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Original Article

The Effect of Topical DLBS 1425 on *Tissue Plasminogen Activator* (tPA) Expression in Trabecular Meshwork of Wistar Rats

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Abstract

Background : *Tissue plasminogen activator* (tPA) in the *trabecular meshwork* (TM) is a serine protease that important to maintain the aquos outflow resistance by activating the *matrix metalloproteinase* (MMP). It can cause a degradation of the extracellular matrix, which can maintain the normal flow of *aquos humor*. However, the use of anti-inflammatory drugs has been shown to reduce the expression of tPA, leading to an increase in the outflow resistance. Therefore, we propose the use of DLBS 1425, an extract of *Phaleria macrocarpa* which has been proven to have anti-inflammatory effects. This study aims to determine the expression of tPA in Wistar rats' TM.
Methods : An experimental laboratory study with *post–test only randomized controlled group design* was performed. Twenty–two Wistar rats were divided into two groups, the control and the experimental. The experimental group was given topical DLBS 1425 at a dose of 6 times / day, for 4 weeks. The control group was given drops of Hyalub Minidose® 6 times / day, for 4 weeks. tPA expression on TM was examined by immunohistochemical staining. Data were collected and processed using the *SPSS 15.0* for Windows.
Results : The mean tPA expression in TM with Allred scores in the experimental group (0.18 ± 0.60) was significantly lower (p <0.001) than the control group (6.27 ± 0.91).
Conclusion : Topical DLBS 1425 suppresses the expression of tPA on the trabecular meshwork of Wistar rats.

Keywords : *tissue plasminogen activator*, trabecular meshwork, DLBS 1425, *Phaleria macrocarpa*

INTRODUCTION

Trabecular meshwork (TM) cells have a role in maintaining the outflow channel patency of *aquos humor*. Tissue plasminogen activator (tPA) is a serine protease encoded by the PLAT gene, synthesized by vascular endothelial cells and in the uvea microvasculature, corneal endothelium, corneal epithelium, and trabecular meshwork found in the eye. TM cells are the first normal cells known to have tPA activity that is more dominant than its inhibitors.[1–3] In TM, tPA has a role to maintain the outflow resistance by changing the plasminogen into plasmin. Plasmin plays a role in activating the pro matrix metalloproteinase (pro–MMP) into a matrix metalloproteinase (MMP), which will cause a degradation of the extracellular matrix so it can maintain the normal flow of *aquos humor*.

A decrease in the expression of tPA can cause the accumulation of extracellular matrix that can clog the trabecular meshwork and causes an increase in the resistance of *aquos humor* outflow.[4,5] This can be caused by anti-inflammatory drugs such as steroids. Ultimately, an increase in the outflow resistance will cause an increase in the intraocular pressure. tPA activity is controlled by endogenous inhibitors, namely the *plasminogen activator inhibitor 1* (PAI–1). An increase in PAI–1 can occur in response to various inflammatory cytokines such as IL–1, IL–6, TNF α, and TGF β which are known factors that can induce a decrease in the outflow facilities. tPA activity is also influenced by several factors such as thrombin, fibroblasts, mastocyte cells and VEGF.[4,6–8]

DLBS *(Dexa Laboratories of Biomolecular Science)* 1425 is a standardized bioactive fraction of the flesh of Mahkota Dewa fruit *(Phaleria macrocarpa)* which has anti-proliferative, anti-inflammatory, and anti-angiogenic effects. Research conducted by Hutama (2016) with various concentrations of DLBS 1425 concluded that a topical concentration of 1 x 10^1 mg / dl of DLBS 1425 could decrease the expression of COX–2 cornea in Wistar rats after base trauma. The result was proven to be statistically significant and had proven the potential of DLBS 1425 as a topical anti-inflammatory.[9] Moreover, the same research was conducted by Ermawati (2017) and achieved a similar result.[10] Therefore, it is concluded that topical DLBS 1425 has an anti-inflammatory potential. However, up until recently, there is no study available on the side effects of DLBS 1425 to the variables. Therefore, this study is aimed to observe the effect of topical DLBS 1425 on tPA expression in the trabecular meshwork of Wistar rats.

METHODS

This study is a laboratory experimental study with a randomized controlled–group post–test only design using Wistar rats, which are given 1 x 10^1 mg / ml topical DLBS 1425. The output of this study is the changes in tPA expression in the Wistar rats' trabecular meshwork. The manufacture process of topical DLBS 1425 is done by adding a DLBS 1425 capsule, 150 mg into the eye drops Hyalub Minidose ® 5 ml, the concentration being 1 ml (30 mg). Then, it is diluted again with Hyalub Minidose 3 times, the concentration being 1 ml (10 mg). This study used 22 male Wistar rats, aged 2–3 months, weighing 200–300 grams, appeared active during the adaptation period, and no anatomic abnormalities in the eyes were seen. Rats that were sick (inactive), dead, and had eye disease were excluded from the sample.

A total of 22 rat eyes complying to the sample criteria were acclimatized in the same place and environment, given the same standard feed ad libitum for one week. Then they were carried out randomly and divided into 2 groups, namely the control group and experimental group. Hyalub Minidose® drop was given 6 times/day for 4 weeks in the right eye of Wistar rats in the control group and DLBS 1425 drop 6 times/day for 4 weeks in the right eye of Wistar rats in the experimental group. Euthanasia was carried out using gas ether and cervical dislocation, bulbi enucleation and trabecular meshwork of the tissue extraction. Immunohistochemical staining was performed in all samples and the assessment of tPA expression on trabecular meshwork was done by Anatomical Pathology specialists. The result of tPA smear on cell cytoplasm is positive if there was a dark staining at 400 times magnification with five different visual fields. The average number of cells expressing tPA per field of view was calculated semi-quantitatively and the proportion of specific staining was calculated using Allred scoring that was conducted at the Experimental Animal Laboratory of the Faculty of Medicine, Diponegoro University, Semarang and the Accurate Pathological Anatomy Laboratory, Semarang in August–October 2018. Observations were made in 5 fields of view. Then, the number of immunoreactive cells painted in dark stain on the cell membrane was counted.

Allred score is a summation of the proportion and intensity scale of the immunoexpression with a total value in the range of 0–8. The numerical value for overall intensity [intensity score (IS)] is based on a 4 point system: 0, 1, 2, and 3 (for none, light, medium, or dark staining). The numerical value for percent stained [proportion score (PS)] is determined by a geometric rather than linear division; no stain = 0; ≤ 1/100 cells stained = 1; ≤ 1/10 cells stained = 2; ≤ 1/3 cells stained = 3; ≤2/3 cells stained = 4; all cells stained = 5. The total value of the two results obtained by Allred score indicates the effect of the expression of the tPA, with the criteria 0–1 indicates no effect, 2–3 indicates small effects (20%), 4–6 indicates moderate effects (50%), 7–8 l indicates large effects (75%). The actual color intensity for the Allred score can be seen in Figure 1.[11] Data was collected and processed using the

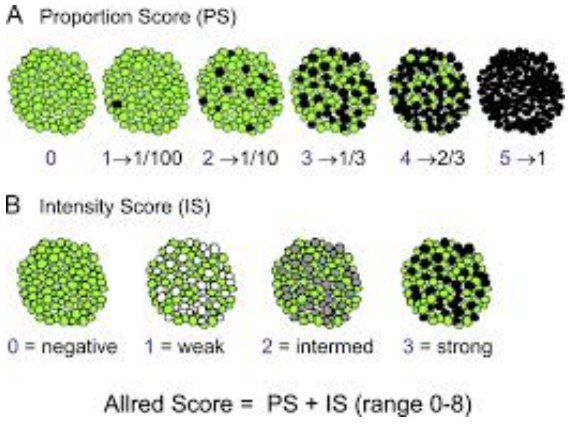

Figure 1. The methodology for calculation of the Allred score. The green color identifies unstained cells, whereas the gray, dark gray, and black colors identify cells stained to different intensities. (A) Series in which the stain intensity is constant (at maximum), and the proportion of stained cells increases from left to right. (B) Series in which the proportion of stained cells is constant (at 1/3), and the stain intensity increases from left to right (from none to maximum).[11]

TABEL 1
Characteristics of expression of tPA in trabecular meshwork wistar rats in the control and experimental groups

Groups	Control	Experimental	Total
N	11	11	22
Mean	6.27	.18	3.23
SD	.905	.603	3.206
Median	7.00	.00	3.50
Min	5	0	0
Max	7	2	7

SD: standard deviation; N: normal; Min: minimal; Max: maximal

Statistical Program for Social Sciences (SPSS) 15.0 for Windows program. Management and treatment of experimental animals has been approved by the Health Research Ethics Commission of the Faculty of Medicine with No.108/EC/H/FK-RSDK/IX/2018, Diponegoro University, Semarang.

RESULTS

The aim of this study is to determine the presence and/or the absence of the effect of DLBS 1425 1 x 10^1 mg / ml concentration on the expression of *tissue plasminogen activator* (tPA) on the trabecular meshwork of Wistar rat. Characteristics of the expression of tPA is depicted in Table 1.

As seen in Table 1 and Figure 2, it is found that there are some differences in tPA expression in the experimental group compared to the control group. The average of tPA expression in the experimental group was lower than in the control group.

Figure 3 shows a darker color in TM on control group compared with experimental group in Figure 4. The dark color indicates a tPA expression in imunohistochemistry examination on TM control group.

The normality of data distribution was tested using the Shapiro–Wilk test. Data is indicated as normal if $p > 0.05$, while $p < 0.05$ indicate an abnormally distributed data. Table 2 shows the normality test in the control and experimental groups which both obtained that the data were not normally distributed with a value of $p < 0.05$.

tPA expression in the control group had a significant difference compared to the experimental group, using the Mann Whitney test ($p < 0.001$). The difference in the expression of tPA on the trabecular meshwork of Wistar rat can be seen in Table 3.

DISCUSSION

This study compares the effect of topical DLBS 1425 suppression on the expression of tPA on the trabecular meshwork of Wistar rats in the control group and experimental group. There is a statistically significant

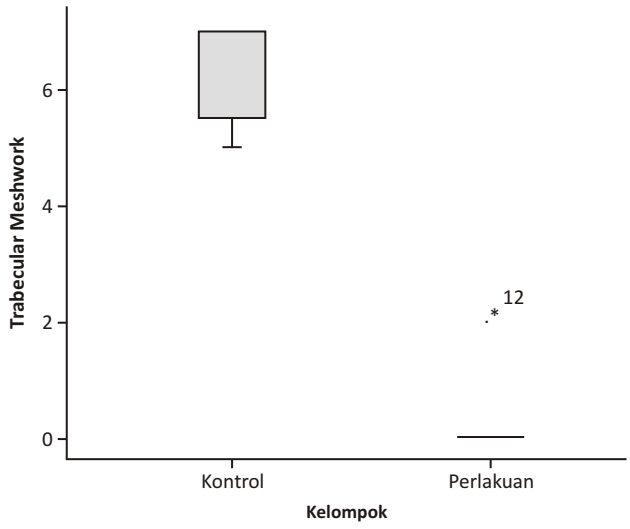

Figure 2. *Box plot* of the expression of tPA in trabecular meshwork of Wistar rats in the control and experimental groups

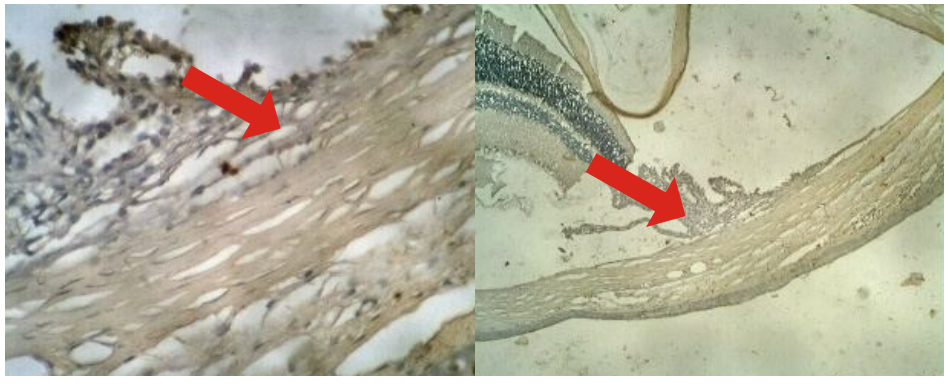

Figure 3. IHC tPA on TM control group

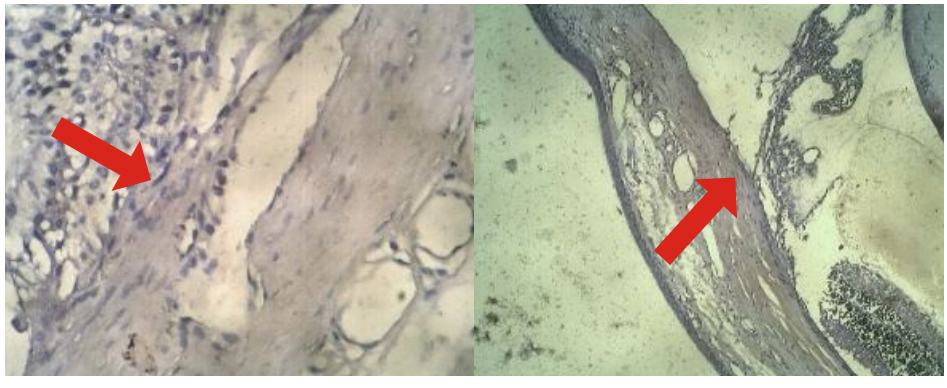

Figure 4. IHC tPA on TM experimental group

difference in tPA expression between both group with a value of p <0.001. Studies regarding the effect of topical preparations of DLBS 1425 for the eye have not been found, but the results of this study indicates that DLBS 1425 has the potential to penetrate the intraocular.

tPA is a serine protease that plays a role in the fibrinolytic system. In an inflammatory condition it is known that pro–inflammatory cytokines such as IL–6 and TNF–α will be released. The increase of IL–6 and TNF–α can cause an increase in fibrinogen and other

TABEL 2
Descriptive and normality of data

Groups	Control	Experimental
Mean ± SD	6.27 ± 0.91	0.18 ± 0.60
Median (min – max)	7 (5 – 7)	0 (0 – 2)
p¥	0.001	0.000

Note : * Normally distributed ($p > 0.05$); ¥ Shapiro–Wilk

TABEL 3
Differences in the expression of tPA on trabecular meshwork of wistar rats in the control and experimental groups

Groups	Control	Experimental
Median (min – max)	7 (5 – 7)	0 (0 – 2)
p‡	<0.001*	

Note:* Significant ($p < 0.05$); ‡ Mann Whitney

inflammatory mediators which ultimately cause an increase in PAI–1 and tPA expression.[7] DLBS 1425 topical has a phalerin content which is an extract from pericarp and mesocarp that has significant anti-inflammatory activity with inhibitory percentage of 63.4% and 69.5%. The anti–inflammatory effect can reduce the expression of IL–6 and TNF–α, causing a decrease in the expression of tPA, with the highest tPA activity is found when it binds with fibrin.[12,13]

tPA can modulate the NF–kB pathway through the expression of NF–kB influenced by chemokines in macrophages. This is done via the interactions between membrane receptor Annexin2 with CD11b that was induced by macrophages. However, it can cause phosphorylation and degradation of IkB, resulting in the release and nuclear translocation of p65/p50, DNA binding, and other gen–transcription. DLBS 1425 contains proliverenol, which is a bioactive fraction of Phaleria macrocarpa fruit. Proliverenol can reduce the expression of NF–kB.[14] Tjandrawinata (2014) assessed the hepatoprotective proliverenol activity by increasing cell resistance up to 53-69% and decreasing NF–kB, TNF–α and caspase 8. A decrease in NF-kB exposure will result in decreased tPA expression.[15]

Silymarin and flavonoids are the components of DLBS 1425 which have antioxidant effects by fighting free radicals and NO.[12] In a study conducted by Giannarelli (2007) in hypertensive and normotensive subjects it was found that hypertensive patients had endothelial dysfunction due to a decrease in nitric oxide (NO) availability and a decrease in tPA release. Moreover, in normotensive subjects, it is found that the normal tPA activity is affects NO availability which plays a role in the fibrinolytic system and as anti-thrombotic.[16] Therefore, an emphasis on nitric oxide is followed by a decrease in tPA expression.

Some studies showed a relationship between the plasminogen activator system and COX 2 with VEGF. These three factors are known to have an important role to be involved in tumor growth, tumor angiogenesis, tumor differentiation and in the metastatic process.[17,18] Phalerin content in DLBS 1425 has the effect of suppressing VEGF expression so that it can act as anti-angiogenesis, anti–proliferative and induce apoptosis.[12] Mandarana's study (2016) showed that DLBS 1425 topical concentrations of $1x10^{-1}$, $1x10^{0}$ and $1x10^{1}$ have an effect on the corneal VEGF expression of Wistar rats after base trauma. VEGF expression in the treatment group was lower than the control group, but it was not statistically significant.19 Therefore, an emphasis on VEGF expression also affects the decrease in tPA expression which plays a role in the plasminogen activator system.

This study shows the effect of the suppression of tPA expression in topical DLBS 1425 administration. This suppression can inhibit changes in plasminogen to plasmin so that it inhibits MMP activation resulting in the accumulation of debris which will increase outflow resistance. The increase in outflow resistance can cause an increase in intraocular pressure. The weakness of this study is that no intraocular pressure measurement after the treatment to prove that the effect of suppression of tPA expression on trabecular meshwork can be clinically influential. Moreover, there is no comparison of the effect of tPA suppression after topical DLBS 1425 treatment

with standard anti-inflammatory drugs on intraocular tissue.

CONCLUSION

Topical DLBS 1425 has a significant suppressive effect on tPA expression on TM of Wistar rats given DLBS 1425 topically compared to the controls that may cause increase outflow resistance. For future research, a measurement of intraocular pressure after DLBS-1425 treatment is necessary to prove that the effect of suppression of tPA expression on TM can be clinically influential. Significant differences between the tPA expression of the control and treatment groups show that DLBS 1425 has the potential for penetration into the intraocular tissue, so other studies need to be conducted that can prove the potential effect of DLBS 1425 on intraocular tissue other than TM. Study comparing the effects of tPA suppression between topical DLBS 1425 with standard anti–inflammatory drugs in intraocular tissue also need to be carried out.

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