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RESEARCH ARTICLE

Matrix Metalloproteinase-3 Down Regulation and Cell Migration Inhibition in Human Pterygium Fibroblasts by Mitomycin-C, Curcumin and Fibrin Glue

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Abstract

BACKGROUND: Pterygium is an ocular surface disease that often occurs in tropical countries with a high recurrence rate. Matrix metalloproteinase-3 (MMP-3) play a key role in the inflammatory process of pterygium. This study aims to investigate the ability of curcumin and fibrin glue (FG) in suppressing the expression of MMP-3, and whether can be expected as adjuvant therapy to reduce pterygium recurrence.

METHODS: Human pterygium fibroblasts (HPF) obtained from primary cultured of pterygium were treated with no treatment, curcumin, mitomycin-C (MMC), and FG. MMP-3 expression was analyzed using immunocytochemistry and the intensity measurement was done using ImageJ software. Cell migration was measured by scratching and stratification of fibroblast culture after cell confluence, and assessed for 48 hours.

RESULTS: The expression of MMP-3 were lower in the HPF treated with 100 mol/mL curcumin, 200 mol/mL, and FG (2205.84±86.1 pg/mL, 1002.51±25.22 pg/mL, 1131.55±17.71 pg/mL, respectively) in comparison with untreated HPF (4703.49±108.9 pg/mL). The expression of MMP-3 were significantly different between groups ($p<0.001$). Cell migration of HPF after scratching with curcumin intervention at 200 mol/mL decrease from 178.67±2.85 (24 hours) to 88.83±1.48 (48 hours). Meanwhile the migration in FG group also decrease from 180.4±2.56 (24 hours) to 72.45±1.25 (48 hours).

CONCLUSION: Curcumin and FG able to reduce the expression of MMP-3 and inhibit the migration of HPF cells.

KEYWORDS: curcumin, mitomycin C, fibrin glue, human pterygium fibroblast, MMP-3

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Introduction

Pterygium is a degenerative and inflammatory disease of the conjunctiva with the clinical appearance of triangular fibrovascular tissue on the nasal side of the conjunctiva leading to the limbus. Risk factors associated with the development of pterygium include UV exposure, viral infections such as human papillomavirus, herpes simplex virus, and cytomegalovirus.(1-3)

The prevalence of pterygium around the world is quite alarming (ranging from 7% to 15%) and the term band pterygium is known for the region with the highest prevalence of pterygium across the equator. According to the 2010 Indonesian Basic Health Study, among the prevalence of pterygium among all detected eye disease cases in Indonesia reached 3.2% in both eyes and 1.9% in one eye.(1,3)

The relatively high recurrence rate after pterygium resection is another issue that clinicians must face. The

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incidence of postoperative recurrence varied from 3.8% to 89%. The pathophysiology of recurrence is not fully understood, but the most likely causes are genetic factors, repeated exposure to risk factors, and surgical technique. Bare sclera technique was the first to be adopted and is still quite high in practice in Indonesia as a modality for the treatment of pterygium, but it is positively associated with a high recurrence rate (up to 24-89%). Treatment modification by using conjunctiva autografts or by adding adjuvant therapy, such as the antimetabolites 5-fluorouracil (5-FU) or mitomycin-C (MMC), as antimitotic and antiapoptotic are often done by clinical practitioners. The use of antimetabolites was successful in reducing the recurrence rate of pterygium, but some serious complications including corneal edema, corneal perforation, or scleral calcification have became new obstacles.(1,3,4)

Recent studies have shown that herbs and spices have a significant attention as natural antioxidants. (5,6) Curcumin, which is a polyphenol derivative from turmeric, has pharmacological properties as antioxidants, antiapoptotic and antiproliferative properties. Curcumin has been found to have anti-inflammatory action through inhibition of nuclear factor kappa-B (NF-κB), antioxidant action through inhibition of free radicals, suppression of lipid peroxidation, increase of antioxidant molecules in tissues and stimulation of the activity of antioxidant enzymes. Curcumin is able to inhibit the expression of matrix metalloproteinases (MMP), especially MMP-3, which is expressed by human pterygium fibroblasts (HPF), as well as the suppression effect of reactive oxidative species (ROS) of fibroblasts due to UV exposure, making curcumin a potential adjuvant therapy for the prevention of pterygium recurrence in the future.(7-9)

Fibrin glue (FG) is a blood-derived product that is used as a biological adhesive and was first introduced in 1909. FG acts by imitating the final step of the coagulation cascade when soluble fibrinogen is activated by thrombin. In the field of ophthalmology, it is an alternative therapy option that is often used in pterygium cases to prevent postoperative recurrence, accelerate wound healing by releasing anti-inflammatory cytokines. FG forms a seal on the wound after pterygium excision to accelerate the wound healing process, minimize recurrence, and prevent more postoperative complications.(10,11)

In this study, HPF cell cultures were treated with curcumin and FG *in vitro* to evaluate their ability in suppressing the expression of MMP-3 and fibroblast migration, and whether can be expected as adjuvant therapy to reduce pterygium recurrence.

Methods

Preparation of Curcumin and FG

Curcumin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved with complete media Dulbecco's Modified Eagle's Medium (DMEM) then sonicated and filtered to create stock solutions at the concentrations of 100 and 200 mol/L.

FG was derived from graded centrifugation of peripheral blood with anticoagulants. A total of 40 mL of peripheral blood was taken from the cubital vein using a sterile syringe containing CPDA in a ratio of 9:1, aseptically. Blood was put into a sterile 10 mL centrifuge tube then centrifuged at 3000 rpm for 15 minutes. The plasma was stored in a sterile centrifuge tube and stored at -20°C for 24 hours, then centrifuged at 4°C, 3000 rpm for 15 minutes. Ten mL of the upper 2/3 of plasma was stored for the preparation of the fibrinogen component, and the platelet-rich plasma (PRP) was stored in sterile micro-tubes to be prepared as material for thrombin production. The plasma was then added with 1 mL ethanol, then incubated at 4°C for 30 minutes followed by centrifugation at 4°C at 3000 rpm for 15 minutes. The supernatant was discarded and the sediment was used as a component of fibrinogen. Thrombin was made by mixing the PRP component with 0.05 mL of 10% $CaCl_2$. FG was made by mixing the fibrinogen and thrombin in a ratio of 1:1.(12,13)

Sample Collection

Pterygium tissue was taken in the surgical installation of the Rumah Sakit Mata Masyarakat Jawa Timur. The tissue then transported directly to Biomedical Laboratory of Universitas Brawijaya for further *in vitro* evaluation and analysis. The protocol of this study was approved by Health Research Ethics Committee, Faculty of Medicine Universitas Airlangga (No: 131/EC/KEPK/FKUA/2021).

Culture and Passage of HPF

Local anesthesia with subconjunctival injection of 2% lidocaine was performed before removal of the pterygium tissue. A sterile cloth was placed on the operating area and the eyeball was disinfected using 5% povidone iodine and then rinsed with balance salt solution. Pterygium tissue retrieval was carried out with bare sclera technique according to the protocol developed by previous study (Figure 1).(7)

After the pterygium tissue was removed, the top of the specimen was taken about 2 mm from the edge and then cut into small pieces (<0.5 mm^2). After being washed

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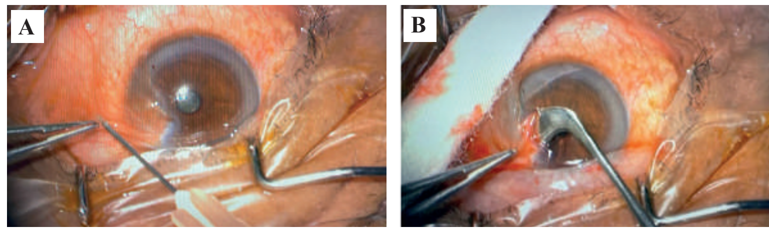

Figure 1. Pterygium excision technique with bare sclera. A: administration of lidocaine 2% before tissue excision; B: pterygium excision using a pterygium spoon.

using phosphate buffer saline (PBS), then the specimen was given red blood cell lysis buffer for 5 minutes and placed on a 100 mm culture dish. One mL of DMEM containing 15% fetal bovine serum (FBS), and penicillin-streptomycin were added to cover the tissue then placed overnight in an incubator at 37°C, and humidified. The medium was replaced daily with fresh culture medium + FBS. When the cells began to reach 80% confluency, FBS was reduced to 10% concentration and then sub-passed. P3-7 cultures were used in this study. Figure 2 showed the primary culture on pterygium tissue.

Cell's Characteristics and Vimentin Staining

The confluent cells were transferred to 24-well plate with a density of 2.5x10^4 cells/plate and then waited until the cells were adhered to the bottom of the plate, then washed 3 times with PBS for 5 minutes. Cells were washed 3 times with PBS for 5 minutes and with 0.1% PBS Triton-X 100 for 1 minute before incubated with 1% bovine serum albumin (BSA) for 30 minutes. The BSA solution was then discarded and incubated with primary antibody overnight. Cells were incubated with Goat Anti Rabbit IgG & LTIRTCVimentin (Cat No. ab6718, BD Biosciences, San Jose, CA, USA) and incubated with DAPI 1: 1000 for 5 minutes. Cells were then photographed with a fluorescence microscope at 40x magnification.

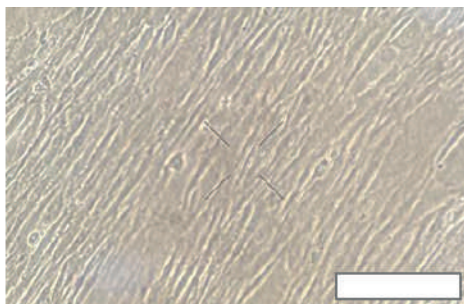

Figure 2. Primary culture on pterygium tissue. Fibroblast growth reached a confluence rate of 90-100% on day 14. White bar : 100 µm.

Measurement of MMP-3 by Immunofluorescence Staining

Cells that were already confluent were harvested and planted on 24 well plate with a density of 2.5x10^4 for 24-48 hours. Then the wells were grouped into untreated, treated with MMC 0.2 and 0.4 mg/mL, treated with curcumin 100 and 200 mol/mL, and treated with FG. Untreated wells only contained cells and medium; MMC 0.2 and 0.4 mg/mL were given for 5 minutes to cells with medium, then washed once with PBS; curcumin was given at doses of 100 and 200 mol/mL to cells with medium; and FG was given until clots formed, then removed and washed once with PBS. Each well was then added 3-4% formaldehyde for 15 minutes and washed with PBS.

Every well were washed again with 0.1% of PBS Triton-X 100 (Cat No. T8787, Sigma Aldrich) for 5 minutes and incubated with 1% BSA for 30 minutes at room temperature. The fluid was discarded and then incubated with primary antibody MMP-3 Rabbit Polyclonal Antibody (Cat No. bs-0431R, Bioss Antibody, Woburn, MA, USA) overnight at 4°C. Antibody was washed with PBS, incubated with MMP-3 secondary antibody 1:1000 for 1 hour. Results were read using a fluorescence microscope, and the expression levels were analyzed using ImageJ software version 1.51 (National Institutes of Health, Bethesda, MA, USA). The MMP-3 expression level was counted as corrected total cell fluorescence (CTCF), determined using the formula: integrated density - (area of selected cells x mean fluorescence of background values).(2)

Cell Migration and *in vitro* Scratch Wound Assay

Confluent pterygium fibroblasts were implanted on 24-well plate with a density of 5x10^4 cells per well and incubated for 24-48 hours to achieve 70-80% confluence. The wells were grouped into untreated, treated with 0.4 mg/mL MMC, treated with 200 mol/mL curcumin, and treated with FG.

The cells that had been given the intervention were then scratched perpendicular to the plate using a 200 µL micropipette and photographed using an Olympus

CKX53 microscope (Olympus Corporation, Tokyo, Japan). Migration distance was measured with imageJ software version 1.51 (National Institutes of Health).

Statistical Analysis

Comparative data between inhibition of fibroblast migration with the interventions and comparison of MMP-3 expression were tested using the one-way Anova test. Tukey Post-Hoc test was carried out to determine the significant difference between one group and another. The *p*-value is considered significant if $p<0.05$. All statistical data was processed using SPSS 26.0 software (IBM Corporation, Armonk, NY, USA).

Results

Expression of MMP-3 After the Intervention of MMC, Curcumin, and FG

The results of analysis showed that the highest expression of MMP-3 was found in the untreated group, and the lowest was in the 0.4 mg/mL MMC group. Groups treated with curcumin showed lower MMP-3 expression compare to the untreated group as the negative control, where 200 mol/mL curcumin group showed lower MMP-3 level than 100 mol/mL curcumin group (Table 1). This showed that 200 mol/mL curcumin had a stronger MMP-3 suppression effect that is quite comparable with FG (1002.51±25.22 *vs.* 1131.55±17.71).

One-Way Anova test followed by Post-Hoc Tukey analysis showed that the comparison of all treatment groups was $p<0.001$, which suggested that the comparison of MMP-3 expression in all groups was statistically significant. Figure 3 showed pterygium fibroblast cells were stained with MMP-3 antibody and DAPI to assess the comparasion of MMP-3 expression in all treatment groups. MMP-3 was obviously seen in untreated group, meanwhile was found less in 0.4 mg/mL MMC group.

Inhibition of Fibroblast After the Intervention of MMC, Curcumin, and FG

The migration distance showed that the fastest migration happened in the untreated group, while the slowest migration happened in the 0.4 mg/mL group after 48 hours of intervention (Table 2). The 200 mol/mL curcumin and FG groups had less significant differences after 24 hours of intervention. The group that was given 200 mol/mL curcumin had slower migration inhibition after 48 hours when compared to FG group (figure 4).

Statistical test using One-Way Anova followed by Post-Hoc Tukey showed a significant comparison ($p<0.05$) in all intervention groups. This suggested that the comparison of HPF migration rate after stratching with and without intervention were statistically significant.

Discussion

Pterygium additionally exhibits several tumor-like features including invasion, metaplasia of epithelial cells, presence of oncogenic viruses, inactivation of tumor suppressor genes, and absence of heterozygosity. The pathogenesis of pterygium and its recurrence after excision is still not completely understood, identification of effective drugs that able to suppress the proliferative system of pterygium is urgently required.(2,14)

MMPs have crucial function in pterygium formation because of the excessive fibroblast proliferation and invasion happens at the apex of the pterygium with destruction of the corneal stroma and basal lamina. Pterygium cells will produce MMP which dissolves the basal lamina and triggers the growth of stromal fibroblasts.(2,14) Overexpression of MMP on basal limbal epithelial cells is the primary reason of pterygium development. Extracellular matrix (ECM) modulation through MMP occurs in the early phase of pterygium invasion, numerous studies have proven a sturdy affiliation among MMP and tumor development/

Table 1. MMP-3 expression in human pterygium fibroblasts after intervention.

Group	Mean±SD	Minimum	Maximum	*p*-value
Untreated	4703.49±108.98	4532.22	4892.56	
0.2 mg/mL MMC	593.99±24.4	546.72	641.32	
0.4 mg/mL MMC	270.60±15.88	245.41	288.32	<0.001
100 mol/mL Curcumin	2205.84±86.1	2072.31	2303.21	
200 mol/mL Curcumin	1002.51±25.22	973.42	1034.45	
FG	1131.55±17.71	1113.56	1152.36	

p-value tested with One-Way Anova.

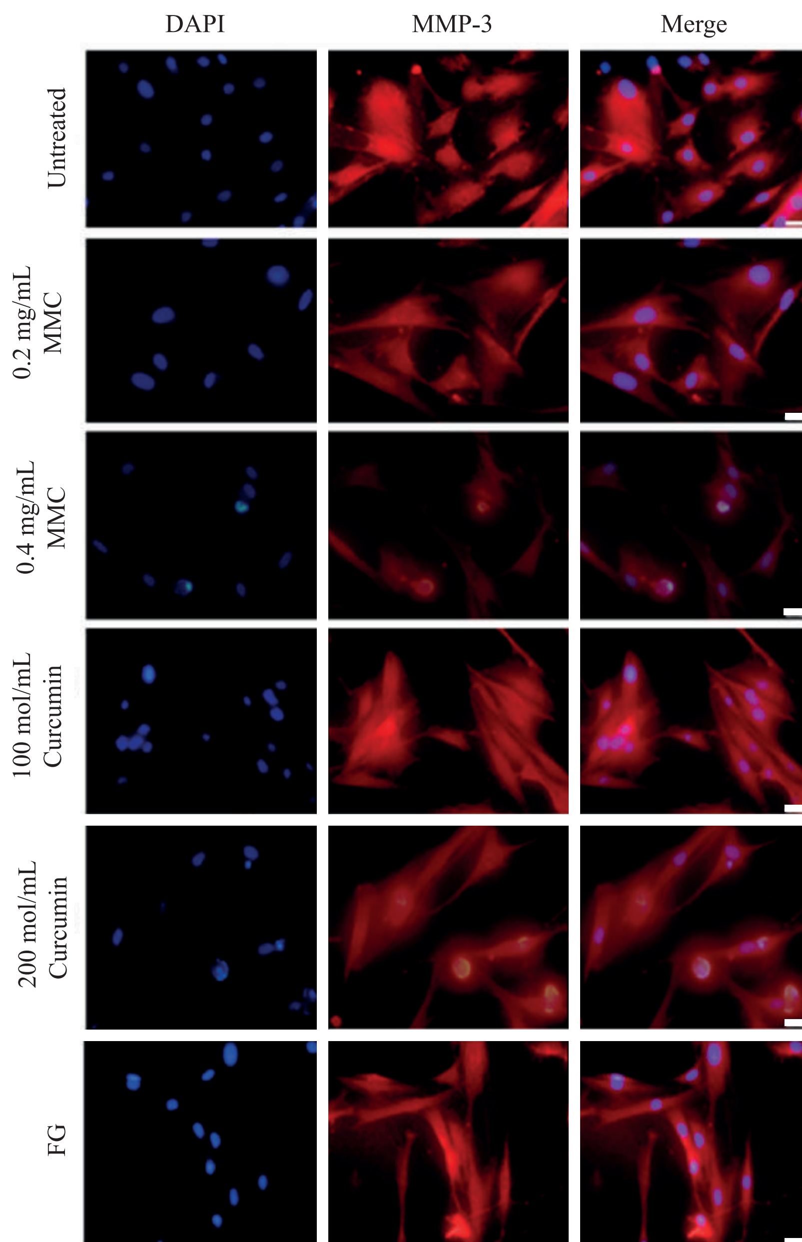

Figure 3. Pterygium fibroblast cells stained with MMP-3 antibody and DAPI. Observed with inverted fluorescence microscope; White bar: 30 µm.

invasion. Invasion of pterygium lesions will growth MMP activity.(15-19).

Pterygium will result in activation of fibroblast tissue, specifically on the top of the pterygium, ensuing in cleavage of fibrillar collagen within the basal lamina, particularly because of MMP-1 and MMP-3. Degradation of the ECM results in the release of stimulating cytokines which includes vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) that cause angiogenesis and MMP-3 has a major effect on angiogenesis because it triggers angiostatin. MMP-3 additionally has large outcomes due to the fact it may activate pro-MMPs such as MMP-1, MMP-9 and MMP-13 so that they play a role in cell migration, proteolysis and angiogenesis.(2,14,19) In this study, it was proven that the highest MMP-3 expression was found in the untreated group, while the lowest expression was found in the 0.4 mg/mL MMC group, followed by the 0.2 mg/mL MMC group. The curcumin intervention also had a suppressive effect on MMP-3 that was more than 50% while compared to the untreated group, especially higher in the 200 mol/mL curcumin group. The suppressive impact of FG was quite similar to of curcumin, so it may be suggested that in this study curcumin and FG have been able to exert a suppressive effect on the expression of MMP-3 in pterygium fibroblast tissue.

Curcumin has been extensively studied as an antifibrosis agent. Previous *in vitro* and *in vivo* study showed curcumin is able to inhibit liver fibrosis and prevent liver

Table 2. Migration distance of human pterygium fibroblast cells after scratching within 48 hours with intervention.

Group	Treatment Period	Mean±SD	Minimum	Maximum	*p*-value
Untreated	0H	221.33±3.57	216.23	225.34	0.000
	24H	141.59±3.12	137.32	147.22	0.003
	48H	32.46±1.25	30.28	34.35	0.001
0.4 mg/mL MMC	0H	233.60±2.87	230.38	237.32	0.000
	24H	210.34±2.57	206.52	215.08	0.000
	48H	132.05±1.65	129.88	135.06	0.002
200 mol/mL Curcumin	0H	228.89±3.75	222.99	233.87	0.000
	24H	178.67±2.85	174.34	183.21	0.001
	48H	88.83±1.48	85.28	91.21	0.001
FG	0H	231.62±3.17	227.16	235.88	0.000
	24H	180.40±2.56	175.32	183.52	0.001
	48H	72.45±1.25	70.77	74.67	0.002

p-value tested with One-Way Anova.

disease progression. Curcumin dampen the inflammation and reduce fibrosis through inhibition of the transforming growth factor (TGF)-β1/Sma and drosophila MAD (Smad) signaling pathway.(19) Other studies reporting the use of curcumin on skin fibroblast cells to prevent photoaging showed that ROS are the basis for overexpression of UVB-modulated MMP-3. Curcumin has a robust antioxidant effect that may suppress ROS by means of suppressing the effects of NF-κB and AP-1 DNA binding activity. UVB induces phosphorylation of the janus kinase pathway and p38 and curcumin has been shown to regulate the activation of NF-κB and AP-1 through the MAPK signaling pathway.(8,20)

The migration rate showed that the untreated group had the fastest migration, while the slowest migration rate was found in the of 0.4 mg/mL MMC group after 48 hours. The curcumin and FG groups had much less substantial differences at 24 hours and 200 mol/L curcumin

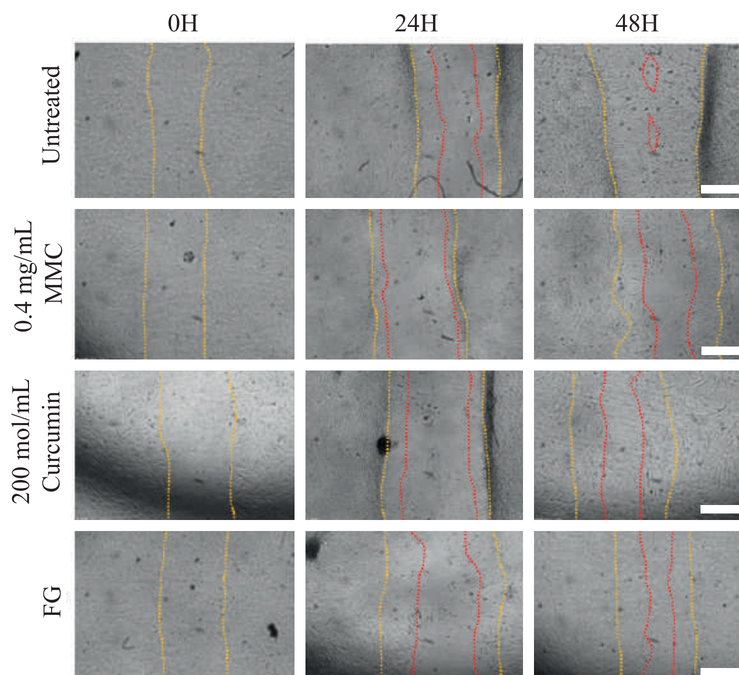

Figure 4. Fibroblast migration distance after scratching analysis. Comparison of pterygium fibroblast cells were scanned to assess cell migration at 0, 24 and 48 hours. Observed with inverted fluorescence microscope; Yellow line: the scratch border; Red line: as fibroblast migration; White bar: 100 µm.

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group had slower migration inhibition after 48 hours when compared to fibrin glue. This indicates that the comparison of the HPF migration rate with and without intervention is statistically significant. The scratching method performed in this study is an easy and effective step to trigger inflammation.(21) This is in accordance with the outcomes of previous study of fibroblast cells in the tenon conjunctival tissue model that was scratched with the conclusion the less the rate of migration of tenon's fibroblast cells to the wound place, the decrease the opportunity of extracellular matrix manufacturing resulting in fibrosis in that place.(12,22)

Another study confirmed the relationship among MMP expression and activity on pterygium fibroblast migration and its suppressive effect on Bevacizumab and cyclosporine A (CsA). The fibroblast migration ratio was assessed to investigate the recovery migration process which confirmed the migration speed suppression of MMP-3 and MMP-13 after bevacizumab and/or CsA intervention.(12)

An *in vitro* trial on cardiac fibroblasts and stated that curcumin had a higher inhibitory impact on fibroblast migration when compared to the control group and the MTT assay showed that the curcumin group also had a decrease proliferative effect than the control group, which is in line with the study.(23) This indicates that the rate of migration in the curcumin group might be slower when compared to the control group. This occurs most probably due to the suppressing effect of the mediators including TGF-β, MMP and other profibrotic mediators via the NF-κB through tumor necrosis factor (TNF)-α dependent induction and mitogen-activated protein kinase (MAPK) signaling pathway.(20,23,24)

FG significantly inhibits collagen synthesis and induces collagen degradation in human tenons, in which the wound healing manner is regulated by inflammation. A decrease in the amount of collagen will eliminate the anti-inflammatory response and fibrin glue substantially inhibits collagen in the injured tenon model and is able to reduce wound formation and contractures.(24,25) In line with this study, which showed that in addition to reduce MMP-3 expression, FG was also able to reduce the speed of HPF migration although it was not as significant as the standard for adjuvant pterygium treatment, particularly MMC. Current study is the first study to analyze the inhibition of MMP-3 expression and HPF migration after the intervention of curcumin and FG. However, additional learning about other wound healing mechanisms is necessary to fully define and understand their effects.

Conclusion

Our findings suggest that curcumin and FG are able to suppress pterygium proliferation by inhibiting HPF migration and suppressing MMP-3 expression. Therefore, FG and curcumin have the potential to be an adjuvant therapy for MMC replacement in the management of pterygium.

Acknowledgments

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Authors Contribution

FR, LI and EK were involved in concepting and planning the research; FR and LI performed the data acquisition/ collection; JA interpretated the result of data and designed the figures and table; EF and IJ calculated the experimental data and performed the analysis; FR, IZ and EK drafted the manuscript and took parts in giving critical revision of the manuscript; FR and EK also collected the research funding.

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