Downregulation of Tumor Necrosis Factor-Alpha and Vascular Endothelial Growth Factor Expression by Epigallocatechin-3-Gallate in Human Pterygium Fibroblasts

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Abstract

Objective

To examine the efficacy of Epigallocatechin-3-Gallate (EGCG) in inhibiting the expression of tumor necrosis factor-alpha (TNF-α) and vascular endothelial growth factor (VEGF) in human pterygium fibroblasts (HPF) as an alternative to pterygium adjuvant therapy.

Methods

This experimental in vitro study was conducted using human pterygium fibroblasts (HPF) obtained from pterygium excision, cultured, and divided into 3 intervention groups: untreated, mitomycin-C (as negative control), and EGCG. TNF-α and VEGF expression were analyzed using immunofluorescence at 48 h and with GambarJ software. The results between groups were analyzed using the ANOVA test, and the correlation between the two expressions was analyzed using the Spearman test.

Results

EGCG at a dose of 50, 100, and 150 μM decreased the expression of TNF-α ($p < 0.001$) and VEGF significantly ($p < 0.001$) compared to the untreated group, and a correlation was found ($p < 0.001$) between the two expressions.

Conclusion

EGCG can reduce the expression of TNF-α and VEGF and shows its potential as adjunctive therapy in preventing pterygium recurrence after excision.

Keywords: EGCG, Human Pterygium Fibroblast, TNF-α, VEGF.

Introduction

A pterygium is an invasive growth of the bulbar conjunctival epithelium and subconjunctival connective tissue in a triangular shape where the apex points towards the center of the cornea (Detorakis & Spandidos, 2009; Belmonte et al., 2011). The prevalence of pterygium is reported to be as high as 12%. Pterygium can cause visual impairment and blindness, so it must be excised. The post-excision recurrence rate with conjunctival autograft was recorded at 5.3-13.5%, while the bare sclera technique reached almost 90%. Adjuvant therapy with mitomycin C (MMC) reduces this rate to 4-7%, but serious complications are common (Erry et al., 2011; Fonseca et al., 2018; Rezvan et al., 2018).

Pterygium is a relevant tropical disease, especially in countries with dense populations, low socioeconomic indexes, and inadequate health policies (Abegunde & Owoaje, 2013; Martens, 2013). The etiology of pterygium is thought to be a neoplasm due to inflammation or degeneration. Exposure to ultraviolet radiation is one of the most important risk factors in the pathogenesis of pterygium because it can cause oxidative stress and increase the release of cytokines, such as TNF-α, which plays a role in the inflammatory process, and growth factors, such as VEGF, which plays a role in the process of angiogenesis (Erry et al., 2011; Fonseca et al., 2018; Shahraki et al., 2021)

Dry, windy, sandy, and dusty environments can also increase the risk of pterygium (Guo et al., 2010; Wang et al., 2020). One of the irritants, namely dust, can irritate the eyes, causing the lipid layer in the tear film to lysis; if exposed continuously for a long time, it can affect the stimulation of the conjunctival surface, especially the limbus area. Pterygium can cause several symptoms, namely blurred vision, red eyes, itching, and a feeling of something being stuck caused by the presence of an inflamed lid, adnexa, or anterior segment (Chakrabarti, 2014; Zulkifley et al., 2019).

These symptoms require immediate surgical excision because the pterygium can reach the cornea's middle and cause blindness. Pterygium can affect both eyes, which can be different or the same degree (Erry et al., 2011). Although this pterygium has a good prognosis, the most complications caused by pterygium are postoperative recurrences. Said to happen (Rao et al., 1998).

TNF-α is a pro-inflammatory cytokine that controls the effects of inflammation on numerous cell types. TNF-α is activated by NF-B expression in pterygium tissue, hence regulating cell proliferation, differentiation, migration, and death. In addition to mediating VEGF-C production via HIF1-α, TNF-α may be a crucial angiogenesis stimulant during pterygium formation. TNF-α can regulate HIF1- activity via multiple intracellular signaling pathways, such as phosphatidylinositol-3 kinase (PI3K)/protein kinase B(Akt) (Dong et al., 2016; Kim & Kim, 2017; Malkov et al., 2021; Zidi et al., 2017).

Mononuclear phagocytes, antigen-activated T cells, NK cells, and mast cells are the primary producers of TNF-α. Lipopolysaccharide is a powerful inducer of TNF secretion by macrophages. IFN-α generated by T cells and NK cells, among others, encourages macrophages to boost TNF production. TNF-α has multiple functions in the inflammatory process, which can increase the prothrombotic role and stimulate adhesion molecules from leukocyte cells and induce endothelial cells, play a role in regulating macrophage activity and immune response in tissues by stimulating growth factors and other cytokines, and serve as a regulator of hematopoietic as well as commitogens for T cells, B cells, neutrophils, and macrophages. In addition to its role in the immune response against bacterial, viral, fungal, and

parasitic invaders, TNF-α has additional positive effects (Feghali & Wright, 1997; Salazar et al., 2007; Sprague & Khalil, 2009).

VEGF is a growth factor that plays an important role in angiogenesis and increases vascular permeability. VEGF contributes to tumor neovascularization which responds to an increase in the need for delivery of nutrients and oxygen, which is needed for the survival of tumor cells. VEGF plays an important role in vasculogenesis during embryogenesis, physiological angiogenesis, and malignant neovascularization. VEGF transcription is triggered by hypoxia and various growth signals (Mukhopadhyay & Datta, 2004; Byrne et al., 2005).

Under normal circumstances, VEGF is expressed to varying degrees by various tissues, including the brain, kidney, liver, and spleen. Oxygen pressure can function as a VEGF regulator. Exposure to hypoxic conditions induces rapid expression of VEGF. In contrast, VEGF expression decreases and stabilizes under normal oxygen levels (normoxia). The level of expression of VEGF also depends on the number of inflammatory cytokines and growth hormones, including Epidermal Growth Factor (EGF), Interleukin-1β (IL-1β), Platelet-derived growth factor (PDGF), Tumor necrosis factor-α (TNF-α), and transforming growth factor- β1 (TGF- β1) (Vogel et al., 2004; Lee & Yang, 2012)

Pterygium showed significantly increased levels of VEGF and VEGFR when compared with normal conjunctiva. Increased VEGF expression also promotes angiogenesis in pterygium formation, which influences normal conjunctival metabolism. VEGF expression can be induced by UV light, cytokines (ILs and TNF-α), and other growth factors. The role of TNF-α and VEGF in the pathogenesis of pterygium suggests that they may be targets for disease management (Chui & Coroneo, 2012; Kim et al., 2015; Cardenas-Cantu et al., 2016).

EGCG is the main catechin compound in green tea extract, which is non-toxic and water-soluble, so it is easily excreted in the urine. EGCG has been shown to inhibit inflammatory processes in other diseases and affect cell death mechanisms similar to MMC. EGCG can cause inhibition of the NF-κB pathway, which is activated by UV-B light so that it can regulate TNF-α. The antioxidants contained in EGCG can also inhibit angiogenesis factors by reducing the expression of the VEGF growth factor through inhibition of HIF1-α in the PI3k/Akt phosphorylation pathway. The things mentioned above make EGCG an effective alternative adjuvant therapy in preventing pterygium recurrence (Yang et al., 2021; Li et al., 2021; Jin et al., 2019; Sharifi-Rad et al., 2020).

Method

Preparation of EGCG

EGCG was generously prepared by Professor Djoko Agus Purwanto (Xi'an Rongsheng Biotechnology CO., LTD) and dissolved in 0,01% Benzalkonium Chloride and 0,093% Natrium Chloride for the preparation of broth solutions at concentrations of 50, 100, and 150 µM saved at 2-8 °C. Exhaustive media The EGCG was diluted to the required concentration with DMEM, sonicated, and filtered.

Culture and the passage of HPF

The Tritya Eye Clinic in Surabaya, Indonesia, collected pterygium tissue aseptically from patients who had signed informed consent forms. Before removing the pterygium tissue, a local anesthetic consisting of a subconjunctival injection of lidocaine 2% was administered. The ocular surface was cleaned with povidone-iodine and Balance Salt Solution (BSS). After the pterygium had been surgically removed, a tiny sample (0.5 mm3) was taken and placed in

a 100 mm culture dish, washed with Phosphate Buffer Saline (PBS), treated with RBC Lysis Buffer for 5 minutes, and finally incubated at 37 degrees Celsius. The samples were placed in 1 mL of Dulbecco's Modified Eagle's Medium (DMEM) containing 15% Fetal Bovine Serum (FBS) and Penicillin Streptomycin, and incubated at 37 degrees Celsius with 95% humidity for one full day. The media was replenished daily until the cells achieved 80% confluence, at which point the FBS concentration was decreased to 10%. Non-confluent and contaminated cells were excluded from the study (Ramadhan et al., 2022).

Cell's characteristics and Vimentin staining

At a density of 2.5 x 104 cells per well, the confluent cells were transferred to well plate 24 until they adhered to the bottom of the plate. Cells were washed in PBS three times for five minutes each time. After 30 minutes, the cells were treated with 1% bovine serum albumin (BSA) at room temperature. After removing the fluid, cells were treated with primary antibodies for 24 hours before being washed with PBS. The cells were treated with Goat Anti Rabbit IgG & LTRITC (AB 6718)-Vimentin, rinsed with PBS, and then incubated at room temperature with DAPI (1:1000) for 5 minutes. At 10x magnification, images of cells were taken with a fluorescent microscope (Ramadhan et al., 2022).

Measurement of TNF-α and VEGF by Immunofluorescence Staining

After 48 hours, confluent cells were collected and plated on well plate 24. The wells were then separated into untreated, MMC 0.4 mg/mL, and EGCG 50, 100, and 150 μM. Untreated wells contained only cells and medium, MMC 0.4 mg/mL was added to cells with medium for 5 minutes, and washed once with PBS. EGCG was added to cells with medium at concentrations of 50, 100, and 150 μM, and rinsed once with PBS. Each well was treated with 3-4% formaldehyde for 15 minutes, followed by two PBS washes. The well plate was rinsed three times for 5 minutes with PBS, and then incubated for 30 minutes at room temperature with 1% bovine sodium albumin (BSA). Cells were treated with TNF-α FITC conjugated Mouse Monoclonal Antibody Santa Cruz Biotechnology, Inc® or VEGF TRITC conjugated Rabbit Polyclonal Antibody Santa Cruz Biotechnology, Inc® antibodies at 4 ^{o}C for 24 hours. The well plate was rinsed for 5 minutes with PBS and incubated for 5 minutes with DAPI at a concentration of 1:1000. Using a fluorescent microscope at 40x magnification, the results were read. Using the following formula, expression levels were calculated using ImageJ software and expressed as corrected total cell fluorescence (CTCF). Density - Combined (Area of selected cell x Mean fluorescence of background readings) (Ramadhan et al., 2022).

Statistical analysis

Comparative data between the expression of TNF-α and VEGF within the interventions were tested using the one-way ANOVA test, followed by the Tukey post hoc test. Correlation data between both expressions were analyzed using the Pearson test. All statistical data were processed using SPSS 26.0 software with a p-value < 0.05 is considered significant.

Result and Discussion

Culture, the passage of HPF, cell's characteristics, and Vimentin staining

The recovery of Pterygium tissue followed the protocol established by the prior investigation.[16] The fibroblast cell growth was observed until the confluence had reached 80% on day 10. P4 cultures were used in this study with a confluency rate of 90 – 100% on day 14. The fibroblast cells were confirmed using vimentin, an antibody marking fibroblasts.

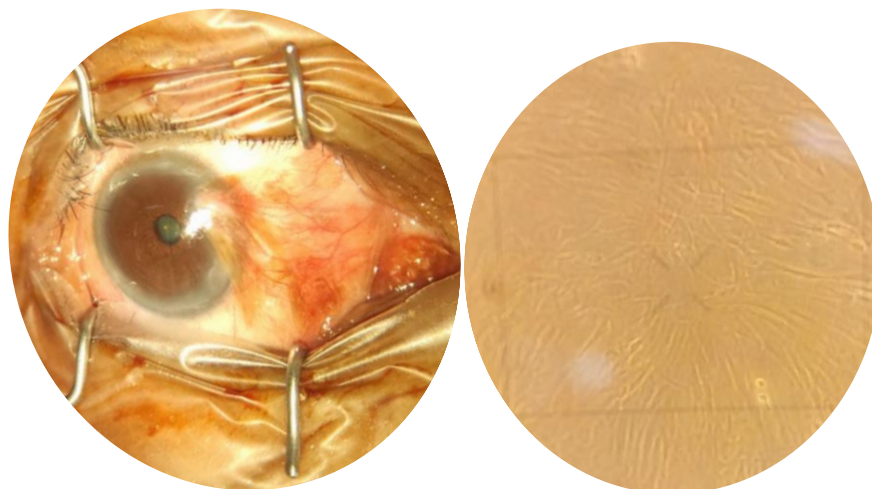

Figure 1. *(Left) pterygium before excision; (Right) primary pterygium tissue culture*

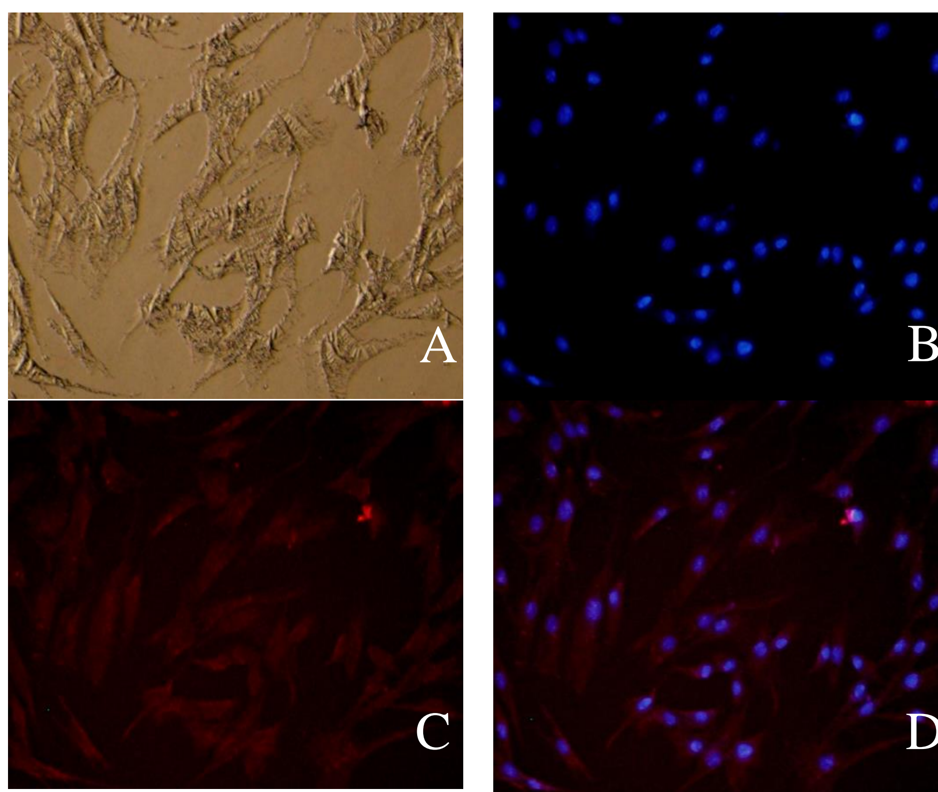

Figure 2. *Human pterygium fibroblast was observed under a fluorescence microscope at 10x magnification in contrast phase (A), DAPI staining (B), vimentin (C), and merge (D)*

Expression of TNF-α and VEGF in EGCG & Mitomycin-C group

The results showed that the highest expression of TNF-α and VEGF was in the untreated group, while the lowest was in the MMC 0.4 mg/ml. TNF-α and VEGF expression of 50 µM showed higher suppression than the untreated group, while 150 µM showed lower suppression than MMC 0,4 mg/mL group. EGCG 100 µM EGCG showed lower expression than the 50 µM EGCG while having stronger expression than 150 µM EGCG. One-way ANOVA test showed a statistical significance of difference among all groups ($p < 0.001$). Tukey tests show that the TNF-α and VEGF expressions between groups are significantly different at $p < 0.05$. Pearson's test showed a significant correlation ($p < 0.001$) between both expressions.

Table 1. *TNF-α expression based on corrected total cell fluorescence*

	Mean CTCF	Standard Deviation	
Untreated	23.738.727	2.519.470,114	
EGCG 50 µM	17.955.308	2.436.117,91	
EGCG 100 µM	10.517.833	1.441.698,329	p < 0,001*
EGCG 150 µM	4.443.017	1.139.406,713	
MMC 0,4 mg/mL	1.083.169	229.620,7551	

*tested by one-way Anova, considered significant if p< 0.05

Table 2. *VEGF expression based on corrected total cell fluorescence*

	Mean CTCF	Standard Deviation	
Untreated	99.072.597	5.876.010,459	
EGCG 50 µM	77.440.835	2.274.680,478	
EGCG 100 µM	41.104.888	1.743.271,169	p < 0,001*
EGCG 150 µM	22.720.365	2.794.650,466	
MMC 0,4 mg/mL	7.119.833	804.646,729	

*tested by one-way Anova, considered significant if p< 0.05

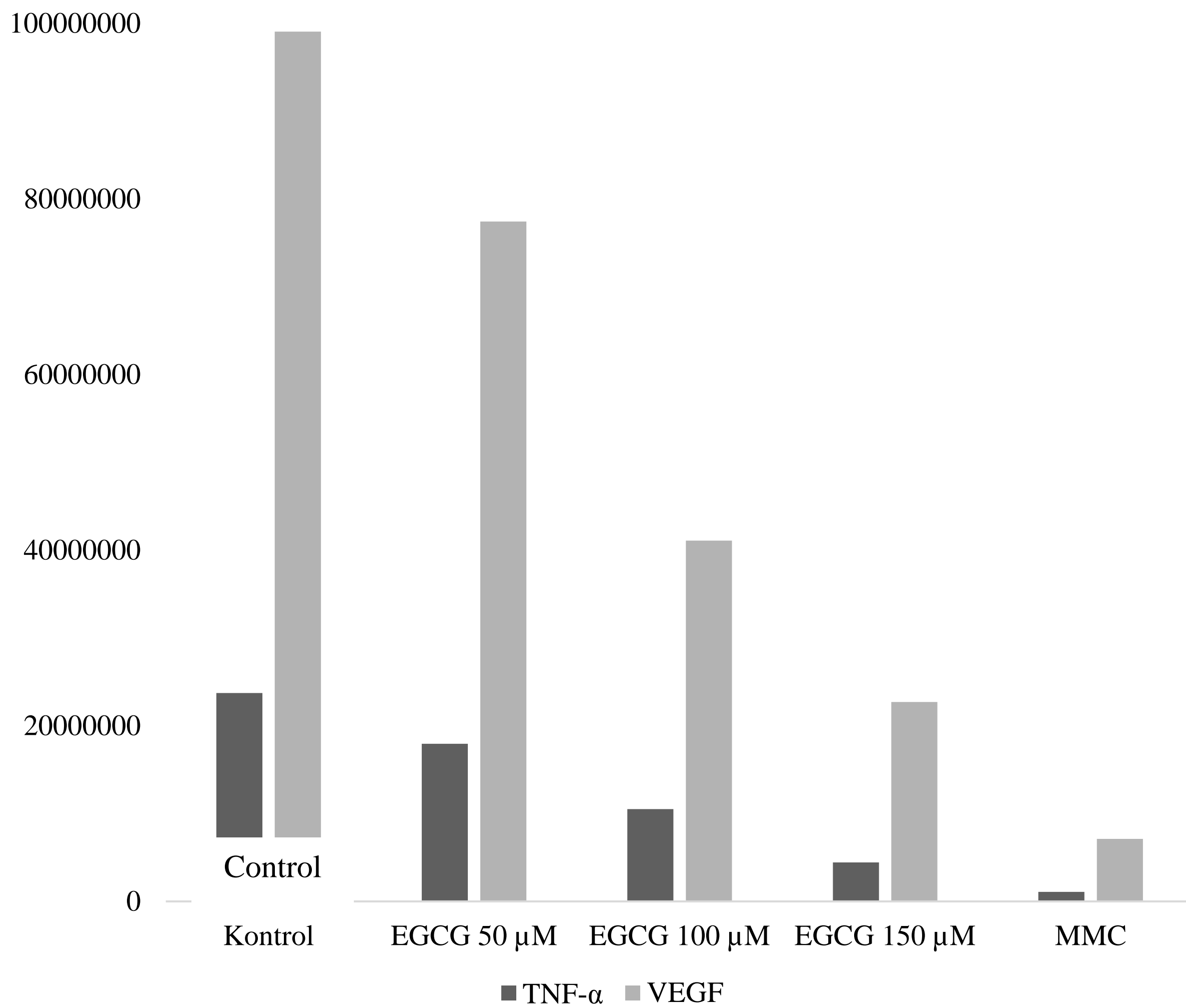

Figure 3: *Correlation between TNF- α and VEGF expression across different group*

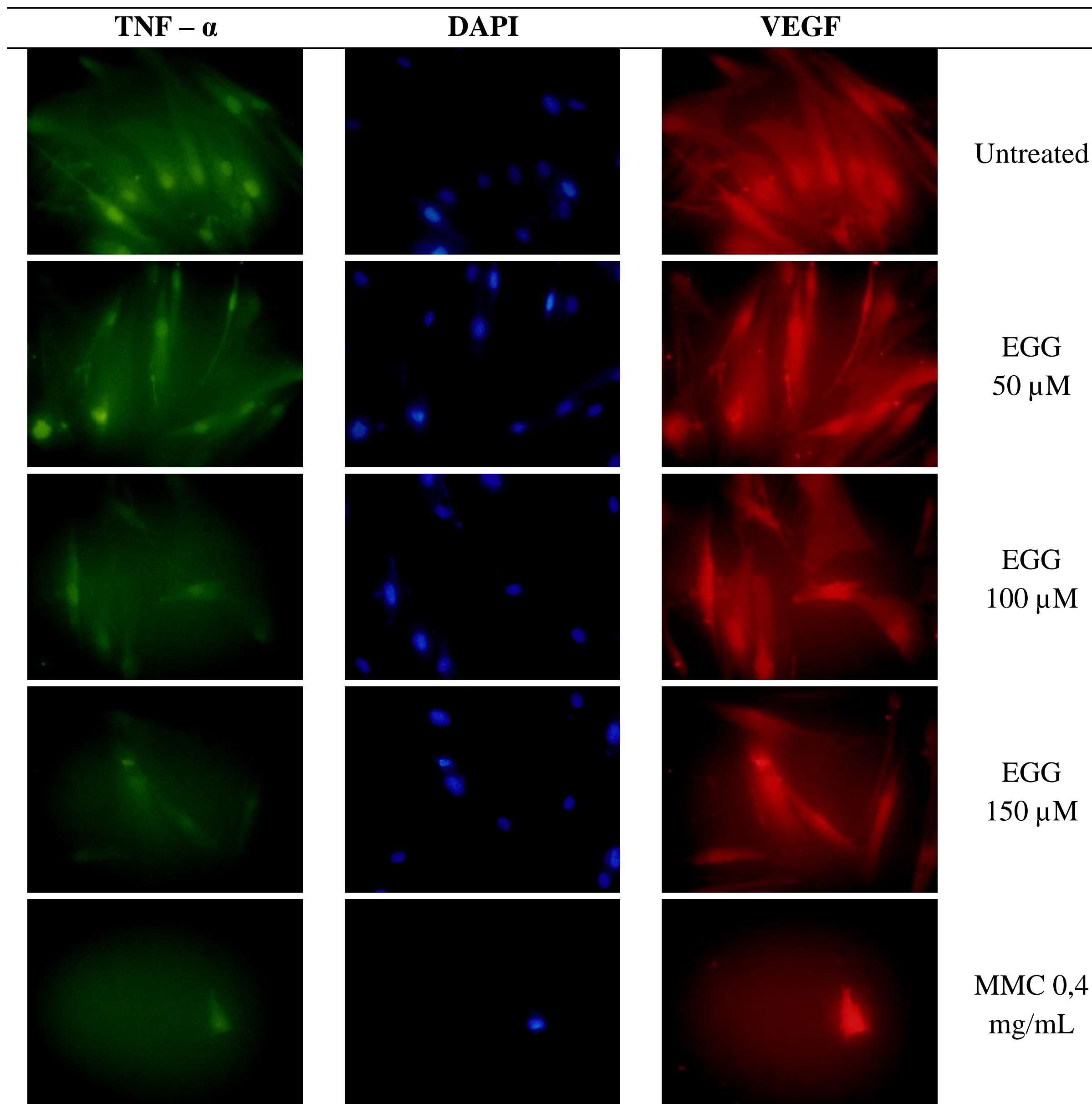

Figure 4. *Human Pterygium fibroblast cells stained with TNF-α, DAPI, and VEGF antibodies, photographed with a fluorescence microscope at 40x magnification, comparing the untreated group, EGCG 50µM, EGCG 100µM, EGCG 150µM, and MMC 0.4 mg/ml*

Pterygium is a disease of the ocular surface that can result in vision loss. The pathogenesis of pterygium and its recurrence are thought to be due to numerous pathways, including cell inflammation, proliferation, migration, and angiogenesis. Identification of medication capable of suppressing these pathways could hugely affect pterygium treatment.

Recurrence after pterygium excision depends on several factors, such as younger age, because TNF–α and VEGF expression are higher than in older people. UV-B rays effect also plays an important role in inducing TNF–α and VEGF expression in pterygium through inflammation and angiogenesis. MMC as adjuvant therapy is often used to reduce the recurrence rate in pterygium, but complications and ocular surface toxicity are still debatable; thus, safer and more effective treatment is needed to replace MMC.

The CCK-8 assay was performed in the pre-experimental study to assess the IC 50 dose of EGCG 102,17 µM. Therefore, the EGCG doses used in this study were 50, 100, and 150

μM. This is in line with research by Yang et al., 2021 that the treatment of 25 and 50 μM EGCG could significantly reduce human primary pterygium cells survival compared to control. Another study by Wu et al., 2019 stated that EGCG with doses ranging from 25−200 μM could also inhibit cell proliferation, viability, migration, and increased apoptosis activity (Yang et al., 2021; Wu et al., 2019)

TNF – α and VEGF expression in human pterygium fibroblast cells that had been given the intervention were observed within 48 hours, then performed immunofluorescence staining with TNF – α and VEGF antibodies. The dose of EGCG used in this study is 50 − 150, and it concluded that it has a dose-dependent inhibitory effect. A study by Toju et al., 2018 stated that TNF-α was significantly increased after 30 minutes of UV exposure, and suppression was found in the dose-dependent EGCG group. Another study by Dong et al., 2016 found that VEGF protein significantly increased 48 hours after TNF-α stimulation (Dong et al., 2016; Toju et al., 2018).

This study showed that EGCG and MMC significantly decreased TNF–α and VEGF expression in human pterygium fibroblast cells after 48 hours of intervention. There were differences in TNF – α and VEGF expression of the three EGCG doses, MMC and untreated group. MMC had the lowest TNF – α and VEGF expression compared to the untreated group and EGCG. This is similar to the experiment by Abdurrauf et al., 2022 where MMC was the most potent agent in reducing pterygium recurrence. However, this result slightly differs from a study by Guo et al. 2019 that showed MMC 0.4 mg/mL effectively reduced VEGF levels in pterygium tissue but had no effect on TNF – α level (Abdurrauf et al., 2022; Guo et al., 2019).

EGCG had a higher suppression effect of reducing TNF – α and VEGF expression when compared to the untreated group hinting that EGCG may have anti-inflammatory and anti-angiogenic activity. An experiment conducted by Li et al., 2019 supports this idea because the result shows that EGCG effectively downregulated TNF-α expression and other interleukins in retinal tissues from experimental autoimmune uveitis mice by targeting the Th17-associated pro-inflammatory gene expression. In another study by Koh et al., 2014 using corneal neovascularization animal models receiving topical EGCG, VEGF expression was effectively suppressed, suggesting that it has an anti-angiogenic effect (Li et al., 2019; Koh et al., 2014).

TNF – α and VEGF expression inhibition might have some mechanism affecting each other, shown by significant correlation in this study. This is consistent with an experiment by Dong et al., 2017 where TNF-α mediates VEGF expression in cultured human pterygium cells. A study by Kim et al., 2017 also concluded that human pterygium fibroblast showed increased expression of HIF-1α and VEGFR upon stimulation with TNF-α. Another study by Xu et al., 2020 found similar results where pro-EGCG could suppress the expression of HIF-1α and VEGF in the inner segment/outer segment and RPE layers while could also downregulate the expression of TNF-α. A review by Rashidi et al., 2017 even stated that EGCG inhibited TNF-α induced activation of NF-κB, which also inhibits VEGF binding to its receptor through blocking ERK and Akt phosphorylation by repressing the expression of HIF-1. However, in an experiment conducted by Lee et al., 2011 there seemed to be no association between TNF-α level and EGCG treatment. The levels of VEGF were lower with EGCG treatment, but the differences were not statistically significant (Xu et al., 2019; Rashidi et al., 2017; Lee et al., 2011; Dong et al., 2016; Kim et al., 2017).

Conclusion

In conclusion, this study shows that EGCG could be an adjuvant treatment for pterygium recurrence by downregulation of TNF–α and VEGF expression as one of the pathways causing pterygium growth. This showed the potential of EGCG as an alternative therapy for post-pterygium excision besides MMC. This is the first study to compare and correlate the effects of EGCG with MMC in reducing TNF–α and VEGF expression on human pterygium fibroblast in vitro. In the future, it is necessary to test other expression markers, such as those that induce cell proliferation, migration, or apoptosis as one of the pathogeneses of pterygium recurrence, and the use of EGCG as topical eye drop preparations that may be possible.

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