

Curcumin Alters VEGF Expression in Human Pterygium Fibroblast

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Abstract

Background: Recurrence remains a great challenge for pterygium management. Recurrence pterygium is also known to be more difficult to manage. Thus, this study focuses on recurrence prevention by exploring the potential of curcumin through its anti-angiogenesis properties.

Methods: An experimental study was performed using cultured Human Pterygium Fibroblast (HPF). HPF was cultured to 90% confluency rate on cell culture and divided into three groups. Control group as a negative control, curcumin 100 µmol/L and curcumin 200 µmol/L were applied to each group of cell cultures. After 48 hours, VEGF expression was analyzed using immunofluorescence staining and fluorescein microscope, then the intensity was measured using ImageJ software.

Results: Curcumin successfully decreases VEGF expression with a mean in control group 88.61 ± 20.05 pixels; curcumin 100 µmol/L 52.64 ± 2.74 pixels; curcumin 200 µmol/L 36.30 ± 2.74 pixels on HPF. The Tukey HSD posthoc test found significant decrease between control group and each treatment group ($p=0.000$, $p=0.000$, and $p=0.004$; respectively)

Conclusions: In conclusion, Curcumin has a potential effect on reducing VEGF expression in human pterygium fibroblast.

Keywords: Vascular Endothelial Growth Factor, VEGF, Human Pterygium Fibroblast

1. Introduction

A pterygium is a wing-shaped growth of conjunctival and fibrovascular tissue that extends to the surface of the cornea. Pterygium is one of the eye diseases that often occur in tropical and subtropical areas that are exposed to a lot of sunlight. The pathogenesis of pterygium itself is currently not clearly understood, but it is highly correlated with exposure to ultraviolet light, outdoor activities, environmental exposure to dust, windy weather, or other infections on the surface of the eyeball that can cause chronic inflammation. (Cantor et al., 2019; Cantu et al., 2014; Veena et al., 2013)

Pterygium can be affected by exposure to ultraviolet, which makes in higher cell DNA damage resulting in inflammation and proliferation of fibrovascular tissue. Several growth factors influence the formation of pterygium, one of them is Vascular Endothelial Growth Factor (VEGF) and basic fibroblast growth factor (bFGF). VEGF is present in pterygium fibroblast cells. (Lu et al., 2017; Cantu et al., 2014; Girolamo et al., 2004)

Recurrence is one of the complications of pterygium treatment that must be considered. In recent studies, there is another alternative to prevent the recurrence of pterygium, namely curcumin. Curcumin (*Curcuma longa*) has a turmeric component, belonging to the Zingiberaceae family which has long been used as a kitchen

ingredient, cosmetic and medicinal ingredient. Curcumin has potential as an alternative adjuvant therapy as a promising treatment for pterygium recurrence in the future, this is because curcumin has antioxidant, anti-inflammatory wound-healing effects, inhibits angiogenesis (anti-VEGF), and induces apoptosis of pterygium fibroblast cells. (Lu et al., 2017; Sancilio et al., 2017; Fu et al., 2015)

This study aims to analyze the effect of curcumin in the reduction of VEGF expression compared to the control group. The results of this study are expected to be an alternative adjuvant therapy and reduce the recurrence rate of the occurrence of postoperative pterygium.

2. Material and Method

The primary reagent included secondary antibody VEGF Mouse Monoclonal Antibody Santa Cruz Biotechnology, Inc. Dulbecco's eagle medium (DMEM) (GIBCO – Life Technologies, USA). Trypsin-EDTA (GIBCO). Fetal Bovine Serum (GIBCO – Life Technologies, USA). Curcumin (Sigma Aldrich).

Study Design

This is an in vitro study using human pterygium fibroblast, conducted at Biomedical Laboratory Brawijaya University and Rumah Sakit Mata Masyarakat Surabaya. All experiments conformed to the local ethics review board, Health Research Ethics Committee, Universitas Airlangga School of Medicine, Surabaya Indonesia. Pterygium fibroblast cells were cultured on growth media and divided into three groups, the control group, and treatment group of curcumin 100 $\mu\text{mol/L}$ and 200 $\mu\text{mol/L}$.

Isolated of HPF

Human Pterygium Fibroblast was isolated from a patient with grade 3-4 pterygium aged 46 years old without ocular abnormality. Tissue was placed in 100 mm culture petri containing 1 mL Dulbecco's Modified Eagle's Medium (DMEM) that consist 15% Fetal Bovine Serum (FBS), dan Penicillin Streptomycin, stored in an incubator 37°C, 95% humidity and 5% CO₂ was stored for 48 hours. Culture media were changed every day prior to 90% confluency, which were sub cultured by warm trypsinization techniques. This method was based and modified on the protocol developed by Lu et al 2017.

Characterization of HPF

Cells were characterized with diamidinophenylindole (DAPI) and VEGF antibody. Vimentin was applied to identify fibroblast cells.

Curcumin preparation

Curcumin (Sigma Aldrich®) was dissolved at 0.05% DMSO to make broth solutions at concentrations 100, and 200 $\mu\text{mol/L}$ stored at 2-8 OC. Complete media DMEM was added to dilute the curcumin to the desired concentration then sonicated and filtered. (Lu et al 2017)

VEGF expression assessment

Cells that are confluent (P3-P7) and have been treated with curcumin, and VEGF expression was assessed after 48 hours then analyzed with a fluorescence microscope at 400x magnification. Expression levels were analyzed using ImageJ software and expressed in corrected total cell fluorescence (CTCF) determined using the formula: Integrated Density - (Area of selected cell x Mean fluorescence of background readings).

Statistical analysis

Comparative data between VEGF expression with curcumin intervention were tested using ANOVA if the data were normally distributed and continue with Tukey HSD post hoc. All descriptive data of the HPF criteria were presented as $\pm$ standard deviation. The p-value is considered significant if the p-value is < 0.05 . The data was analyzed using SPSS version 26.0 where $P < 0.05$ was considered to be statistically significant.

Results

HPF Isolation and characterization

This study managed to isolated HPF cultured and produced sprouting cells on day 10 and successfully at 90% confluency in 14 days. Morphology cell were analyzed using Inverted Fluorescence Microscope. Fibroblast cells was showed positive on vimentin.

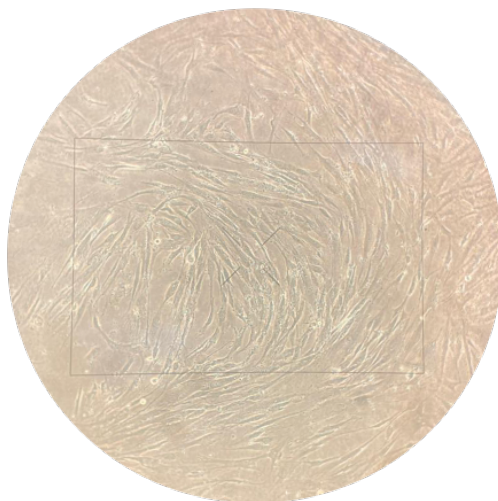


Fig. 1 Primary culture on pterygium tissue showed fibroblast growth with 90-100% confluency rate on day 14 Inverted Fluorescence Microscope, magnification 100x

Effect of curcumin on VEGF expression

The attenuation of curcumin to VEGF expression was observed in all treatment group with curcumin concentrations 100, and 200 $\mu\text{mol/L}$. Control group mean 88.61 ± 20.05 pixel, curcumin 100 $\mu\text{mol/L}$ 52.64 ± 2.74 pixel and curcumin 200 $\mu\text{mol/L}$ 36.30 ± 1.10 pixel (table 1). ANOVA test showed a significant difference in VEGF expression between each groups ($p=0.000$, $p=0.000$, and $p=0.004$; respectively). The highest mean VEGF expression was found in the control group and the lowest mean was in the curcumin group 200 $\mu\text{mol/L}$ followed with curcumin group 100 $\mu\text{mol/L}$. Immunofluorescence staining was analysed with fluorescence microscope 400x magnification. (Fig. 2)

Table 1. VEGF Expression

Group	N	Mean (pixel)	SD (Pixel)	P (One-way ANOVA)
Control	12	88.61	20.05	0.000
Curcumin 100 µmol/L	12	52.64	2.74	
Curcumin 200 µmol/L	12	36.30	1.10	

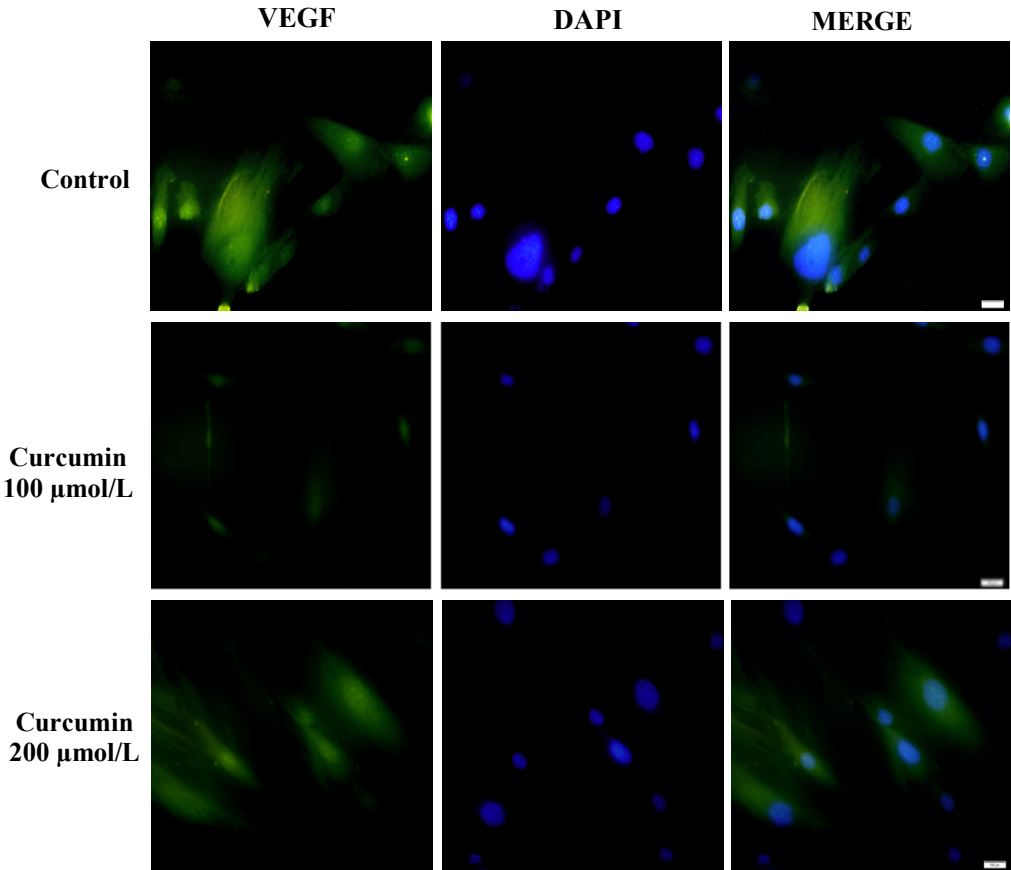


Fig 2. HPF with immunofluorescence staining with VEGF antibody for VEGF expression in control group, curcumin 100 µmol/L and 200 µmol/L (microscope 400x magnification)

4. Discussion

This study isolated fibroblast cells from pterygium tissue of patients with grades 3-4 pterygium aged 46 years, male, with no other abnormalities on ocular surface and had never undergone intraocular surgery. In

this study, our sample criteria of patient aged 46 years and never undergo intraocular surgery or pterygium excision before, this is in line with research by Singh et al, 2015, which showed that the risk of recurrence is higher in patients under 40 years of age. This could be caused by growth factors in patients aged < 40 years which play a greater role than growth factors in patients > 50 years. In a study by Khalfauoui et al., 2011 and Shahraki et al., 2021, it was shown that there was an overexpression of VEGF in recurrent pterygium patients with younger patients ages so that younger patients have higher risk of recurrence. (Shahraki et al., 2021; Singh et al, 2015; Khalfauoui et al, 2011)

HPF cells that have been confluent were characterized using vimentin antibodies, in this study positive vimentin results were obtained in pterygium fibroblast cells. This is in line with research by Zhang et al., 2007 that characterization of fibroblast cells in pterygium showed positive results against vimentin antibodies. In addition, a study by Komaratih et al., 2018 Characterization of Tenon's fibroblast cells also used vimentin antibodies and obtained positive results. (Komaratih et al., 2018; Zhang et al., 2007)

Curcumin is a yellow polyphenol isolated from *Curcuma longa*, which belongs to the Zingiberaceae family. *Curcuma longa* is found in tropical and subtropical regions and is widely used in developing and developed countries as a therapy in medicine. Curcumin has antioxidant, anti-inflammatory, anti-angiogenic, and wound healing effects. Curcumin also provides anti-tumor effects mediated by various mechanisms, both in vitro and in vivo. Curcumin can inhibit the formation of angiogenesis by reducing the overexpression of VEGF and COX-2 as the formation of new cell tissue. Based on research by Bian et al., 2001 Curcumin has the function of inhibiting angiogenesis by downregulating VEGF in corneal disorders, one of which is pterygium and diabetic retinopathy. (Lesnieska et al., 2019; Wang et al., 2019; Lu et al., 2017; Sancilio et al., 2017; Araujo et al., 2001)

In this study, the effect of curcumin studied was the effect in inhibiting VEGF expression in fibroblast cells that had been given intervention of 100 $\mu\text{mol/L}$ and 200 $\mu\text{mol/L}$ and seen the effect of decreasing expression within 48 hours, then Immunofluorescence staining performed using VEGF antibody. VEGF expression with curcumin 200 $\mu\text{mol/L}$ was shown to cause greatest reduction in VEGF expression, followed by 100 $\mu\text{mol/L}$, and control group. Curcumin 200 and 100 $\mu\text{mol/L}$ was found to reduce the fibroblast cells significantly compare to control group. This is in line with research by Zhang et al., 2007 that in vitro, curcumin at a dose of 20 mol/L has an inhibitory effect on cell proliferation where cell proliferation plays an important role in the formation of VEGF, while the dose of curcumin is 20-160 mol/L at 24-72 hours has a dose- and time-dependent inhibitory effect. While in a study by Lu et al., 2017 in vitro, curcumin can inhibit the formation of VEGF depending on the dose and the concentration of 80 mol/L has a strong inhibitory potential effect. (Lu et al., 2017; Zhang et al., 2007)

5. Conclusion

In conclusion, Curcumin significantly reduced the expression of VEGF in Human Pterygium Fibroblast, and curcumin was dose dependent. Due to its effect on VEGF expression reduction in human pterygium fibroblasts, curcumin could be a potential adjuvant therapy to reduce recurrence rates following pterygium excision.

6. Acknowledgements

None

7. Financial Support

This research was financially supported by the Ministry of Research and Higher Education, Indonesia

8. Conflict of Interest

Nil

9. Ethical Satandard

Ethical approval was obtained from Health Research Ethics Committee, Universitas Airlangga School of Medicine, Surabaya Indonesia. (No: 133/EC/KEPK/FKUA/2021). All Procedures were performed with ethical standards.

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