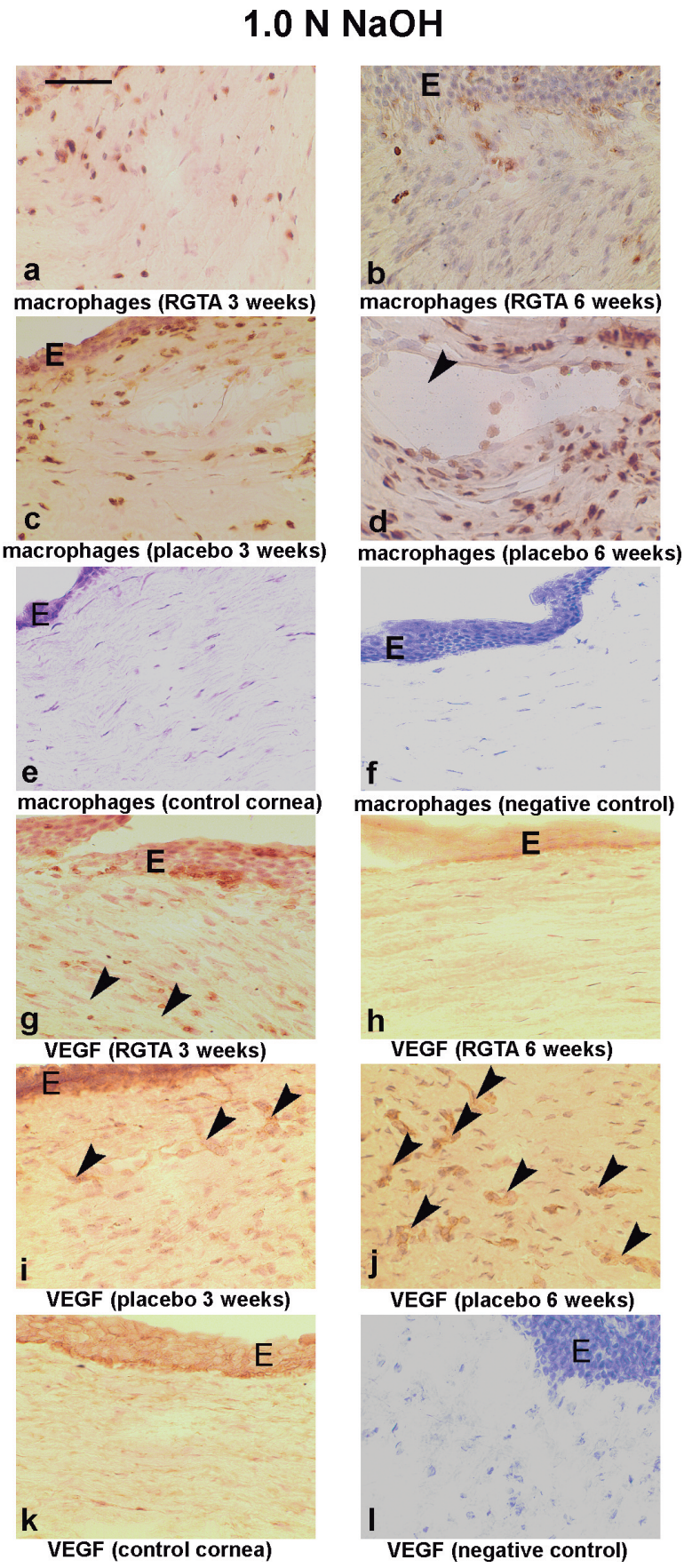

Fig. 8. Immunohistochemical staining of macrophages/ monocytes and vascular endothelial growth factor (VEGF). **a.** An alkali-injured cornea treated with RGTA for three weeks. The number of cells stained with anti-macrophage antibody (macrophages, monocytes) is low in the corneal stroma. **b.** An alkali-injured cornea treated with RGTA for six weeks. Very similar immunohistochemical pattern to in **(a)**. The cornea is reepithelialized (E - epithelium). **c.** An alkali-injured cornea treated with placebo for three weeks. Corneal stroma is vascularized and numerous cells stained with anti-macrophage antibody are present in the corneal stroma. **d.** An alkali-injured cornea treated with placebo for six weeks. A high number of cells stained with anti-macrophage antibody is seen in vascularized (arrow) corneal stroma. **e.** A control cornea. No positive staining with anti-macrophage antibody is seen in the cornea. The cornea is stained with counterstaining only. **f.** Negative control. The primary anti-macrophage antibody was omitted from the incubation medium. The section is stained with counterstaining only. **g.** An alkali-injured cornea treated with RGTA for three weeks. The expression of VEGF is low in the corneal epithelium (E) as well as in the corneal stroma (arrows). **h.** An alkali-injured cornea treated with RGTA for six weeks. The expression of VEGF is low in the corneal epithelium (E) and absent in the corneal stroma. **i.** An alkali-injured cornea treated with placebo for three weeks. The expression of VEGF is more pronounced in the corneal epithelium (E) compared to the epithelium (E) of the control cornea **(k)**. Arrows point to the expression of VEGF in the corneal stroma. **j.** An alkali-injured cornea treated with placebo for six weeks. The expression of VEGF is high in the corneal stroma (arrows). **k**. Control cornea. The expression of VEGF is low in the corneal epithelium (E) and absent in the corneal stroma. **l.** Negative control. The primary antibody was omitted from the incubation medium. The section is stained with counterstaining only. In The sections are lightly counterstained with Haematoxylin. Scale bar: 10 μm.

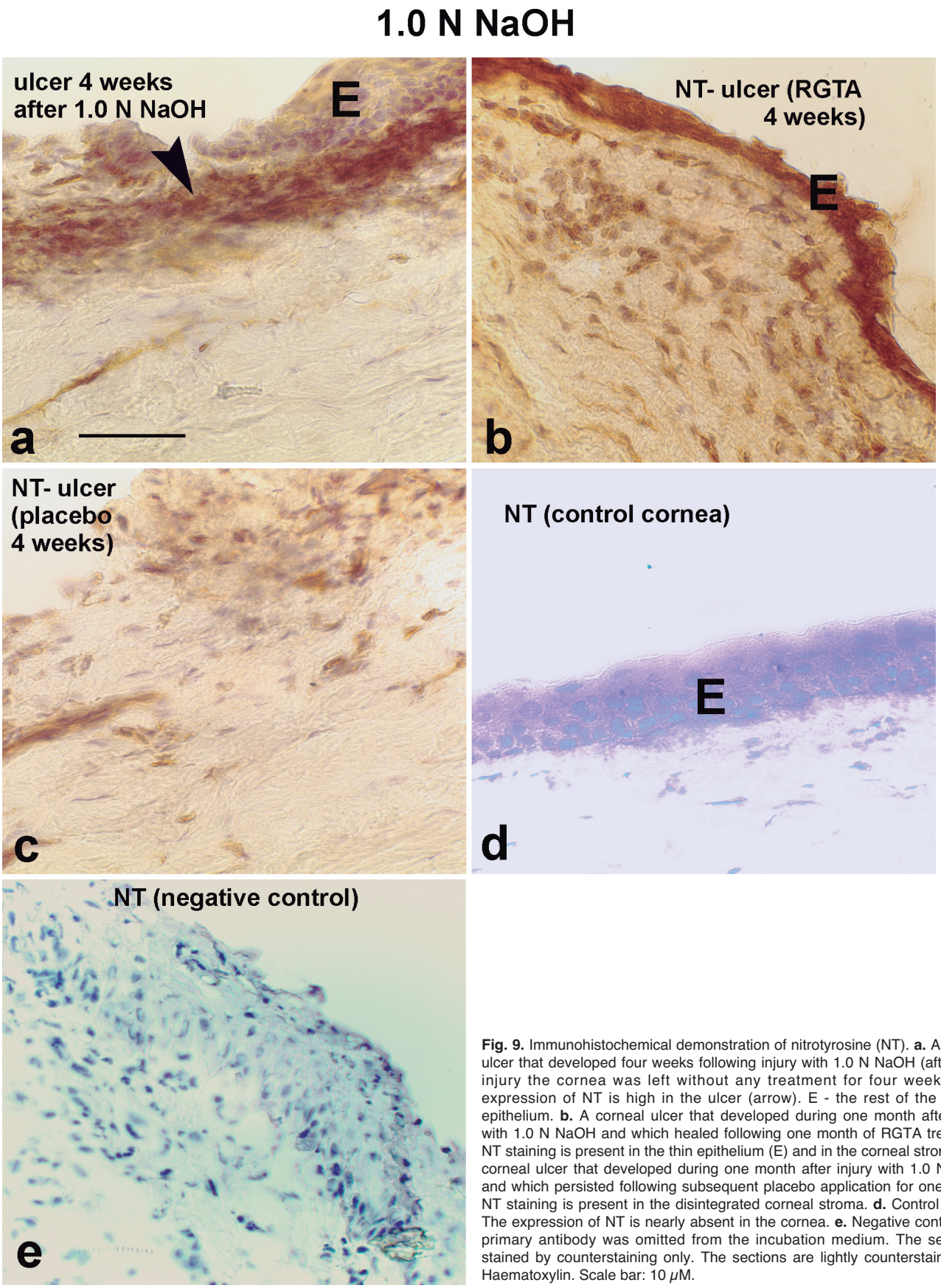

Fig. 9. Immunohistochemical demonstration of nitrotyrosine (NT). **a.** A corneal ulcer that developed four weeks following injury with 1.0 N NaOH (after alkali injury the cornea was left without any treatment for four weeks). The expression of NT is high in the ulcer (arrow). E - the rest of the corneal epithelium. **b.** A corneal ulcer that developed during one month after injury with 1.0 N NaOH and which healed following one month of RGTA treatment. NT staining is present in the thin epithelium (E) and in the corneal stroma. **c.** A corneal ulcer that developed during one month after injury with 1.0 N NaOH and which persisted following subsequent placebo application for one month. NT staining is present in the disintegrated corneal stroma. **d.** Control cornea. The expression of NT is nearly absent in the cornea. **e.** Negative control. The primary antibody was omitted from the incubation medium. The section is stained by counterstaining only. The sections are lightly counterstained with Haematoxylin. Scale bar: 10 μM.

however, staining for nitrotyrosine remained present in the thin corneal epithelium and in the corneal stroma. In contrast, after the treatment of corneal ulcers with placebo, the ulcers persisted (Fig. 8c, nitrotyrosine staining is present in the disintegrated corneal stroma).

In all experiments, a comparison of the results obtained using dextran or saline as the placebo revealed no significant differences between the two solutions.

The measurement of central corneal thickness as an index of corneal hydration.

The central thicknesses of corneas injured with 0.15 N NaOH and treated with RGTA or placebo is shown in Table 1 and representative photographs of corneas in Fig. 10. After RGTA treatment corneal transparency was restored after six days, whereas after placebo treatment corneal opalescency persisted.

Representative photographs of the development of corneal neovascularization in corneas injured with 1.0 N NaOH treated with RGTA or placebo during healing are shown in Fig. 11. Compared to placebo-treated eyes, after RGTA application corneal neovascularization was suppressed. This was most evident during the first three weeks of treatment.

Representative photographs of ulcerated corneas after injury with 1.0 N NaOH subsequently treated with RGTA or placebo are shown in Fig. 12. Following RGTA treatment corneal ulcers were healed, whereas after placebo application ulcers largely persisted. Corneal neovascularization was suppressed after RGTA treatment compared to placebo application, where

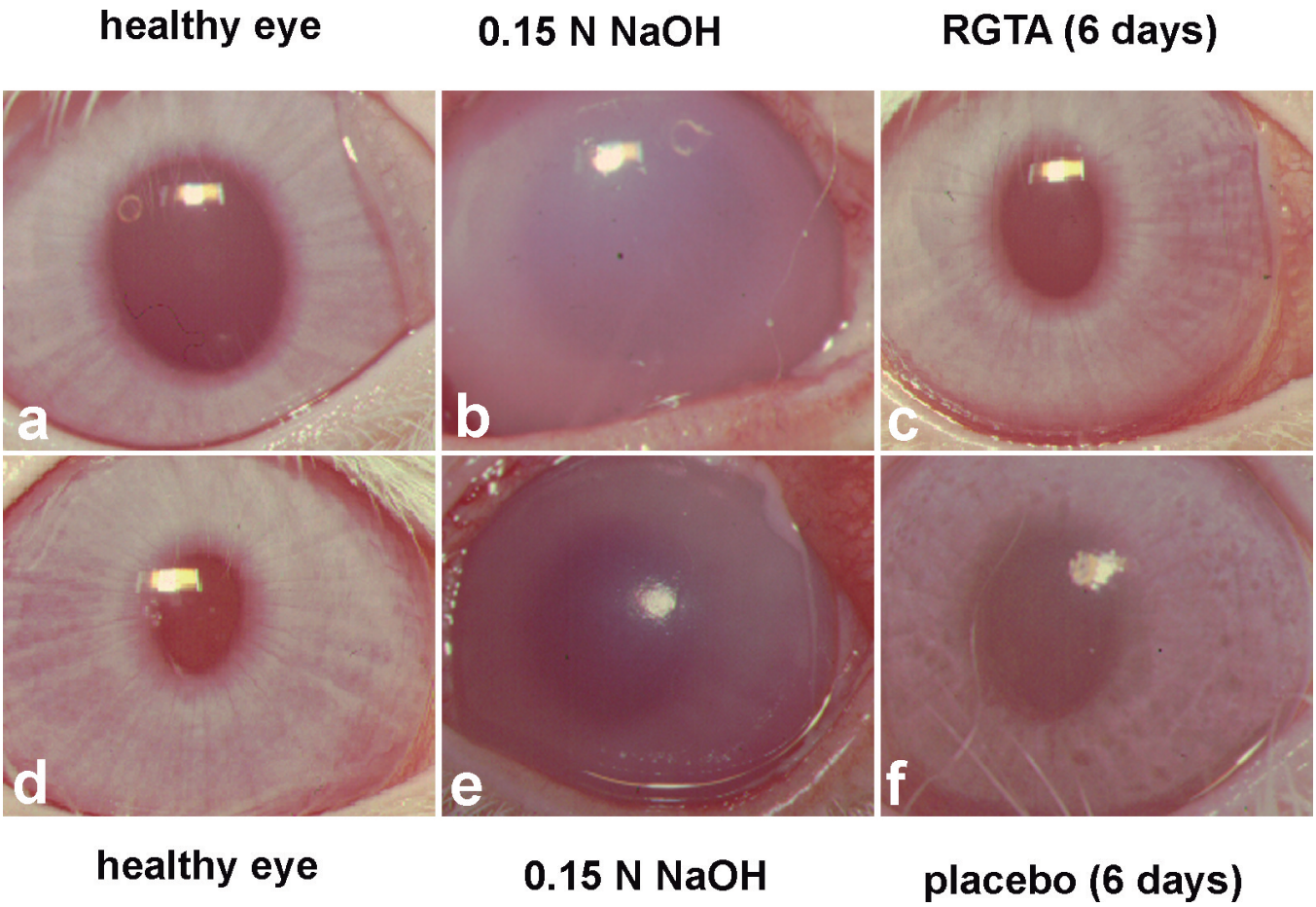

Fig. 10. Representative photographs of corneas injured with 0.15 N NaOH and treated with RGTA or placebo (dextran). **a.** The cornea of healthy eye (the first eye). **b.** The cornea (of the first eye) one day after the alkali injury. The cornea is opalescent.**c.** The alkali-injured cornea (of the first eye) following six days of healing. During this time interval RGTA was dropped on the corneal surface one hour after the injury and then every second day. The cornea is transparent. **d.** The cornea of healthy eye (the second eye). **e.** The cornea (of the second eye) one day after the injury with 0.15 N NaOH. The cornea is opalescent. **f.** The cornea (of the second eye) six days following injury with 0.15 NaOH. During this time interval the cornea was treated with placebo (dextran) which was applied on the corneal surface one hour after the injury and then every second day. Corneal opalescency persists.

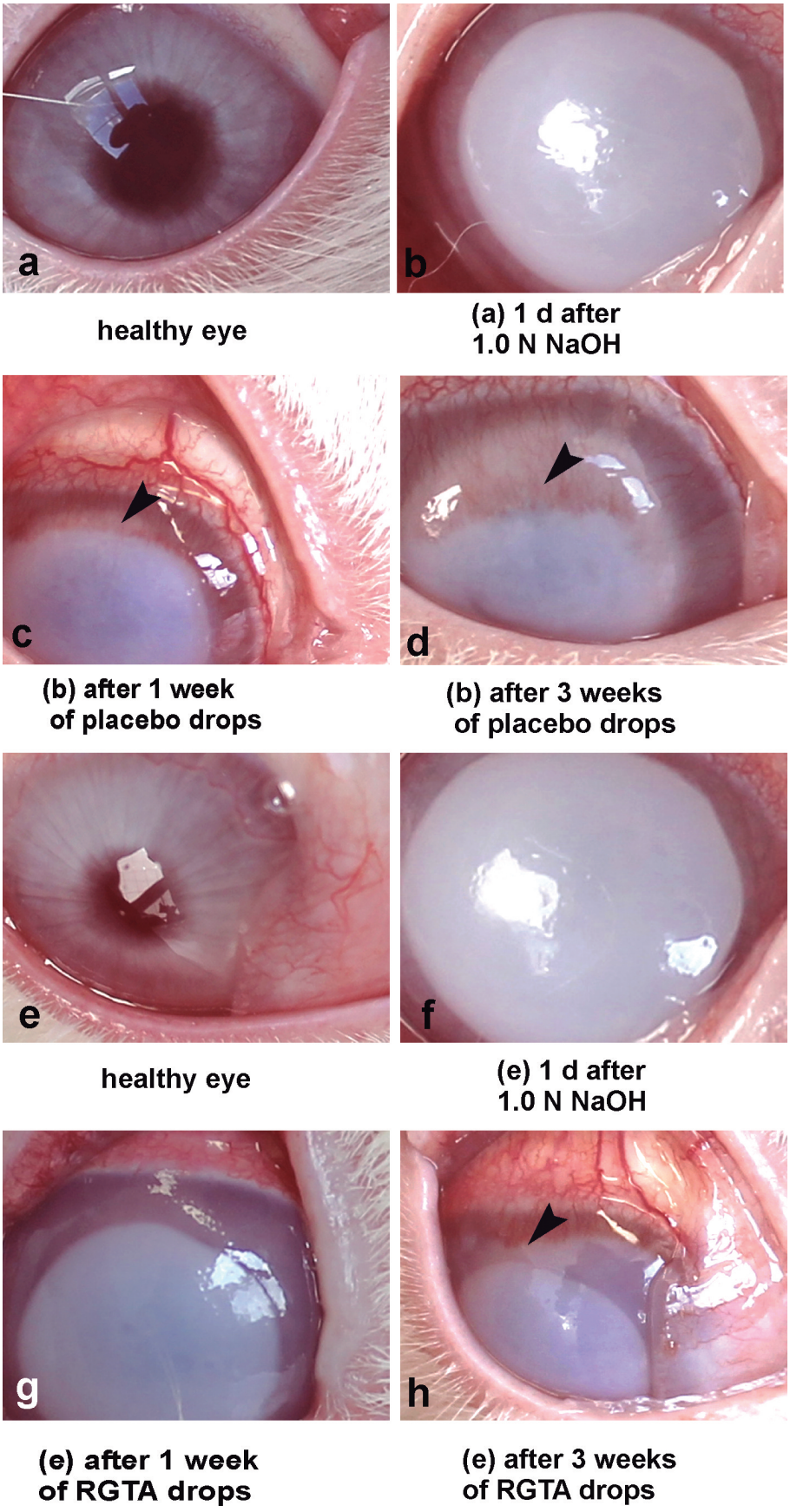

Fig. 11. Representative photographs of the development of corneal neovascularization in eyes injured with 1.0 N NaOH and treated with placebo (dextran) or RGTA during healing. **a.** The cornea of healthy eye (the first eye). **b.** Cornea (of the first eye) one day after injuy. Corneal transparency is lost in the injured area. **c.** Cornea (of the first eye) one week after injury and treatment with placebo. Corneal neovascularization is seen at the limbal region and reaches the injured area (arrow). **d.** Cornea (of the first eye) three weeks after injury and placebo treatment. Corneal neovascularization is highly pronounced (arrow). **e.** The cornea of healthy eye (the second eye). **f.** The cornea (of the second eye) one day after injury. Corneal transparency is lost in the injured region. **g.** The cornea (of the second eye) one week after injury and treatment with RGTA. Corneal neovascularization is not present. Compare with placebo treated eye (c), where corneal neovascularization is seen at the limbal region. **h.** Cornea (of the second eye) three weeks after injury and RGTA treatment. Corneal neovascularization is present only at the limbus. Compare with placebo treated eye **(d)** where corneal neovascularization is highly expressed (nearly in the middle of the injured area).

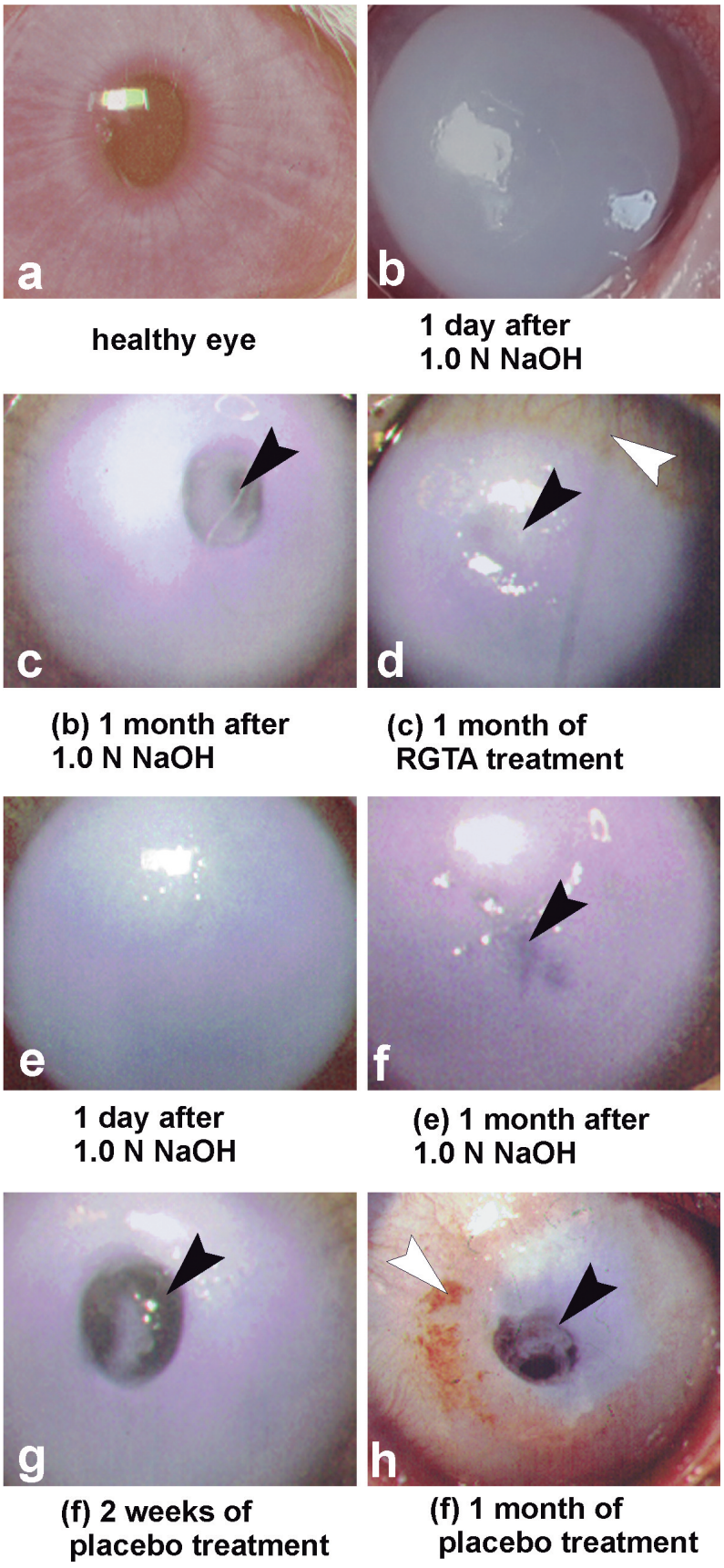

Fig. 12. Representative photographs of eyes with corneal ulcers developed after the injury with 1.0 N NaOH and subsequently treated with RGTA or placebo (dextran). **a.** The cornea of healthy eye (the first eye). **b.** The cornea (of the first eye) one day after injury with 1.0 N NaOH. Corneal transparency is lost. **c.** The cornea (of the first eye) one month after injury (during this time interval the cornea was without any treatment). Corneal ulcer develops (black arrow). **d.** The cornea (of the first eye) one month of subsequent RGTA treatment. The corneal ulcer is healed (black arrow). Cornea is vascularized (white arrow). **e.** The cornea (of the second eye) one day after injury with 1.0 N NaOH. Corneal transparency is lost. **f.** The cornea (of the second eye) one month following the injury (during this time interval the cornea was without any treatment). Central corneal defect develops (black arrow). **g, h.** The cornea (of the second eye) after two weeks of subsequent placebo (dextran) treatment **(g)** and four weeks of subsequent placebo treatment **(h).** Deep central corneal ulcer developed (**g**, black arrow) and largely persisted (**h**, black arrow). The cornea is highly vascularized (white arrow). Compare with RGTA treated eye where corneal neovascularization is less expressed **(d)**.

corneal neovascularization was highly pronounced.

Quantitative determination of gene expression in placebo- and RGTA-treated alkali-injured corneas

Expression of genes for proinflammatory cytokines IL-1ß, IFN-γ and iNOS in alkali-injured and placebo/ or RTGA/treated corneas was determined by quantitative real-time PCR on day 7 (after 0.15 N NaOH) and on day 21 (after 1.0 N NaOH) following the injury. As demonstrated in Fig. 13, a strong expression of all tested genes was found in injured and placebo-treated corneas on days 7 (Fig. 13A) and 21 (Fig. 13B). This gene expression was significantly inhibited in RGTA-treated corneas. In Fig. 13 (a) IL-1ß; (b) IFN-γ; (c) i-NOS.

Discussion

Regenerating agents (RGTA) are biopolymers engineered to replace heparan sulfates, obtained by the chemical substitution of dextran, and have been shown to modulate collagen synthesis in several cell-culture and tissue-explant models (Garcia-Filipe et al., 2007). RGTA is specifically bound to matrix proteins and growth factors that are destroyed after an injury. The RGTA-bound proteins are protected from proteolysis, and this allows the extracellular matrix to restore (Chebbi et al., 2008). RGTA was found to be effective in healing alkali-injured skin in humans (Garcia-Filipe et al., 2007) as well as pressure, diabetic and vascular skin wounds (Groah et al., 2011). Zakine et al. (2011)

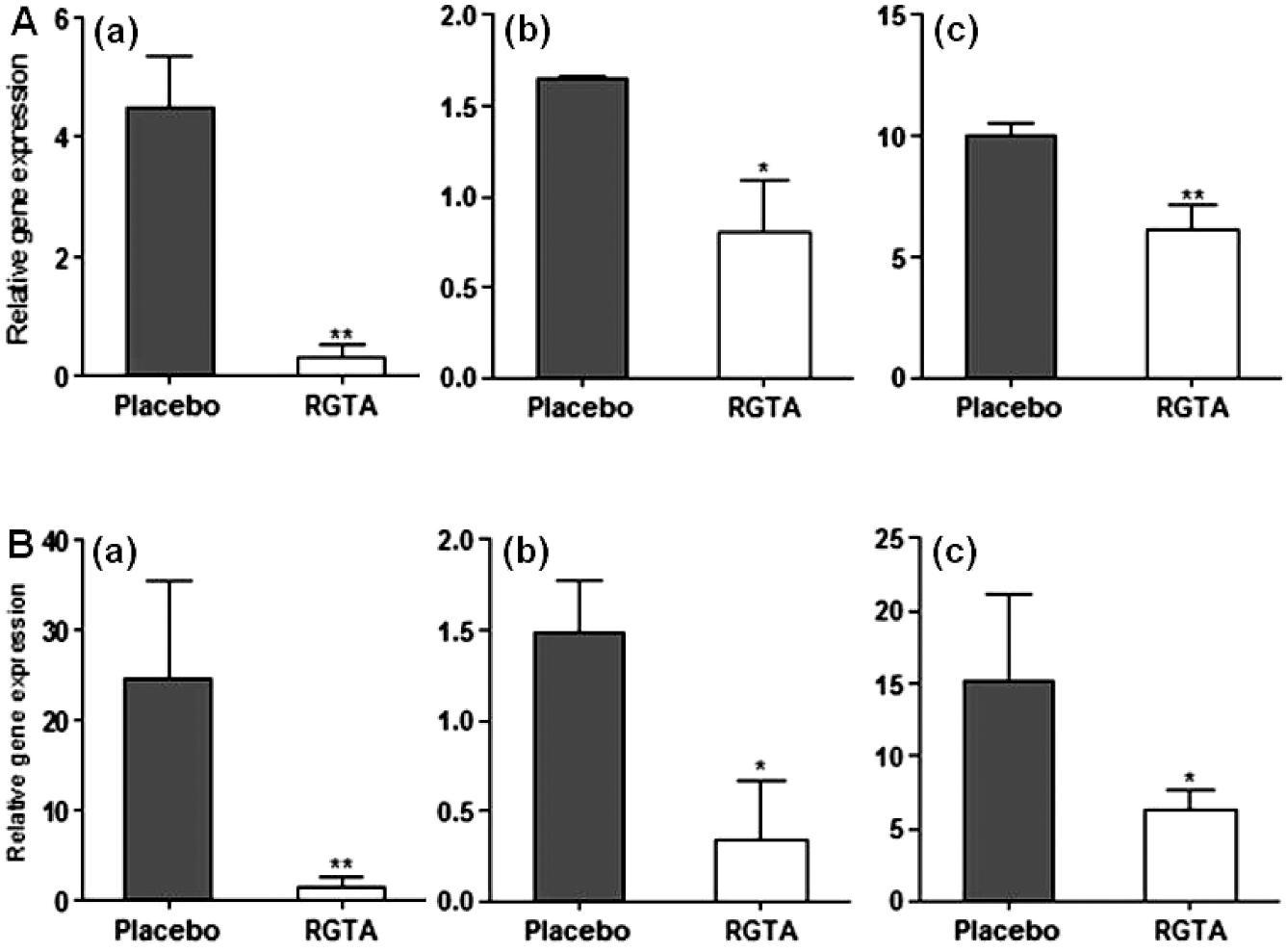

Fig. 13. Expression of genes for proinflammatory cytokines in alkali-injured corneas from placebo- and RTGA-treated eyes. Corneas from the eyes injured by alkali and treated with placebo or RTGA were harvested on day 7 (after 0.15 N NaOH) **(A)** or day 21 (after 1.0 N NaOH) **(B)** and the expression of genes for IL-1ß **(a)**, IFN-γ **(b)** and iNOS **(c)** was determined by real-time PCR. Each bar represents the mean ±S.D. from at least 3 determinations. Values with asterisks are significantly (*P<0.05, **P<0.01) different from the placebo-treated control.

investigated the accelerated remodelling of alkali-injured skin in hairless rats. In clinical ophthalmology, RGTA was successfully used for the healing of chronic and severe corneal dystrophies as well as corneal ulcers resistant to normal treatments (Chebbi et al., 2008; Aifa et al., 2012; De Monchy et al., 2012). Brignole et al. (2005) described preliminary findings with RGTA in vivo in alkali injury of the rabbit cornea and in vitro in benzalkonium chloride-induced cell toxicity. In vivo, RGTA enhanced corneal re-epithelialization, corneal transparency and the histological appearance of alkali-injured corneas. In vitro, RGTA protected conjunctival cells from oxidative stress evoked by benzalkonium chloride by reducing hydrogenium peroxide production and glutathione uptake.

In this study, the efficacy of RGTA treatment was examined in corneal alkali injury induced by low as well as high concentration of NaOH. Kubota et al. (2011) described that alkali evokes oxidative stress, which precedes intracorneal inflammation. These authors investigated (using dihydroethidium fluorescence) reactive oxygen species in the mouse corneal epithelium at early time intervals after the injury with low concentration of alkali (0.15 N NaOH). In the present study, using the same concentration of NaOH, we found in placebo-treated alkali-injured corneal epithelium the increased expression of urokinase-type plasminogen activator, matrix metalloproteinase-9, nitric oxide syntases and xanthine oxidase, enzymes that generate nitric oxide and reactive oxygen species. In contrast, the expression of superoxide dismutase, an important antioxidant enzyme, was markedly decreased in the alkali-injured placebo-treated corneal epithelium. RGTA application suppressed the oxidant/antioxidant imbalance (Fig. 2), reduced the expression of proteolytic enzymes (Fig. 3) and accelerated corneal healing. In alkali-injured corneas the central corneal thickness (considered an index of corneal hydration), measured by an ultrasonic Pachymeter, was increased. This elevated corneal hydration decreased rapidly after one week of treatment with RGTA drops, whereas in placebo-treated alkali-injured corneas, in which the oxidant/antioxidant imbalance persisted and the staining for proteolytic enzymes was increased, elevated corneal hydration decreased slowly (Table 1). Nirankari et al. (1981) hypothetized that superoxide radicals are involved in tissue destruction after alkali injury. These authors injected superoxide dismutase or ascorbic acid subconjunctivally in alkali-injured rabbit eyes. Both antioxidants were effective in preventing corneal perforation. Very similar results were obtained by Alio et al. (1995) utilizing topical treatment with superoxide dismutase or dimethylthiourea. Feng et al. (2004) described decreased aldehyde dehydrogenase 3A1 activity in alkali-injured mouse corneas.

The inhibition of serine proteases (plasminogen activator/plasmin system) and metalloproteinases at the corneal surface is important for protecting the cornea against further proteolysis. The in vitro inhibition of serine proteases by chemically modified dextrans was confirmed by Ledoux et al., (2000). In this study, we demonstrated that the expression of serine protease and metalloproteinase was inhibited by RGTA application in vivo (Figs. 3, 5). Matrix metalloproteinases are a family of zinc-dependent enzymes capable of degrading proteoglycans, fibronectins and laminin present in the interstitial matrix and basement membranes. Matrix metalloproteinase 9 belongs to gelatinases/type IV collagenases. Matrix metalloproteinases are secreted as proenzymes and are modulated by activating proteases (Woessner, 1991). Urokinase-type plasminogen activator is a serine protease that converts plasminogen to plasmin and then activates pro-matrix metalloproteinases. It degrades the extracellular matrix, stimulates cell migration, proliferation and chemotaxis and induces angiogenesis (Chao et al., 2011).

In placebo-treated alkali-injured corneas, peroxynitrite formation (detected by nitrotyrosine residue) and malondialdehyde staining were present in the corneal epithelium. RGTA reduced the staining (Fig. 5). Nitric oxide is generated by nitric oxide synthases. Increased nitric oxide production and elevated reactive oxygen species generation lead to the formation of peroxynitrite, a toxic reaction product between nitric oxide and superoxide (Cejkova et al., 2005). Peroxynitrite is an important marker of oxidative stress (Ceriello, 2002; Chirino et al., 2006) and malondialdehyde a marker of lipid peroxidation (Buddi et al., 2002). Our results are in accordance with those of Kubota et al. (2011), who described that alkali already at low concentrations evokes oxidative stress in the cornea, which precedes the inflammatory response. According to our present findings, the source for toxic oxygen and nitrogen species are xanthine oxidase and nitric oxide synthases, which are highly expressed in the alkali-injured placebo-treated corneal epithelium (Figs. 2, 6, 7).

Injury to the corneal epithelium or endothelium evokes an increase in corneal hydration (Cullen, 2009). Thus, changes in corneal hydration (evaluated by the central corneal thickness using an ultrasonic Pachymeter) are sensitive markers of corneal damage (Doughty and Cullen, 1989; O'Donnell and Efron 2006; Cejka et al. 2007, 2010). Following alkali injury the central corneal thickness was increased and only slowly returned to normal values. RGTA application accelerated the decrease of this elevated corneal hydration (Table 1). Also, the corneal transparency recovered faster (Fig. 10).

Following the injury with high alkali concentration, corneal re-epithelialization of the injured area was accelerated after RGTA application. Moreover, after RGTA treatment the inflammatory reaction, as well as corneal neovascularization, pronounced in placebo-treated corneas, were reduced, very probably via the suppression of nitric oxide synthases and proteases. This was in contrast to placebo-treated alkali-injured corneas where the expression of nitric oxide synthases and proteases was strong and neovascularization highly pronounced (Figs. 5, 7, 11). Suppression of corneal

inflammation and neovascularization was also shown immunohistochemically using anti-macrophage/monocyte and anti-vascular endothelial growth factor antibodies. The number of cells detected with anti-macrophage antibody was less in alkali-injured cornea treated with RGTA, compared to placebo-treated alkali-injured cornea. Very similar results were obtained with the staining of vascular endothelial growth factor (Fig. 8). Moreover, quantitative results with real-time PCR show a significant decrease in pro-inflammatory cytokine as well as i-NOS induction after RGTA compared to placebo (Fig. 13). Because IL-1ß and i-NOS are genes of macrophages, while IFN-γ that of T cells, our findings point to the conclusion that the response of both cell types is reduced after RGTA treatment.

Finally, the efficacy of RGTA for the healing of corneal ulcers was investigated. RGTA application (or placebo) was started four weeks after a more profound alkali injury, when corneal ulcers developed. Subsequent RGTA treatment healed the majority of these ulcers within one month: six superficial ulcers and four deeper ones were completely healed. The remaining two ulcers required longer time of healing (seven weeks). In contrast to RGTA drops, after placebo application the ulcers persisted to a large extent (Figs. 9, 12).

Summarizing our results, RGTA treatment decreased oxidative and nitrosative stress to the cornea, highly expressed in placebo-treated alkali-injured corneas. After RGTA application the prooxidant/antioxidant imbalance was decreased in the corneal epithelium and the elevated expression of nitric oxide synthases were suppressed. The formation of peroxynirite was reduced leading to decreased corneal damage (the staining of malondialdehyde, a marker of lipid peroxidation, was decreased in alkali-injured cornea). Furthermore, RGTA application caused reduced expression of proteolytic enzymes in epithelial cells and keratocytes of alkali injured cornea, resulting in the inhibition of corneal destruction. Both the number of inflammatory cells and corneal neovascularization were decreased (Fig. 8).

To our knowledge, we report for the first time the increased expression of xanthine oxidase and nitric oxide synthases, enzymes that generate superoxide radical and nitric oxide in epithelial cells and keratocytes of alkali-injured cornea and the inhibitory effect of RGTA on these enzymes, although the effect of heparin, RGTA, or heparin-like polymers on the expression of the above mentioned enzymes in other diseased organs have been already described (Matsamura et al., 1998; Ceccarelli et al., 2009; Mu et al., 2012).

Also, the inhibitory effect of RGTA on proteolytic enzymes as first described in our rabbit model of corneal alkali injury was proved previously using heparin-like polymers in in vivo as well as in in vitro non ophthalmology studies (Ledoux et al., 2000; Escartin et al., 2003). The inhibitory effect of RGTA on the above mentioned enzymes is associated with the reduced damage of corneal basement membrane components and also with decreased extracellular matrix breakdown. Serine proteases and metalloproteinases are potent mediators of the inflammatory response (Hojilla et al., 2008; Safavi and Rostami, 2012); the inhibition of these enzymes by RGTA application plays a key role in the decreased intracorneal inflammation and suppressed corneal neovascularization.

Finally, quantitative findings with RGTA or placebo effects on alkali-injured cornea using real-time PCR show that RGTA compared to placebo significantly decreased the pro-inflammatory cytokine and i-NOS induction.

In conclusion, RGTA facilitated corneal healing after alkali injury. RGTA protected the alkali injured corneas from extensive proteolysis caused by the excessive action of proteolytic enzymes and from oxidative as well as nitrosative damage evoked by toxic oxygen and nitrogen products.

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