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GLAUCOMA

Antifibrotic effects of tocotrienols on human Tenon's fibroblasts

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Abstract
Purpose To compare the antifibrotic effect of vitamin E isoforms α-, γ-, and δ-tocotrienol on human Tenon's fibroblasts (hTf) to the antimetabolite mitomycin C.
Methods Antifibrotic effects of α- (40, 60, 80, 100, and 120 μM), γ- (10, 20, 30, and 40 μM) and δ-tocotrienol (10, 20, 30, and 40 μM) on hTf cultures were evaluated by performing proliferation, migration and collagen synthesis assays. Whereas for vitamin E the exposure time was set to 7 days to mimic subconjunctival application, cultures were exposed only 5 min to mitomycin C 100 μg/ml to mimic intraoperative administration. Cell morphology (phase contrast microscopy) as an assessment for cytotoxicity and cell density by measuring DNA content in a fluorometric assay to determine proliferation inhibition was performed on day 0, 4, and 7. Migration ability and collagen synthesis of fibroblasts were measured.
Results All tested tocotrienol isoforms were able to significantly inhibit hTf proliferation in a dose-dependent manner (maximal inhibitory effect without relevant morphological changes at day 4 for α-tocotrienol 80 μM with 36.7% and at day 7 for α-tocotrienol 80 μM with 42.6% compared to control). Degenerative cell changes were observed in cultures with concentrations above 80 μM for α- and above 30 μM for γ- and δ-tocotrienol. The highest collagen synthesis inhibition has been found with 80 μM α-tocotrienol (62.4%) and no significant inhibition for mitomycin C (2.5%). Migration ability was significantly reduced in cultures exposed to 80 μM α- and 30 μM γ-tocotrienol (inhibition of 82.2% and 79.5%, respectively, compared to control) and also after mitomycin C treatment (60.0%). Complete growth inhibition without significant degenerative cell changes could only be achieved with mitomycin C.
Conclusion In vitro, all tested tocotrienol isoforms were able to inhibit proliferation, migration and collagen synthesis of human Tenon's fibroblasts and therefore may have the potential as an anti-scarring agent in filtrating glaucoma surgery.

Keywords Vitamin E · Tocotrienol · Antifibrotic effect · Tenon's fibroblast · Filtrating glaucoma surgery · Mitomycin C

The authors have full control of all primary data and agree to allow the journal to review their data if requested.

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Introduction

Postoperative fibrosis of the filtering bleb or of the surgical fistula is a known cause of failure in glaucoma surgery [1, 2]. Tenon's fibroblasts are assumed to be the

main component of scar tissue in glaucoma surgery [2]. Antimetabolites as mitomycin C and 5-fluorouracil are currently used as anti-scarring drugs in glaucoma surgery, especially in eyes with an elevated risk of bleb scarring, but may hold the risk of postoperative complications, e.g., ocular hypotonia (bleb leakage), conjunctival necrosis of the filtering bleb, or endophthalmitis because of cytotoxic side effects [3–10]. Although recent studies have shown encouraging results for mitomycin C with a relatively low complication rate [11], alternative agents may still be useful. In cancer research, the antiproliferative and apoptotic effects of different vitamin E forms have been shown. Vitamin E acts as an antioxidant with neuroprotective, antithrombotic, anti-inflammatory and antineoplastic effects through its involvement in the intracellular signaling pathway [12–16]. Vitamin E is the generic name for two subclasses (tocopherols and tocotrienols), which itself consist of different isoforms. Vitamin E inhibits the proliferation of human Tenon's capsule fibroblasts in vitro [17]. In a previous in vitro study of our group, only α-tocotrienol showed antiproliferative effects without significant toxicity on human Tenon's fibroblasts compared to α-tocopherol, α-tocopheryl-acetate, and α-tocopheryl-succinate. A 50% growth inhibition could be achieved using 50 μM of α-tocotrienol [18]. Higher (and therefore eventually more effective) concentrations have not been tested. Based on prior studies [19–21] γ- und δ-isoforms of tocotrienol may even have a better antiproliferative effect than α-tocotrienol. Therefore, the aim of this study was to evaluate the tocotrienol isoform with the highest antifibrotic effect compared to mitomycin C in vitro. For this purpose, we performed a proliferation, cell migration, and collagen deposition assay. Degenerative cell changes produced by vitamin E isoforms and mitomycin C were assessed by phase contrast microscopy.

Materials and methods

Reagents

RRR-α-, RRR-γ-, and RRR-δ-tocotrienol (α-T3, γ-T3, δ-T3) were a kind gift from the Malaysian Palm Oil Board (Kuala Lumpur, Malaysia). Vitamin E forms were dissolved in ethanol absolute and then stored light-protected at 4°C. Mitomycin C was purchased from Kyowa (distributed by Roche Pharma, Reinach, Switzerland) and dissolved freshly in phosphate-buffered saline (PBS) before use.

Cell cultures

Explants of human Tenon's capsule were obtained from seven patients at the time of cataract surgery. All patients gave their informed consent before inclusion in the study, which was approved by the local ethics committee and conformed to the provisions of the Declaration of Helsinki. The seven different human Tenon fibroblast cultures were thawed and placed in 25-cm^2 tissue culture flasks containing Dulbecco`s minimal essential medium (DMEM) supplemented with L-glutamine (584 mg/l), sodium pyruvate (100 mg/l), glucose (1,000 mg/l), penicillin (60 U/ml), streptomycin (60 μg/ml) and 10% fetal calf serum (FCS). None of the seven donors, aged 68–93 years, had antiglaucomatous, antimetabolite, or any other ocular treatment, or ocular surgery before the corneo-scleral cataract surgery, when Tenon specimens were taken. Cultures were maintained in a humidified 5% CO_2 incubator at 37ºC. Confluent cultures were trypsinized, centrifuged, and repassaged. The above medium was changed twice per week. As quantified by trypan blue dye exclusion method, viability was always more than 95%. For the assays, third- to fifth-passage cells were used. All assays were performed on all cell lines in triplicate.

Cell proliferation assay

Fibroblasts were seeded at a density of 1,500 cells/well in 96-well tissue culture plates (black/clear-bottom, Corning Life Sciences), with each well containing 200 μl of culture medium. Fibroblasts were washed with PBS 24 h after plating and incubated for another 48 h with DMEM containing 0.2% FCS to induce growth arrest [2]. At day 0, cells were rinsed with PBS and cell growth was restimulated with adding fresh DMEM/10% FCS. Tocotrienols were immediately diluted to the indicated concentrations (40, 60, 80, 100, and 120 μM α-tocotrienol, 10, 20, 30, and 40 μM γ-tocotrienol and 10, 20, 30, and 40 μM δ-tocotrienol) in above media. Cells treated with an equivalent amount of ethanol absolute (0.8 μl/well) were included as control.

Mitomycin C was dissolved in PBS to concentrations of 10, 100, and 400 μg/ml. Growth-arrested cells were exposed for exactly 5 min to 100 μl/well mitomycin C solution. Fibroblasts were then gently washed with PBS alone and fed with 200 μl/well DMEM/10% FCS again. Multiple rinsing with PBS was not performed since a decrease in cell density was observed in preliminary studies. PBS-treated cultures served as control. The plates were then incubated for 7 days at 37ºC in 5% CO_2 in a humidified air atmosphere. No media was changed during this time.

Cell morphology was studied at day 0, 1, 4, and 7 using phase contrast light microscopy (Leica DMIRB research microscope, Leica Microsystems, Wetzlar, Germany). Photomicrographs were obtained with a color camera (Kappa

CF15 MC, Kappa Messtechnik, Gleichen, Germany) connected to a video printer (Sony UP-5200MDP, Sony, Schlieren, Switzerland). Images were evaluated by a masked investigator.

As recently described in detail [18], cell density was finally determined with a fluorometric assay (CyQUANT Cell Proliferation Assay Kit, Molecular Probes, distributed by JURO Supply, Lucerne, Switzerland) measuring DNA content of each well at days 0, 4, and 7.

Collagen synthesis assay

Collagen content was analyzed with a Sircol Soluble Collagen Assay (Biodye Science, Cologne, Germany) for cell cultures 4 days after exposure to α-tocotrienol, δ-tocotrienol, γ-tocotrienol (control: exposure to ethanol abs. 0.8 µl) and mitomycin C (100 µg/ml, exposure time 5 min; control: exposure to PBS). Sirius red binds specifically to soluble collagen and can be quantified with a colorimeter.

Cell migration assay

Effect of tocotrienol and mitomycin C on migration ability of Tenon's fibroblasts was tested with a CytoSelect 96-well Cell Migration and Invasion Assay (Cell Biolabs, Inc., distributed by JuroScience GmbH, Lucerne, Switzerland) 2 days after exposure to tocotrienols or mitomycin C. The migration assay kit contains polycarbonate membrane inserts (8-µm pore size) in a 96-well plate. The membrane is a barrier discriminating cells with migration towards chemoattractants in the medium above the membrane. These cells were then dissociated from the membrane, and then a lysis and DNA-extraction is performed and cell count determined with a cytofluorometer as described above.

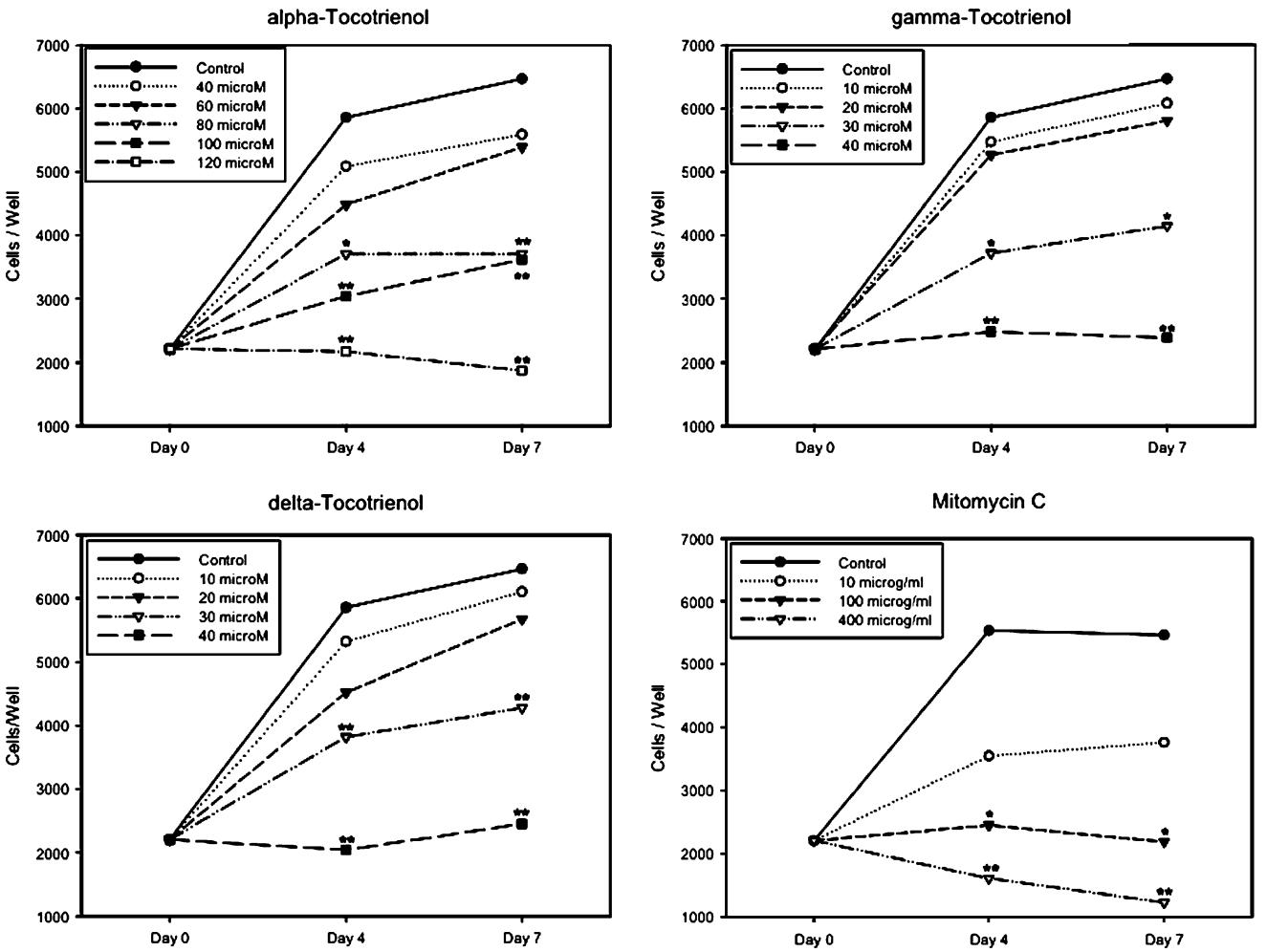

Fig. 1 Effects of incubation with different tocotrienols in variable concentrations for 7 days or exposure to mitomycin C for 5 min on human Tenon's fibroblast proliferation. A fluorometric assay determined proliferation by DNA content quantification at days 0, 4, and 7. Means of cell numbers/well of seven different cell cultures in triplicate are presented. Significantly reduced values, compared to control, are marked with one ($p<0.05$) or two ($p < 0.01$) *stars*

Statistical analysis

The relationship between the fluorescence signal and the number of cells/well was very strongly linear (Pearson correlation coefficient r=0.99). Results of the proliferation assay are therefore presented as the mean number of cells/well. Inhibition of proliferation, migration, and collagen synthesis were also expressed as percentages of inhibition compared to control. Differences between control and compound values were analyzed by Friedman's test followed by the non-parametric Dunnett's test based on rank sum as post-hoc analysis. Wilcoxon rank-sum test was used when appropriate. The criterion for statistical significance was $p<0.05$.

Results

Cell proliferation and morphology

Figure 1 and Table 1 summarize the results of the proliferation assay. All tested tocotrienol forms were able to significantly inhibit fibroblast proliferation at days 4 and 7, and effects occurred in a dose-dependent manner. Strong antiproliferative effects but no relevant degenerative cell changes were observed in cultures treated with 80 µM α-T3, 30 µM γ-T3, and 30 µM δ-T3. At these concentrations, cell densities were statistically comparable at day 4 (α-T3 3,709±1,394 (mean ± SD) cells/well, γ-T3 3,723±2,160 cells/well, δ-T3 3,820±2,063 cells/well; $p=0.87$) and day 7 (α-T3 3,714±1,524 cells/well, γ-T3 4,147±1,781 cells/well, δ-T3 4,281±2,186 cells/well; $p=0.57$) between all three tocotrienol forms. There was, however, considerable variation of the antiproliferative effect of all three tocotrienol forms between the seven different cell lines.

At higher concentrations, all tocotrienol forms led to nuclear and cytoplasmic condensation (Fig. 2).

Five-minute exposure to 100 µg/ml mitomycin C resulted in complete growth inhibition without affecting cell morphology (day 0, control 2,208±720 cells/well; day 7, 100 µg/ml mitomycin C 2,187±647 cells/well; Wilcoxon test: $p=0.81$). Viability of cultures treated with 400 µg/ml mitomycin C was severely compromised (Fig. 2). Since α-tocotrienol 80 µM, δ-tocotrienol 30 µM and γ-tocotrienol 30 µM were the tocotrienol concentrations with the best antiproliferative effect without relevant degenerative cell changes, only these concentrations were used in the further assays.

Table 1 Effects of incubation with different tocotrienols in variable concentrations for 7 days or exposure to mitomycin C for 5 min on human Tenon's fibroblast migration (M), proliferation (P), and collagen synthesis (C) expressed in percentages of inhibition compared to control group at specific time points (days 2, 4, and 7)

	Day 2	Day 4		Day 7
	% M	% P	% C	% P
α-tocotrienol				
40 µg/ml	20.7	13.2	−0.7	13.6
60 µg/ml	57.7	23.5	33.0	16.7
80 µg/ml	**82.2**	**36.7**	**62.4**	**42.6**
100 µg/ml	n.a.	**48.1**	n.a.	**44.1**
120 µg/ml	n.a	**63.0**	n.a.	**71.0**
γ-tocotrienol				
10 µg/ml	n.a.	6.5	n.a.	6.0
20 µg/ml	n.a.	10.1	n.a.	10.3
30 µg/ml	**79.5**	**36.5**	**56.5**	**35.9**
40 µg/ml	n.a.	**33.3**	n.a.	**42.5**
δ-tocotrienol				
10 µg/ml	n.a.	9.2	n.a.	5.6
20 µg/ml	n.a.	22.8	n.a.	12.3
30 µg/ml	57.1	**34.8**	**60.4**	**33.8**
40 µg/ml	n.a.	**65.1**	n.a.	**62.0**
mitomycin C				
10 µg/ml	n.a.	35.8	n.a.	31.2
100 µg/ml	**25.7**	**55.8**	2.5	**60.0**
400 µg/ml	n.a.	**71.0**	n.a.	**77.6**

Significant results ($p<0.05$) are marked in bold

Collagen production

Comparing collagen content of different cell cultures (Fig. 3, Table 1) after exposure to tocotrienol and mitomycin C revealed significant inhibition of collagen synthesis for α-tocotrienol 80 µM, γ-tocotrienol 30 µM and δ-tocotrienol 30 µM (Friedman ANOVA, $p=0.0002$; Dunnmatt post-hoc test $p<0.05$, each) with the highest inhibition for 80 µM α-tocotrienol, whereas mitomycin C did not inhibit collagen synthesis (Wilcoxon one-tailed test; $p=0.23$). Collagen content in the mitomycin C control (PBS) did not differ from that of the tocotrienol control (ethanol) (Wilcoxon two-tailed test; $p=0.47$).

Cell migration ability

Migration ability (Fig. 4, Table 1) was significantly reduced in the proliferation assay exposed to α-tocotrienol 80 µM and γ-tocotrienol 30 µM compared to the ethanol control (Dunnett post-hoc test, $p<0.01$, each; Friedman ANOVA, $p<0.0001$) and also for the mitomycin C assay compared to the PBS control (Wilcoxon one-tailed test; $p=0.02$). No significant inhibition of cell migration could be shown for α-tocotrienol 40 and 60 µM and δ-tocotrienol 30 µM ($p>0.05$, each). There was no significant difference between the tocotrienol (ethanol) and the mitomycin C control group (PBS) (Wilcoxon two-tailed test; $p=0.58$).

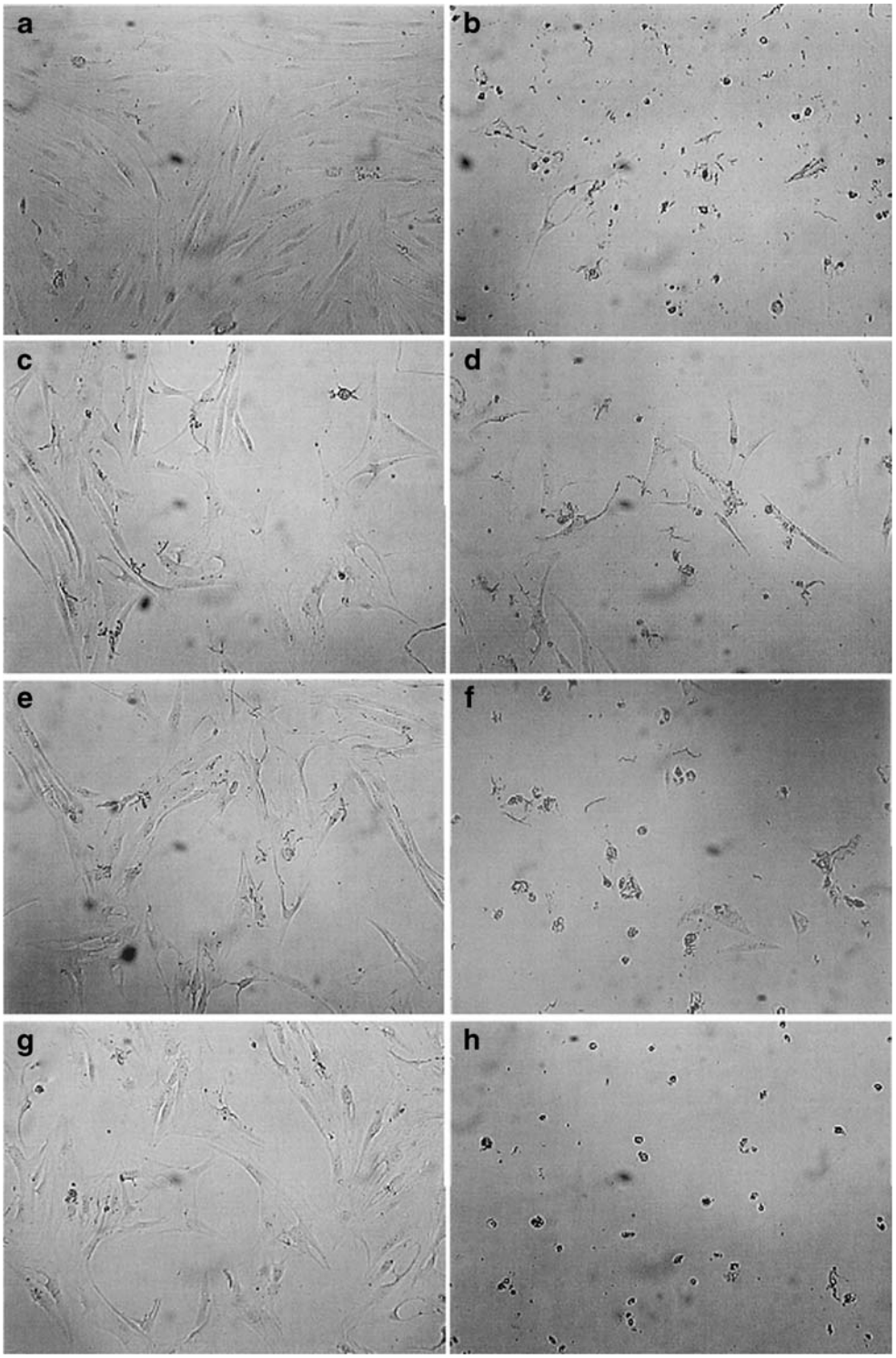

Fig. 2 Phase contrast photomicrographs of human Tenon's fibroblasts on day 7 of the proliferation assay. **a** control; **b** 400 μg/ml mitomycin C; **c** 80 μM α-tocotrienol; **d** 120 μM α-tocotrienol; **e** 30 μM γ-tocotrienol; **f** 40 μM γ-tocotrienol; **g** 30 μM δ-tocotrienol; **h** 40 μM δ-tocotrienol. (representative photographs were obtained from fibroblasts in the center of the well. Magnification: all x 50)

Discussion

The outcome of filtrating glaucoma surgery depends on the amount of wound healing and scarring. In a review of Lama et al., pathways involved in wound healing after glaucoma surgery are described, including vascular leakage, coagulation, cellular migration, granulation tissue, and scar formation [22]. Antimetabolites such as mitomycin C and 5-fluorouracil are used in filtrating glaucoma surgery to reduce bleb scarring in patients at risk for postoperative bleb failure [3–5]. Due to their cytotoxic effectiveness, hypotony and endophthalmitis may occur [10, 23]. The dose-dependent effect of mitomycin C on apoptosis was shown by Crowton et al. [24]. Although experience with mitomycin C revealed an

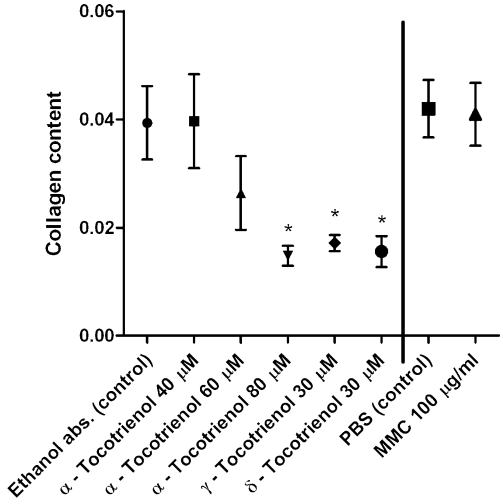

Fig. 3 Collagen content in cell cultures 4 days after exposure to tocotrienol and mitomycin C showing significant collagen synthesis inhibition with α-totoctrienol 80 µM, γ-tocotrienol 30 µM, and δ-tocotrienol 30 µM (* $p<0.05$, each) and no significant inhibition for mitomycin C 100 µM

acceptable safety profile [25, 26], alternative antiproliferative agents with less cytotoxic and cell degenerative effects compared to mitomycin C would still be needed.

In prior studies, vitamin E isoforms have revealed an antifibrotic potential [17–19, 21]. Based on these findings, we evaluated the effects of different tocotrienol isoforms and concentrations on human Tenon's fibroblasts regarding inhibition of proliferation, migration, and collagen synthesis, revealing a significant antifibrotic effect for all tested isoforms.

This study focused on inhibition of proliferation, migration, and collagen synthesis, whereas other possible factors involved in wound-healing are not fully covered. Prior studies have also evaluated the effects of vitamin E isoforms on angiogenesis, apoptosis, and cytotoxic effects

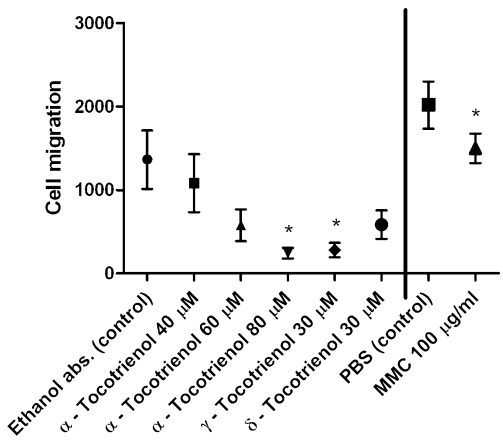

Fig. 4 Cell migration assay (8 µm pore size; migration time 48 h) for different tocotrienol isoforms/concentrations and mitomycin C. (* $p<0.05$)

[27–30]. In a previous study of our group, the application of tocotrienol at a concentration of 100 µM did not lead to an increase of glucose 6-phosphate dehydrogenase release as a measure for dying cells [18]. However, synthetic forms of vitamin E such as α-tocopheryl-succinate led to significant increased G6PD levels. Considering α-tocotrienol, degenerative cell changes were only observed after repeated application of 50 µM. In the present study, 80 µM α-tocotrienol showed a significant anti-proliferative effect without relevant morphologic changes as well.

Exposure times of hTf to tocotrienols (7 days) and mitomycin C (5 min) have been chosen on purpose to be different in our study. During glaucoma filtration surgery, the application time of mitomycin C is commonly limited to a few minutes. Such a short-time application is unlikely to be suitable for tocotrienols. To the best of our knowledge, it is difficult to formulate a type of tocotrienol capable of realizing a sufficiently high and stable concentration such as needed in an intraoperative application of a few minutes as cellular uptake of tocotrienols is rather a matter of hours than minutes [31]. We therefore used a long-term application approach, which clinically could be obtained for example with a subconjunctival injection of the compound. Our results should encourage for further dose-finding and toxicity studies, as in-vitro concentrations are not directly portable to in-vivo use. As mitomycin C at a concentration of 100 µg/ml revealed the best effect without significantly toxicity, we decided to chose only this concentration for migration and collagen synthesis assays.

Whereas in our study mitomycin C as well as α-tocotrienol 80 µM and γ-tocotrienol 30 µM significantly reduced cell migration of hTf, the antiproliferative effect of mitomycin C was higher compared to tocotrienol. On the other hand, mitomycin C (100 µg/ml, 5 min exposure time) did not reveal significant inhibition of collagen production in contrast to tocotrienol (α-tocotrienol 80 µM, γ-tocotrienol 30 µM and δ-tocotrienol 30 µM). Because of this advantage, tocotrienol may have a certain potential as an agent for filtrating glaucoma surgery as collagen synthesis is an important factor for bleb and surgical fistula scarring in glaucoma filtrating surgery [32, 33]. The results of our study are encouraging for further research as tocotrienols may offer the potential as an alternative agent to currently used anti-scarring drugs as mitomycin C or 5-fluorouracil. As tocotrienol isoforms are lipid-soluble agents, subconjunctival drug application could be difficult and an appropriate carrier substance may have to be used.

In vitro, all tested tocotrienol isoforms have revealed good antiproliferative effectiveness on human Tenon's fibroblasts. Further in vitro and in vivo studies will be necessary to evaluate the safety and the potential role of tocotrienol as a mean to prevent bleb failure in filtrating glaucoma surgery.

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