BIOCHEMICAL AND CELLULAR ARCHIVES

Volume No. : 19 (2019) **Issue No. :** Supp-02 (December)

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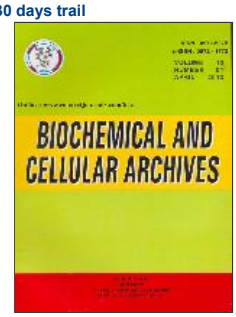

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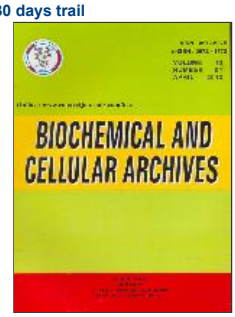

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DOI : 10.35124/bca.2019.19.S2.4695

Biochem. Cell. Arch. Vol. 19, Supplement 2, pp. 4695-4701, 2019 | www.connectjournals.com/bca | *ISSN 0972-5075*

ALTEPLASE IN PREVENTING POSTERIOR CAPSULE OPACITY BY PLASMINOGEN ACTIVATOR INHIBITOR-1 AND TYPE I COLLAGEN EXPRESSION INHIBITION IN A POSTERIOR CAPSULE OPACITY MODEL WITH FIBRIN REACTION *IN VITRO* : A EXPERIMENTAL LABORATORY STUDY

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(Received 11 August 2019, Revised 22 October 2019, Accepted 25 October 2019)

ABSTRACT : To investigate the effect of alteplase on plasminogen activator inhibitor -1 (PAI-1) and type I collagen expression in a posterior capsule opacity (PCO) model with fibrin reaction *in vitro*. Cultured human lens epithelial cells (HLEC) were isolated from the anterior lens capsule following a capsulotomy during cataract surgery. Cultured HLEC went into an *in vitro* wound scratch assay on fibrin coated dishes and were then divided into 4 groups consisting of a FBS 10% control group, and treatment groups of alteplase 25, 50 and 100 µg/ml. The effect of alteplase on plasminogen activator inhibitor (PAI-1) and type I collagen expression in PCO and its reaction with fibrin was investigated, determined by fluorescein intensity, labelled with fluorescein isothiocyanate (FITC) antibody of PAI-1 and type I collagen. The difference between expression levels among the groups was analyzed using a Kolmogorov-Smirnov test followed by a posthoc test showing a significance level of $p<0.05$. The lowest PAI-1 expression was obtained in the group treated with alteplase 100 ìg /ml ($1.26x10^6 \pm 3.31x10^5$) compared to the control group ($6.27x10^6 \pm 2.37x10^5$, p= 0.00); a significant result was also obtained among treatment groups, and showed that inhibition behaves in a dose dependent way. All treatment groups significantly inhibited type I collagen as compared to control group ($3.54x10^6 \pm 8.28x10^5$, p= 0.00), but the result is not significantly different among treatment groups. The inhibition effect of PAI-1 and type I collagen were influenced from the affects of several another pathways in type I collagen synthesis other than PAI-1. Alteplase showed an inhibition effect on PAI-1 and type I collagen with fibrin reaction *in vitro* in anterior lens capsule fibrosis, despite the inhibition effect on type I collagen not aligning with the inhibition of PAI-1.

Key words : Alteplase, lens epithelial cell, HLEC, PCO, fibrosis, PAI-1, type I collagen.

INTRODUCTION

Cataracts are the leading cause of blindness in the world, with the prevalence of cataracts in Indonesia seen in those over 50 years old, reaching 64-95%. Predictably, cataract surgery is the most common operative procedure performed on the eyes. Posterior capsular opacity (PCO) complications are seen at a rate of 5-50% after cataract surgery. The incidence of PCO increases in patients with a potential risk of post-operative fibrin;this is seen in children with cataracts, surgery involving excessive iris manipulation, patients with a history of diabetes mellitus, uveitis, vitrectomy, eyes treated with silicon oil, traumatic cataracts, pseudoexfoliation syndrome and patients with a history of iris and trabecular surgery (Dotan *et al*, 2013; Yoshino *et al*, 2012).

The occurrence of PCO is thought to be a wound healing response to tissue injury. Fibrosis type PCO from lens capsule fibrosis, occurs due to the remaining lens epithelial cells (LEC) migrating as a result of mesenchymaltrans differentiation (EMT) into myofibroblast cells. Fibrin, a provisional matrix of fibroblasts that later undergo transdifferentiation into myofibroblast cells, goes on to produce a pathological extracellular matrix, one of which is type I collagen. Various methods and treatments have been carried out to prevent capsule fibrosis, with only satisfactory results. Alteplase, for example, a recombinant tissue plasminogen activator (r-tPA), has a clinical role in ophthalmology by being applied to the anterior chamber to degrade fibrin formation by inhibiting PAI-1. Based on this fact, it is expected that administration of alteplase will incite a fibrinolysis process, whereby fibrin causes a provisional matrix of fibroblasts able to decrease, thus inhibiting the occurrence of anterior lens capsule fibrosis. The aim of

this study was to analyze the alteplase effect to PAI-1 and type I collagen (Nibourg *et al*, 2015; Tripathi *et al*, 2005).

MATERIALS AND METHODS

Materials

The primary reagents included type I collagenase (Roche USA), alpha modified eagle medium (α-MEM, Gibco-Life Technologies, USA), nonessential amino acid (NEAA, Sigma, USA), trypsin (Gibco - Life Technologies, USA), fetal bovine serum (FBS, Biowest, USA), basic fibroblast growth factor (bFGF, Gibco- Life Technologies, USA), anticoagulant citrate phosphate dextrose acid (CPDA), $CaCl_2$ (sigma). Primary FITC antibodies for vimentin, p63, PAI-1 and type I collagen (BIOSS, USA).

Methods

Study design

This is an *in vitro* study using human LEC (HLEC), conducted at the Stem Cell Research and Development Center, at the Universitas Airlangga. All experiments conformed with the local ethics review board, Dr. Soetomo Hospital. After performing *in vitro* wound scratch assays on fibrin coated dishes, HLEC were divided into 4 groups consisting of a FBS 10% control group and treatment groups of alteplase at 25, 50 and 100 mcg/ml. Analysis of PAI-1 and type I collagen were evaluated on the 10th day after scratching and treatment.

Isolation of HLECs

Lens anterior capsule tissue was obtained following capsulotomy during cataract surgery from subjects whose lens capsuleswere eligible for processing. The tissue was then transported to the Stem Cell Research and Development Center, at Airlangga University in Surabaya, Indonesia. It was isolated according the protocol established by Ibaraki with modification (Ibaraki, 2002); the anterior lens capsule tissue was cultured in 60 mm dishes using media containing aMEM, bFGF, NEAA, 10% FBS, 5 ng/ml FGF, 1% penicillin streptomycin and 1% amphotericin B until reaching 80-90 confluence; then cells were distributed over 96 well plates.

Fibrin Coated Dish preparation

Fibrin coated dishes weremade according to protocol established by Komaratih *et al* (2019) with a modification of using blood from the subject. Fibrin made from 9 ml of blood taken with 10 ml spuit containing 1 ml of acid citate dextrose was placed in sterile tubes and left overnight in -4°C. The following day, the blood was further centrifuged at 40 g for 10 minutes to obtain an amount of plasma; 500 µl of this was placed into each well, with the addition of calcium chloride in 1:10 ratio to create fibrin at the bottom of the well. Isolated HLEC were then placed on top of the fibrin layer (Komaratih *et al*, 2019).

Characterization of HLECs

Cells were characterized with diamidinophenylindole (DAPI) and fluorescein isothiocyanate (FITC) staining with vimentin to identify mesenchymal cells, and p63 to identify epithelial cells.

Alteplase preparation

The treatment groups of alteplase were made using extrapolation from cultured human corneal endothelial cells, as described in previous research conducted by Yoeruek *et al* (2008). Alteplase was then dissolved in culture medium until it reached 25, 50 and 100 mcg/ml (Yoeruek *et al*, 2008).

In vitro scratch assay

HLEC were seeded into 24 wells, at a density of 5×10^3 cells/well (6 wells for each group) in a 96 well plate. The cells were then scratched using yellow pipette tips and washed with culture medium to remove loose and dead cells.

PAI-1 and type I collagen expression assessment

Using a fluorescein microscope, measurements to assess the influence of alteplase were taken 10 days after scratching by analyzing the intensity of fluoresce in using FITC antibody of PAI-1 and type I collagen. The microscopic images were then measured and assessed using ImageJ software.

RESULTS

HLEC isolation and charaterization

This study successfully isolated HLEC, reaching its first confluent monolayer of 80-90% in 14 days. NEAA and bFGF supplementation promoted proliferation while maintaining viability of the cells. Cells were placed in a fibrin coated dish and characterized using DAPI to identify cells with a nucleus and FITC staining with vimentin and p63in order to identify mesenchymal and epithelial cells (Figs. 1 and 2).

Expression of PAI-1

The effect of alteplase in a culture of PAI-1 of HLEC is presented in Table 1, Figs. 3 and 4. The lowest PAI-1 expression was obtained in the group treated with 100 ìg/ml ($1.26x10^6\pm 3.31x10^5$) compared to control group ($6.27x10^6\pm2.37x10^5$, p = 0.00), alteplase 25 mcg/ml ($3.83x10^6\pm6.64x10^5$, p = 0.00), and alteplase 50 mcg/ml($2.58x10^6\pm5.87x10^5$, p = 0.006). The Games-Howell posthoc tests showing inhibition of PAI-1 resulted as significant in all treatment groups compared to controls (Table 2).

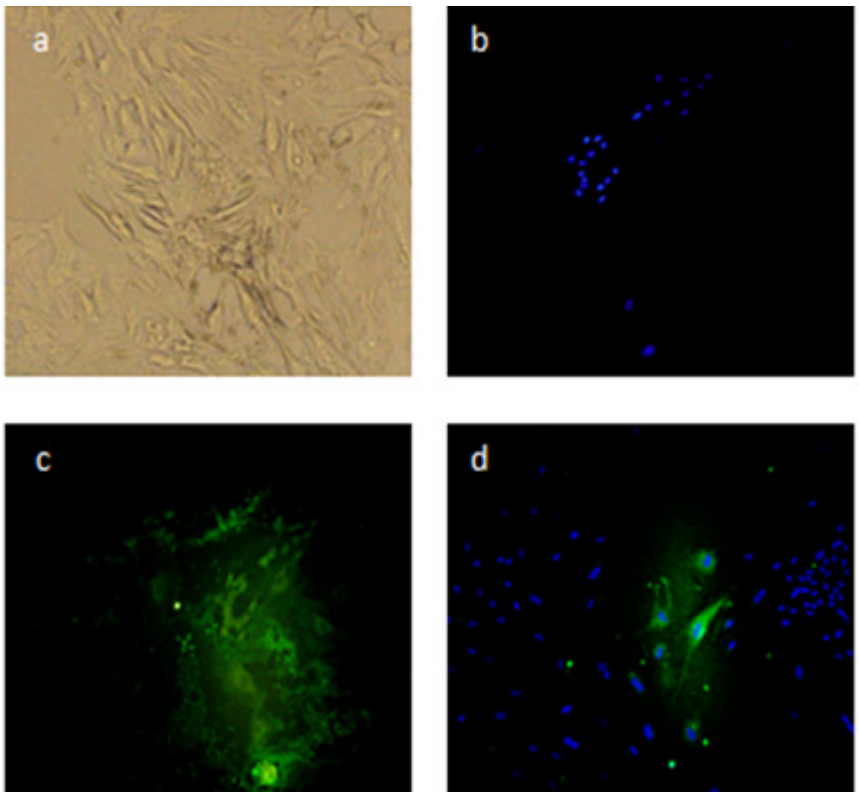

Fig. 1 : Series of vimentin staining a) contrast phase; b) nucleus showing blue fluorescence with DAPI staining; c) green fluorescence showing positive vimentin FITC; d) merge (positive vimentin cells).

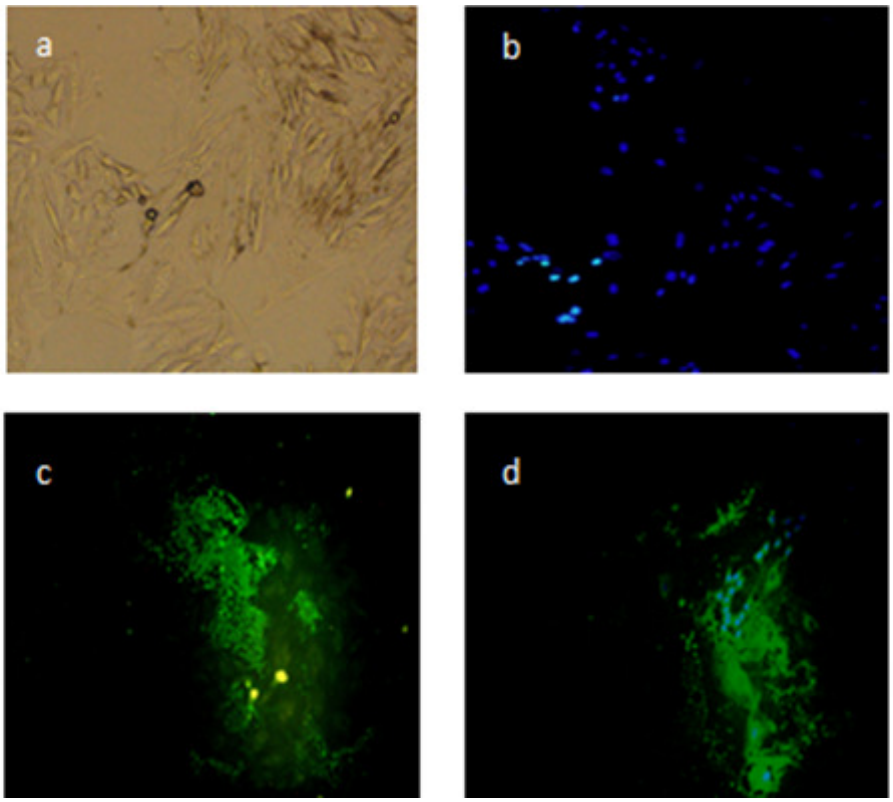

Fig. 2 : Result a) contrast phase; b) DAPI staining showing blue fluorescent nucleus; c) FITC postitive p63 shown in green fluorescent; d) merge (p63 cells positive).

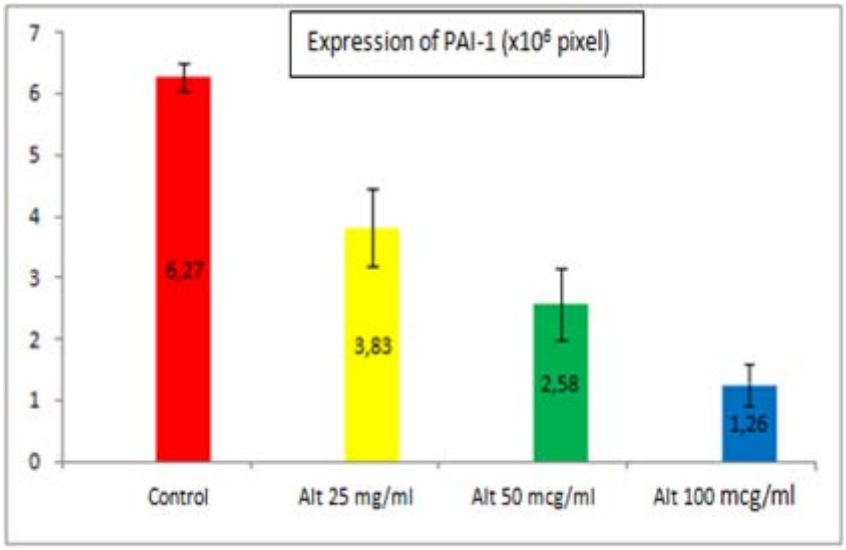

Fig. 3 : Effect of alteplase on PAI-1 compared to FBS 10% control group and treatment groups.

Table 1 : Type I collagen expression (Tukey HSD).

Group	N	Mean (pixel)	SD	P (One wayAnova)
K1	6	$6.2740x10^6$	$2.3782x10^5$	0.000
P1	6	$3.8327x10^6$	$6.6424x10^5$	
P2	6	$2.5834x10^6$	$5.8785x10^5$	
P3	6	$1.2600x10^6$	$3.3173x10^5$	

Table 2 : PAI-1 expression (Games-Howell).

	Control	Alt 25 mcg/ml	Alt 50 mcg/ml	Alt 100 mcg/ml
Control	-	0.00*	0.00*	0.00*
Alt 25 mcg/ml	0.00*	-	0.028*	0.00*
Alt 50 mcg/ml	0.00*	0.028*	-	0.006*
Alt 100 mcg/ml	0.00*	0.00*	0.006*	

*p< 0.05; statically sigificant.

Table 3 : Mean of type I collagen expression.

Group	N	Mean	SD	P (Anova)
K1	6	$3.5440x10^6$	$8.2818x10^5$	0.000
P1	6	$1.9988x10^6$	$5.1209x10^5$	
P2	6	$1.4336x10^6$	$6.1268x10^5$	
P3	6	$1.4334x10^6$	$5.3063x10^5$	

Table 4. Type I collagen expression (Tukey HSD)

	Control	Alt 25 mcg/ml	Alt 50 mcg/ml	Alt 100 mcg/ml
Control	-	0.02*	0.00*	0.00*
Alt 25 mcg/ml	0.002*	-	0.431	0.430
Alt 50 mcg/ml	0.00*	0.431	-	1.00
Alt 100 mcg/ml	0.00*	0.430	1.00	

*p< 0.05; statically sigificant.

Expression of type I collagen

The inhibition of type I collagen can be seen in Table 3, Figs. 5 and 6 and compares the group treated with alteplase100 mcg/ml ($1.4334x10^6 \pm 5.30x10^5$) to the control group ($3.54x10^6 \pm 8.28x10^5$, p= 0.00), alteplase 25 mcg/ml ($1.99x10^6 \pm 5.1x10^5$, p = 0.43) and alteplase 50 mcg/ml($1.43x10^6 \pm 6.12x10^5$, p=1.00). The effect of alteplase on PAI-1 of HLEC culture is presented in Figs. 5 and 6. The Tukey post hoc test showed that all of the treatment groups had a significant result compared to the control group (Table 4), with an especially significant type I collagen inhibition shown in the alteplase100 mcg/ml group; however, no significant differences resulted between comparisons of the treatment groups.

DISCUSSION

The wound healing response consists of four phases: homeostasis, inflammation, proliferation and remodeling. The homeostasis phase is initiated within seconds, lasting up to a few hours after injury; its activity is also known to decrease rapidly, moving on to the next phase. Several factors influence this phase, especiallyregardingplatelet and fibrin involvement. Fibrin formation is regulated by fibrinolysis, occurs in less than one minute, and becomes stable in 30 minutes (Uluer *et al*, 2010; Kattula *et al*, 2018; Guo *et al*, 2010).

The occurrence of PCO is higher in cataract surgery patients at risk for intraocular inflammation, which can cause fibrin formation due to the presence of LEC lesions. The lesions induce inflammatory mediators such as IL-1 and PGE_2, which can further alter the balance of the blood aqueous barrier, including interfering with the balance of plasminogen, tPA, and thrombin. When a fibrin formation occurs, it naturally increases the expression of

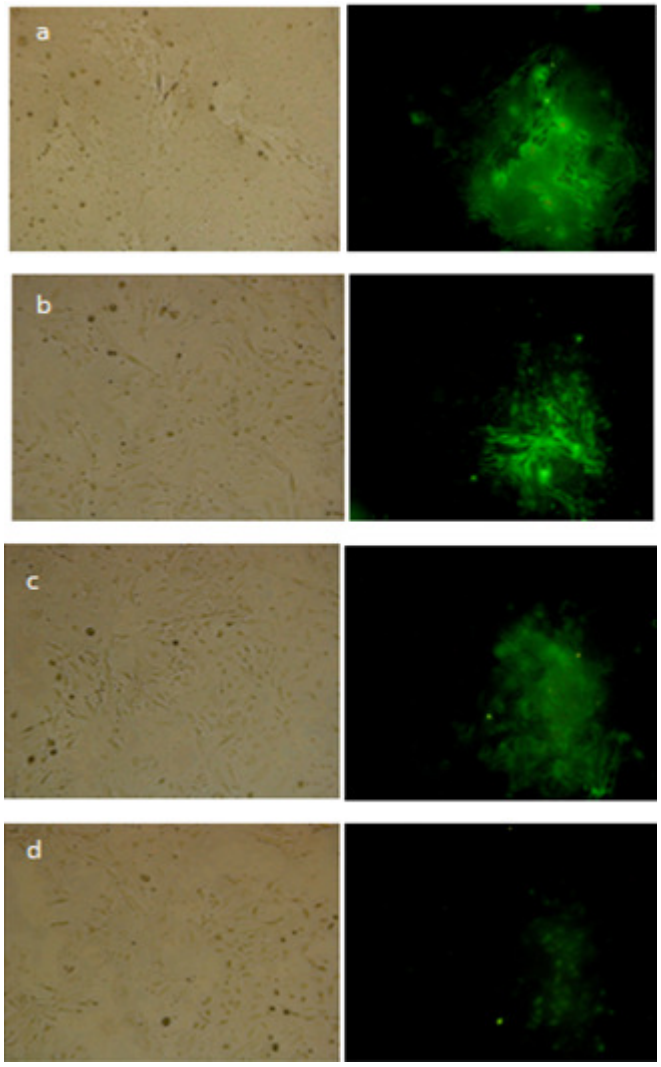

Fig. 4 : Assessment of PAI-1 in contrast phase and fluorescence of HLEC culture a) control; b) alteplase 25 mc/ml; c) alteplase 50 mcg/ml; d) alteplase 100 mcg/ml; decreasing green intensity by increasing dosage.

PAI-1 to inhibit the activity of uPA and tPA,both of which function to induce fibrinolysis. Persistent fibrin can be a provisional matrix for fibroblasts playing a role in tissue fibrosis. Myofibroblasts have a transdifferentiation response with contractile components and are able to bring wound edges closer; one of the pathological ECM that containmyofibroblasts is type I collagen (Ghosh and dan Vaughan, 2012). Fibrinolysis occurs from the degradation of fibrin, as carried out by uPA and tPA produced endogenously or exogenously by recombinant techniques and namely from r-tPA, one of which is by alteplase. Alteplase hasbeen useful in the fields of cardiology and neurology as an emergency intravenous therapy, when rapid occlusion of blood vessels to induce fibrinolysis is needed (Nishi *et al*, 2018; Song *et al*, 2010; Ghatak *et al*, 2018; Gurman *et al*, 1992).

As an exogenous tPA produced by recombinant techniques, alteplase is known as an r-tPA that works by inhibiting PAI-1. This study assessed the effectiveness of alteplase in the prevention of fibrin formation in PCO *in vitro*, which was achieved by inducing cellular injury and using fibrin to mimic clinical conditions during cataract surgery. In this study, a procedure modification from the one presented by Stamm *et al* (2016) was performed, in that the technique used for scratching a culture dish represented the mechanical injury that occurs clinically. To that end, this study followed a mechanical injury model to form a discontinuity of cellular components such as those that occur in the LEC sequence in the anterior lens (Stamm *et al*, 2016).

TGF-β is a major growth factor in the PCO process and in the EMT process of lenses and other organs that are also associated with PAI-1. TGF-β has two pathways,

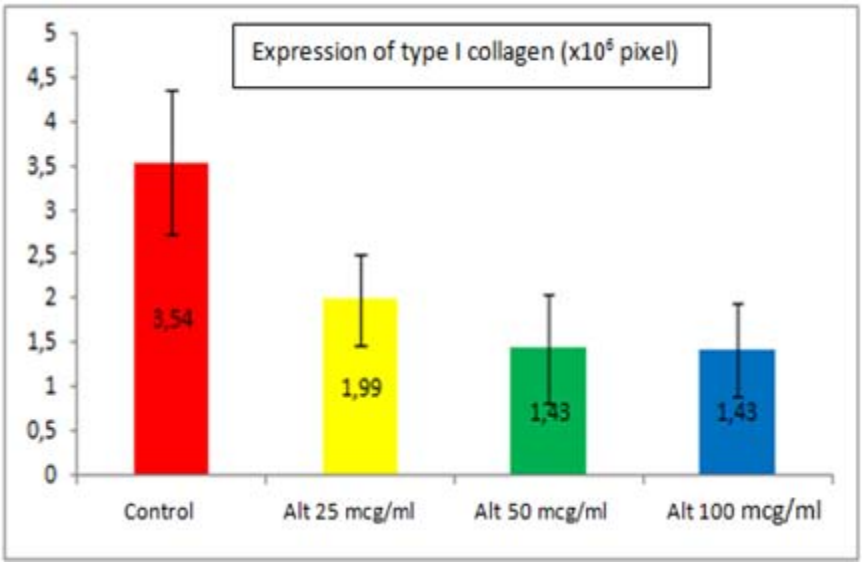

Fig. 5 : Effect of alteplase on type I collagen compared to FBS 10% control group and treatment groups.

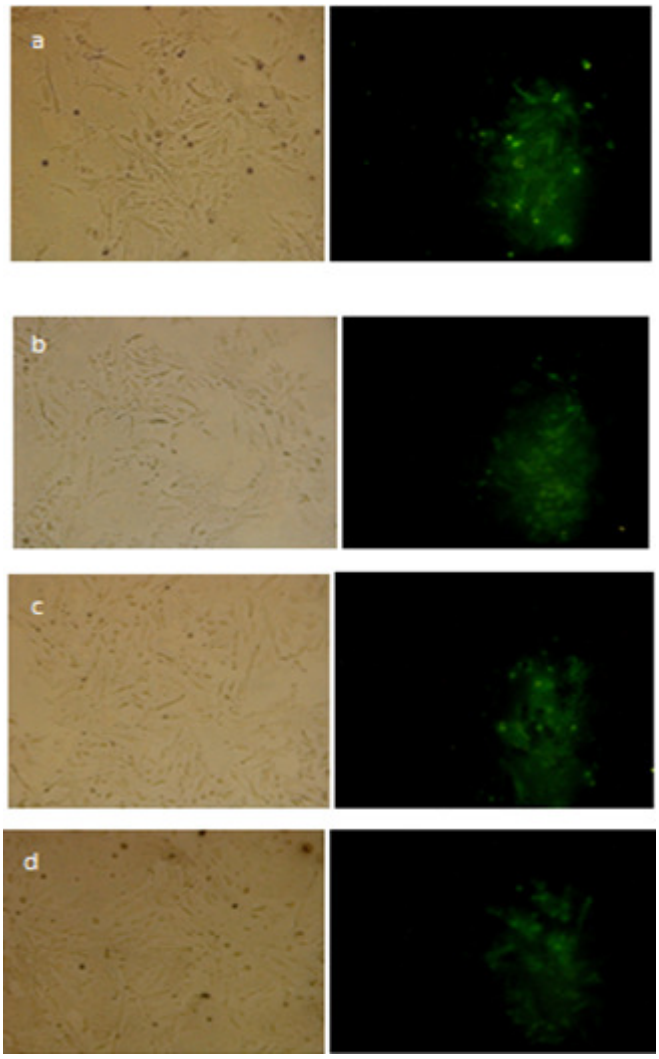

Fig. 6 : Assessment of tye I collagen in contrast phase and fluorescence of HLEC culture a) control; b) alteplase 25 mc/ml; c) alteplase 50 mcg/ml; d) alteplase 100 mcg/ml.

named Smad dependent which has Smad 1 to Smad 5 components and Smad independent which consists of other pathways, namedRho-GTPase,phosphatidilinositol 3-kinase and MAPK. The Smad dependent pathway is more dominant in the induction of PAI-1 expression, but the independent pathway could also induce the PAI-1 (Dotanet *et al*, 2013; Song *et al*, 2010; Ghosh *et al*, 2012).

Assessment in this study was carried out on the 10th day post-treatment following a graph of collagen formation in the wound healing process, which began on the 2nd day and the levels went up quite far on the 10th day. It can be seen the lowest to highest dose treatment group, can significantly inhibit PAI-1 expression compared to the control group. Significant inhibition of expression occurred between treatment groups, and the effect of PAI-1 inhibition was also dose dependent, with the lowest expression of PAI-1 found in the largest dose group, which is 100 mcg/ml. Also found that the control group had the highest type I collagen expression, which was significant for the other three control groups. This is in line with clinical research conducted by Heiligenhaus *et al* (1998), who conducted a study with 41 control group subjects and compared with 44 treatment group subjects who received 10 ìg intracameral alteplase at the end of cataract surgery, with 41 control group subjects, found significantly higher PCO fibrosis in the control group at 90 days after surgery with visual acuity was also significantly better than the control group with the PCO inhibition mechanism that had not been explained in the study (Heiligenhaus *et al*, 1998).

In this study, the inhibition to PAI-1 is not in line to the magnitude of the inhibition in type I collagen, and this can be caused by several reasons. Guo *et al* (2010) stated that there are growth factors that function as powerful inflammatory mediators other than TGF-β, which are PDGF, FGF, EGF and all four can be found in the lens. These four inflammatory mediators can provide an inflammatory response in one stimulus, then they will attract neutrophils which will increase cellular ROS and increase cell damage, macrophages as fibroblast attractant, and lymphocytes at the end of the inflammatory phase to trigger remodeling (Nibourg *et al*, 2015; Wang *et al*, 2017).

PDGF has four types of molecules, namely PDGF-A, PDGF-B, PDGF-C and PDGF-D. A study conducted by examining mesothelial peritoneal cells of rats that were given adenoviruses as stress and shut down the TGF-β pathway. The final results of the study stated that PDGF-B is a powerful growth factor for the process of fibrosis, and PDGF-B can work independently without the need for TGF-β and can initiate EMT with α-SMA end markers, a marker of pathological EMT (Patel *et al*, 2010).

Bastiaans *et al* (2014) performed retinal pigment epithelium (RPE) cell culture and analyze its association with proliferative vitreorethinopathy (PVR). In the event of a PVR, a blood retinal barrier breakdown occurs firstly with RPE network damage that activates factor X to factor Xa that converts prothrombin into thrombin. Thrombin will provide a response from PDGF, and make EMT process in RPE, marked by the discovery of stress fiber in RPE. Wang *et al* (2017) performed a culture of mouse LEC, and blockade TGF-β/Smadpathwat and then did FGF administration. it was found that with FGF, LEC experienced a meaningful EMT process without passing through a TGF-β, pathway. Another study used corneal epithelial cells which were injured, then given EGF 15 minutes later, it was found that TGF-β level were increased, so it is an inflammatory process and has a

synergistic effect with TGF-βfollowed by a proliferation phase can occur more strongly as a wound healing response (Bastiaans *et al*, 2014; Shu *et al*, 2019; Wang *et al*, 2017).

PAI-1 is a response in the homeostasis phase of wound healing and runs for a short time, whereas type I collagen is in the proliferation phase which is initiated by the inflammatory phase, which occurs after the homeostasis phase. PAI-1 expression can be suppressed by giving alteplase in the phase of homeostasis, while the process of formation of type I collagen as a pathological ECM from the EMT process can continue due to the pathway of TGF-β, whose position is more proximal than PAI-1, which has other branching pathways, and also due to activation of other growth factor pathways such as PDGF, EGF and FGF, which can continue the inflammatory phase and goes to the proliferation and remodeling EMT phases.

CONCLUSION

In this study, it is concluded that alteplase can significantly reduce the expression of PAI-1 as a mediator of the fibrosis process with reaction to fibrin, as well as reduce the expression of type I collagen as a pathological ECM from the lens anterior capsule fibrosis process, and PAI-1 is not directly related to type collagen -1.

ACKNOWLEDGEMENT

We thank to the Airlangga Health Science Institute Research grant.

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