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ORIGINAL ARTICLE

Mitomycin C, curcumin, and fibrin glue inhibit the cell proliferation and expression of TGF- β in human pterygium fibroblast (<https://www.balimedicaljournal.org/index.php/bmj/article/view/3315>)

Muhammad Abdurrauf, Ferdian Ramadhan, Nurwasis, Ismi Zuhria, Betty Agustina Tambunan, Hari Basuki Notobroto, Budi Surahman, Evelyn Komarath

Online First: Apr 14, 2022 |



Abstract

pdf (<https://www.balimedicaljournal.org/index.php/bmj/article/view/3315/2016>)

ORIGINAL ARTICLE

The clinical pictures of COVID-19 pediatric patients in dr. R. Soedarsono Regional General Hospital, Pasuruan, East Java, Indonesia (<https://www.balimedicaljournal.org/index.php/bmj/article/view/3046>)

Peter Prayogo Hsieh, Hans Kristian, Allison Joseasta Marsya Permana, Monique Wongsodharjo, Pramita Anindya Nugraheni, Pherenice Charisti, Wenta Darsvitri

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Abstract

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ORIGINAL ARTICLE

The efficacy of probiotics supplementation on the lipid profiles of obese adolescents : a randomized trial (<https://www.balimedicaljournal.org/index.php/bmj/article/view/3163>)

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ORIGINAL ARTICLE

Hematology profile of under five years old children suffered from acute diarrhea at Idaman Banjarbaru Hospital, Indonesia (<https://www.balimedicaljournal.org/index.php/bmj/article/view/2668>)

Harapan Parindungan Ringoringo, Jun Rahmawati Surya Mentari, Roselina Panghiyangan, Edi Hartoyo, Rahmiati Lao

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Mitomycin C, curcumin, and fibrin glue inhibit the cell proliferation and expression of TGF- β in human pterygium fibroblast



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ABSTRACT

Introduction: High recurrence of a fibrotic disease, pterygium, following the surgical procedure is perceived as the primary challenge of its management. As the standard procedure, adjuvant therapy of using mitomycin C could dramatically reduce the recurrence of pterygium but could cause multiple serious complications. Our study aimed to investigate curcumin and fibrin glue as alternative candidates for adjuvant therapy in pterygium surgery.

Methods: Human pterygium fibroblast (HPF) was isolated from the patient and cultured *in-vitro*. The HPF culture was then exposed with mitomycin C (0.4 mg/mL), curcumin (200 μ mol/L), and fibrin glue, respectively, for 48 hours. The outcomes were determined by the proliferation of HPF and the expression of transforming growth factor-beta (TGF- β) which were obtained from 2,5-diphenyl-2H-tetrazolium bromide assay and immunofluorescence staining analysis with TGF- β antibody.

Results: The experiment revealed that mitomycin C, curcumin, and fibrin glue could significantly inhibit the proliferation of HPF ($p < 0.05$) suggesting their antifibrotic effect. Further analysis with immunofluorescence staining showed that mitomycin C, curcumin, and fibrin glue could significantly reduce the level of TGF- β as compared with control group ($p < 0.05$).

Conclusion: Mitomycin C was the most potent adjuvant agent to reduce the recurrence of pterygium, followed by curcumin and fibrin glue. Taken altogether, curcumin and fibrin glue have role as adjuvant therapy to prevent recurrence in pterygium surgery.

Keywords: Curcumin, Fibrin glue, Mitomycin C, Pterygium, TGF- β .

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INTRODUCTION

Pterygium is an uncontrolled fibrovascular tissue overgrowth of the conjunctiva which could cause repeated inflammation, vision reduction, and cosmetic problems.¹ Bare sclera was first proposed as the first pterygium surgical technique in the early 1900s, but this technique has a high pterygium recurrence rate (24–89%).² Clinicians have now developed new techniques to reduce the recurrence rate: conjunctival autografting (recurrence: 8.3–33%), amniotic membrane transplantation (3.8–40%), and the administration of antimetabolites.² Mitomycin C is the most widely employed antimetabolite which could reduce to the recurrence rate up to 7–9%.² Complications were found following the administration of mitomycin C, including cataract formation, uveitis anterior, scleral plaque

and necrosis, corneal edema and ulcer, and inflammation in anterior chamber depth corneal defect, and non-curable sclera.³ Therefore, researchers nowadays are seeking antimetabolite alternatives that could be an option for the adjuvant therapy.^{4,5}

The development of this fibrotic disease on the ocular surface is multifactorial, yet it is strongly associated with the expression of transforming growth factor-beta (TGF- β).⁶ Fibrosis could be induced by the stimulation of transcriptional regulation concomitant to the TGF- β -associated Smad complex formation.^{7,8} Other than that, TGF- β could induce the fibrosis via non-Smad pathway by activating Extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway which regulates the trans-differentiation of myofibroblast, proliferation, and cell survival.⁹ Moreover,

TGF- β is a pro-inflammatory factor which could increase the risk of pterygium growth.¹⁰ Therefore, The adjuvant therapy for pterygium should inhibit the proliferation of fibroblast and reduce the production of TGF- β .

A major bioactive compound from *Curcuma longa L.*, curcumin, is one of natural products with high tolerability and rich with medicinal benefits such as antioxidant, antiinflammation, antimutagen, and anticancer.^{11,12} In ophthalmic management, curcumin has been proposed as a therapy for glaucoma, cataract, age-related macular degeneration, diabetic retinopathy, and dry eye syndrome.¹³ More importantly, curcumin has been reported to possess antifibrotic and anti-TGF- β activities.¹⁴ The investigation was also carried out on a biological adhesive, fibrin glue, in which its usage in ophthalmology has

been introduced since early 1940s for corneal transplantation in rabbit model.¹⁵ Fibrin glue could reduce inflammation and improve proliferation, hence it has been used widely in ophthalmic surgery.¹⁵ Herein, higher curcumin concentration than previously reported and a comparative study among mitomycin C, curcumin, and fibrin glue in inhibiting HPF proliferation are the novelty of this research.¹³

METHODS

Study design and sample collection

This study used human pterygium fibroblast (HPF) isolated from the head of pterygium of a patient undergoing the excision procedure who had priorly signed the informed consent in Mata Masyarakat Hospital (RSMM), Surabaya, Indonesia. The patient was 50 years old, had never undergone other intraocular surgery, and had no other disorders on the eyeball surface. The pterygium fibroblast tissue should had reached cornea and exceeded the corneal limbus. The isolated HPF was then cultured and divided into four groups: control, curcumin, mitomycin C, and fibrin glue. Into each group, 12 HPF cultures were assigned. Contaminated HPF cultures were excluded or dropped out from the study. Group allocation was performed with randomization using Randomizer, an online web-based randomizer tool. Effects of curcumin, mitomycin C, and fibrin glue were determined based on the HPF proliferation and level of TGF- β .

Isolation and culture of HPF

The peak of the specimen (2 mm from the border) was collected from its tissue, cut into small pieces (1 mm³), washed with Hanks solution, and placed into a 100-mm culture dish. The sample was drop-wise with 1 mL Dulbecco's Modified Eagle's Medium (DMEM) containing fetal bovine serum (FBS) 15% and gentamicin 50 g/mL. Incubation of the sample was carried out overnight at 37°C with 95% humidity and 5% CO₂ content.

To confirm, the fibroblast subculture was separated from the epithelial cells by reducing the FBS concentration as much as 10%, allowing epithelial cells to be retained in the well. HPF morphology was then investigated on Olympus microscope

with fluorescence staining. Cultured cells in complete medium were grown up to 3–7 generations. Only stable cells were used in this study.

Preparation of curcumin

Curcumin was dissolved into 0.05% dimethyl sulfoxide (DMSO) to prepare the stock solution with 10 mmol/L concentration, stored at -200°C. DMEM was added to the stock solution to dilute curcumin into a desired concentration.

Preparation of fibrin glue

Peripheral blood (40 mL) was collected from cubital vein with venipuncture containing citrate-phosphate-dextrose-adenine (CPDA) with a ratio of 9:1. The blood was centrifuged at 3000 rpm for 15 minutes. The collected plasma was then stored in a sterilized tube (-20°C; 24 hours), and subsequently centrifuged again at 4°C and 3000 rpm for 15 minutes. Thereafter, 2/3 of the top part of the plasma (10 mL) was collected to prepare fibrinogen component. The other 1/3 part (the PRP) was stored in a sterilized microtube for thrombin preparation. The collected 2/3 part was added with 1 mL ethanol 95% and incubated at 4°C for 30 minutes. Centrifugation was performed once again at 4°C and 3000 rpm for 15 minutes. The supernatant was removed and the pellet was collected to prepare fibrinogen. Thrombin was prepared by mixing its PRP component with 0.05 mL CaCl₂ 10%. Fibrin glue was prepared by mixing the fibrinogen and thrombin with a ratio of 1:1. This procedure followed the previously published reports.^{16,17}

Exposures of curcumin, mitomycin C, and fibrin glue

Stable HPFs were grown in 96-wells plate with a cell density of 5x10³/mL with a 200 μ L/well volume until the confluence of 90% was reached. Confluent cells were separated into control, curcumin, mitomycin C, and fibrin glue groups. Curcumin group was exposed with curcumin 200 μ mol/L, mitomycin C group – mitomycin 0.4 mg/mL for 5 minutes, and fibrin glue group – fibrin glue for as long as it took until the fibrin formed. Control group was only grown in culture medium and did not receive any treatment.

Determination of HPF proliferation

HPF proliferation was measured at 0, 24, and 48 hours after the treatment. A solution of 2,5-diphenyl-2H-tetrazolium bromide (MTT) with a 1 mg/mL concentration was added into each well prior to 4-hours incubation. MTT solution was then aspirated and added with 100 μ L DMSO. Well plate was read under microplate spectrophotometer (BioTek Synergy H1; BioTek, Winooski, VT, USA) at 540 nm.

Determination of TGF- β

Level of TGF- β was measured concomitant to the 48-hours incubation after the treatment. Into each well, formaldehyde 3–4% was added and left for 5 minutes, followed by washing with PBS (2x). The level of TGF- β was observed using immunocytochemistry by sequentially wash the well plate with PBS (5 minutes; 2x), PBS Triton-X 100 0.1% (5 minutes; 1x), and bovine sodium albumin (BSA) 1% (30 minutes; 1 x). The solution was removed from the culture and incubated with primary antibody of TGF- β for 24 hours at 4°C. Thereafter, the antibody was washed with PBS for 5 minutes (3x), and followed by an incubation using secondary antibody of TGF- β Mouse Monoclonal Antibody (Santa Cruz Biotechnology Inc, TX, USA) 1:1000 for 30 minutes at room temperature. The well plate was then re-washed with PBS for 5 minutes for 3x and incubated with DAPI 1:1000 for 5 minutes before washed again with PBS for 5 minutes. Results of fluorescence microscope were obtained at 400x magnification. The expression level of expression of TGF- β was measured by taking a picture of the well with electron microscope, then analyzed using ImageJ 1.53e software and formulated as corrected total cell fluorescence (CTCF), obtained by: Integrated Density – (Area of selected cell x Mean fluorescence of background readings). This procedure followed the suggestion from a previous report.¹⁸

Statistical analysis

The data were presented descriptively in mean \pm standard deviation (SD). Normality of the data distribution was determined based on Shapiro-Wilk test. Normally distributed data were processed with a parametric one-way ANOVA

followed by post hoc Tukey test for the TGF- β expression and t-test with Welch's correction for the proliferation. All the statistical analysis was carried out on IBM SPSS 21.0 (Armonk Corporation, NY, USA).

RESULTS

Culture of the isolated HPF isolated

Images collected from the fluorescence staining of the cultured HPF collected from a patient are presented (Figure 1). Pterygium fibroblasts were isolated through an excision using hockey mesh, with protocol following the suggestion from a previous report.¹⁹ The cell was observed to generate sprouting cells on day 10, where the confluence had reached 90-100% on day 14. The fibroblast cells were confirmed by antibody targeting vimentin, the fibroblast marker.

Effect of mitomycin C, curcumin, and fibrin glue on the proliferation

Following the exposure of mitomycin C, curcumin, and fibrin glue on HPF, its proliferation was calculated based on MTT assay, where the data have been presented in Table 1. All groups were observed to experience an increase on cell density after 24- and 48-hours incubation, respectively.

Control group had the highest cell density among others within 24- and 48-hours observation. Mitomycin C had the lowest increase on cell density, especially on 24-hours post-exposure observation (0.411 ± 0.101), where the difference was very significance in comparison with control ($p<0.001$). After the incubation for another 48 hours, the inhibition persisted as the strongest (0.563 ± 0.207 ; $p=0.016$). Curcumin had the second-lowest cell density (0.588 ± 0.152) after mitomycin C on 24-hours post-exposure observation, where the difference is statistically significant as compared with control ($p=0.022$). Significant, yet the lowest proliferation inhibition, was found in fibrin glue group following the incubation for 48 hours (0.601 ± 0.123 ; $p=0.016$).

Effect of mitomycin C, curcumin, and fibrin glue on the TGF- β

Images from the immunofluorescence staining with TGF- β antibody on control, mitomycin C, curcumin, and fibrin glue groups have been presented (Figure 2). The expression of TGF- β post-48-hours exposure was presented in a form of corrected total cell fluorescence (CTCF), calculated using ImageJ 1.53e software.

The expressions of TGF- β obtained from above calculation in control,

mitomycin C, curcumin, and fibrin glue groups have been presented in Figure 3. The highest TGF- β expression was observed in control group with a value of 59.10 ± 9.38 pixel. The number was significantly lower in mitomycin C group (19.59 ± 8.93 pixel), followed by curcumin group (35.63 ± 6.34 pixel), and lastly by fibrin glue (46.55 ± 9.69 pixel). The P value obtained from the one-way ANOVA showed a statistical significance of difference among all groups ($p<0.001$). Tukey tests show that the TGF- β expressions between groups are significantly different at $p<0.05$.

DISCUSSION

The unstopped proliferation of fibroblast causes recurrence of pterygium during the remodeling phase attributed to the mutation at p53 gene.^{19,20} Hence, the mechanisms in cell proliferation are being targeted to prevent the recurrence of pterygium. Mitomycin C has been reported to possess antifibrotic effects against HPF.²¹ In this present study, mitomycin C was revealed resulting in significant inhibition of HPF proliferation. Unfortunately, mitomycin C could affect other cells and cause multiple complications (for example, the occurrence of scleral perforation).²² Herein, curcumin and fibrin glue were alternatively proposed as antifibrotic agents against the fibroblast, where they were shown to significantly reduce the proliferation.

Curcumin is a promising candidate with minimum safety concern. A previous report suggested that curcumin intake as much as 8000 mg/day for three months did not cause severe side effects to patients.¹² Curcumin itself has been reported to have the significant ability of inhibiting fibroblast proliferation as compared with

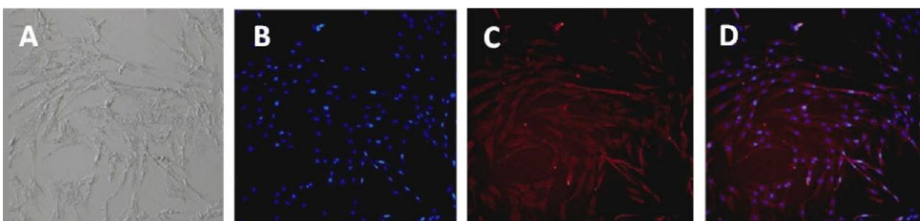


Figure 1. Fluorescence staining of human pterygium fibroblast (HPF) with contrast phase (B), diamidino-2-phenylindole (DAPI) (C), vimentin (D), and merge (E), observed under inverted fluorescence microscope with 200x magnification.

Table 1. Human pterygium fibroblast (HPF) proliferation observed in control, mitomycin C, curcumin, and fibrin glue after 0, 24, and 48 h exposure.

Group	Cell density, Mean \pm SD					
	0 h	p	24 h	p	48 h	p
Control	0.363 ± 0.006	NA	0.534 ± 0.112	NA	0.655 ± 0.008	NA
Mitomycin C	0.376 ± 0.127	0.456	0.411 ± 0.101	$<0.001^{**}$	0.563 ± 0.207	0.016^*
Curcumin	0.372 ± 0.004	0.239	0.469 ± 0.142	0.018^*	0.588 ± 0.152	0.022^*
Fibrin glue	0.367 ± 0.003	0.552	0.497 ± 0.007	0.043^*	0.601 ± 0.123	0.016^*

SD: standard deviation. NA: Not applicable. p -value was calculated from t -test with Welch's correction.

* Significant at $p<0.05$ as compared with control;

** significant at $p<0.001$ as compared with control

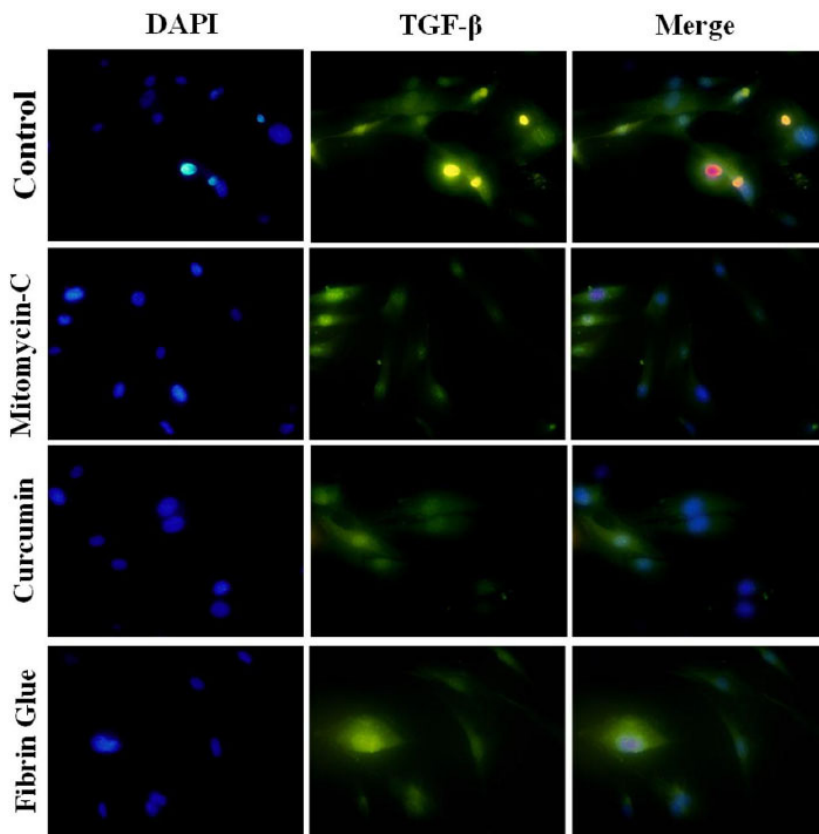


Figure 2. Immunofluorescence images of HPS in control, mitomycin-C, curcumin and fibrin glue groups in diaminido-2-phenylindole (DAPI), TGF- β , and merge staining observed under fluorescence microscope with 400x magnification.

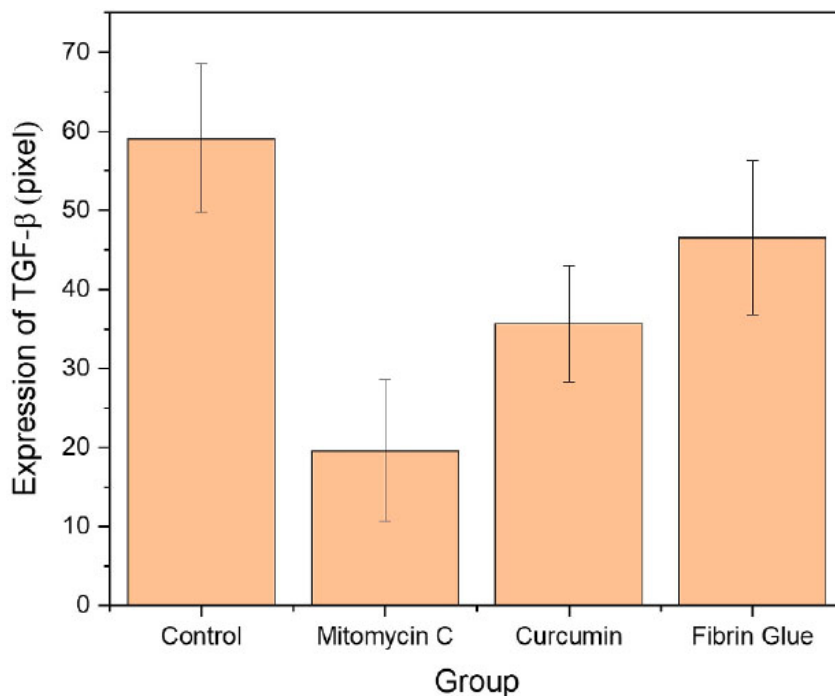


Figure 3. Expression of TGF- β in control, mitomycin C, curcumin, and fibrin glue groups observed after 48-hours exposure. Based on one-way ANOVA among all groups.

control group.¹⁴ In this present study, curcumin was found to significantly reduce the proliferation of HPF compared to the control group. After mitomycin C group, the curcumin group was also shown with the second least number of cell densities. In a previous report, curcumin was depicted to inhibit the fibroblast proliferation in time- and dose-dependent manners.²³ Thus, in the future study, it is interesting to investigate further concentration range (>200 $\mu\text{mol/L}$) of curcumin.

In the ophthalmic management, fibrin glue has been widely used, especially in the pterygium operation.²⁴ It is considered a safe and effective method to attach the conjunctival autograft on the wound closure following the surgery.²⁴ Herein, we have proven that fibrin glue is an effective antifibrotic agent against HPF proliferation. It is in line with the findings reported, where previously, fibrin glue was reported to reduce the proliferation of scar tissue of a focal chord of animal models. In ophthalmic treatment itself, fibrin glue had been witnessed to reduce fibroblast proliferation on conjunctiva post trabeculectomy.^{17,25}

TGF- β plays a significant role in initiating and maintaining proliferation phase during wound healing by converting fibroblast into actively proliferating myofibroblast.^{20,26} The mainstay therapy for proliferation-based diseases, Mitomycin C, was reported to attenuate the secretion of TGF- β via DNA synthesis. Reductions of TGF- β expression have been particularly observed in cultured HPF cells²¹ and patient conjunctiva.²⁷ The proposed mitomycin C alternative, curcumin, was also reported capable of inhibiting the synthesis of TGF- β via its negative regulator –Transforming growth-interacting factor (TGIF).^{14,28-30} In the case of fibrin glue, its main antifibrotic mechanism has been associated with TGF- β modulation.^{17,25} In line with previous studies, this present study also suggested that TGF- β levels were attenuated in mitomycin C, curcumin, and fibrin glue groups as shown by the immunofluorescence staining images (Figure 2). Mitomycin C was found to be the most effective TGF- β suppressing agent, followed by curcumin and fibrin glue, respectively.

CONCLUSIONS

Administrations of mitomycin C, curcumin, or fibrin glue could significantly inhibit the proliferation of HPF *in-vitro*. The main factor in fibroblast proliferation, TGF- β , was found attenuated in mitomycin C, curcumin, or fibrin glue group suggesting their anti-TGF- β properties. Mitomycin C was revealed to be the most potent agent in preventing the proliferation of pterygium fibroblast, but its safety should be a matter of concern. Further study should be carried out *in-vivo* with higher concentration range of curcumin.

ETHICAL APPROVAL

Ethical approval was obtained from the Ethical Committee, Faculty of Medicine, Universitas Airlangga, Indonesia (No. 128/EC/KEPK/FKUA/2021). The patient provided the written informed consent.

COMPETING INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

All author had contributed for manuscript writing and agreed for the final version of the manuscript for publication.

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