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Effect of Kt6 Variant Cowpea (*Vigna Unguiculata*) Extract on Matrix Metalloproteinase-9 and VEGF Expression of Corneal Inflammation Rat Model (*Rattus novergicus* Strain Wistar)

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Abstract: Matrix metalloproteinase-9 (MMP-9), a specialized enzymes capable of proteolytically degrading extracellular matrix proteins, have been postulated to play an important role in angiogenesis. VEGF expression caused activation of angiogenesis stages includes proteolysis, proliferation of endothelial cell, migration and tubulogenesis. Genistein has been reported to be the most potent inhibitor of cancer cell growth in vitro but also impair the proliferation of vascular endothelial cells in corneal neovascularization. In this interventional experimental study, we evaluate the effect of genistein in cowpea (Vigna unguiculata) methalonic extract on MMP-9 and VEGF expression in rat corneal-alkali induced inflammation. Sixty three rats were randomly choosen, 3 rats as control, 60 rats were induced with NaOH 1M and administered with 25 µM, 50 µM and 100 µM of genistein from cowpea methanolic extract four times daily. Alkali burn as inflammation model using NaOH 1M infiltration through filter paper which were aplicated on the center cornea of the right eye for 60 seconds as positive control and cowpea methalonic extract were administered immediately after alkali burn by NaOH. MMP-9 and VEGF expression was observed at 6, 24, 48, 96, and 168 hours by immunohistochemistry. Each dose of cowpea methalonic extract significantly decreased the MMP-9 and VEGF expression (p=0.000). Interaction between time of administration and dose of administration had influenced the MMP-9 and VEGF expression. Genistein in cowpea methalonic extract decreased the MMP-9 and VEGF expression on NaOH alkali burn corneal inflammation in rats but time of administration not affected.

Keywords: extracellular matrix (ECM), MMP-9 expression, VEGF expression, cowpea (Vigna unguiculata) methalonic extract, NaOH alkali burn.

Introduction

Healthy cornea does not contain blood vessel and lymph, but cornea is prone to various trauma, either physical, environmental stress, infection, or genetic mutation. The condition causes cornea inflammation or cornea cell death.[1] Cornea inflammation is the most eye disease in humans and animals that able to cause blindness and eyes organ loss.[1-3] Angiogenesis often relates with cornea inflammation, is complex process that involves endothelial cell proliferation, migration, extra cellular matrix remodeling, and formation of tubular structure. In the migration process and tubulogenesis is not free from the role of matrix metalloproteinase (MMPs) especially MMP-9. MMP-9 has role in degrading extra cellular matrix (ECM) of endothelial basal membrane cell of

blood vessel. MMP-9 also has role in destructing the cornea epithelial integrity, and also has role in releasing the angiogenic factors, cytokine receptor, and molecule adhesion.[4-6] Some researches concluded that VEGF has important role than growth factor in angiongenesis. Excessive VEGF expression caused activation of angiogenesis stages that includes proteolysis, proliferation of endothelial cell, migration and tubulogenesis.[7,8]

Genistein, grouped in isoflavone serves to inhibit angiogenesis. Fotsis et al, found that genistein inhibits the vascular endothelial cell proliferation and angiogenesis at concentration of 5 and 150μM/L.[9-11] Genistein has effect to decrease cornea neovascularization and decrease the blood vessel leakage, through inhibiting mechanism of tyrosine kinase protein as important component in the biological tissue control that determine the cell growth and differentiation. The inhibition of tyrosine kinase protein activation for intracellular signal of endothelial cell cause inhibition of MMP-9.[9-13] The giving of genistein also causes the VEGF expression decrease that inhibits cellular proliferation, angiogenesis decrease, and apoptosis.[14-20]

Cowpea, *Vigna unguiculata*, is legume plants as protein source for tropical and subtropical areas.[21,22] Cowpea contains isoflavone as the fitoestrogen, with main component of daidzein (4',7-dihydroxyisoflavone), genistein (4',5,7-trihydroxyisoflavone), and glycetin (7,49-dihidroxy-6-methoxyisoflavone). Cowpea, *Vigna unguiculata,* variant KT6 according to Yuaris et al (2012) that grow in Nusa Penida Bali contain high isoflavonoid.[7,23-26]

To know the isoflavonoid effects of genistein from cowpea (*Vigna unguiculata*) extract at cornea then in research it was done induction of rat cornea neovascularization by using chemical cauterization. Based on research of Zhou et al the giving of NaOH 1 M topical able to induce cornea neovascularization in 48-72 hours. At the research, it will be observed the MMP-9 expression and VEGF to know the inhibiting effect of isoflavonoid genistein of *Vigna unguiculata* extract in the cornea angiogenesis process.

Research Method

Research design

The research was experimental using randomized single blind method for eyes group under treatment with control group.

Determination of cowpea extract content

Cowpea that is used in the research obtained from Nusa Penida Bali. Cowpea extraction according to *Figallo* method.

The standard to investigate the genistein and daidzein content in the cowpea extract of aglycon form that is genestein and daidzein, so the extraction yield will be hydrolyzed first with acid. The hydrolysis aimed to cut the glycoside bonding at the extract so will be formed aglycon extract.

The genistein and daidzein content determination in the cowpea extract yield is done by high performance liquid chromatography (KCKT). After known the ganistein and daidzein content, then the extract total content is determined by adding the genistein and daidzein. Then the total extract content is used as the foundation to calculate the dosage. In this research, the isoflavonoid genistein amount that obtained is 147 mg/1000 ml (147 μM). Then the extract is solved in the aquadest to get the topical preparation with concentration of 25μM, 50μM, and 100μM.

Determination of cowpea dosage

The research of Pomvrey et al (2003) found that genistein in dosage of 50μM is able to inhibit MMP-9 expression after 72 hours. According to Lee at al (2012), Teruko et al (2007), Kim et al (2008) genistein decreased the proangiogenic factor expression through inhibition of tyrosine

kinase protein (PTK). The giving of genistein of dosage 50 μM induced VEGF-loaded endothelial apoptosis through production inhibition and MMP activitity. The MMP production decrease is caused by the inhibition to VEGF in stimulating the PTK and MAPK activities. Then in the research, it was used genistein as the reference dosage to give the cowpea extract. While the used dosage was 25, 50, and 100μM, with extract PH between 6.9-7.5.

Samples treatment

- The white rat's eyes were topically anesthetized by using *pantocain* eye drop 0.5%
- At the central cornea of rat is adhered filter paper of 5.5 mm diameter that have been immersed in the NaOH solution of 1 M for 60 seconds.
- Group I (negative control) : aquadest application of right eye cornea central of 4 times/day.
- Group II (positive control): 15 right cornea of rats + NaOH 1 M application at the cornea central during 60 seconds. After that it is given aquadest 4 times/day.
- Group III : 15 right cornea of rats + NaOH 1 M application at the cornea central during 60 seconds. After that it is given flavonoid genistein of cowpea extract of eye drop 25 μM of 4 times/day.
- Group IV : 15 right cornea of rats + NaOH 1 M application at the cornea central during 60 seconds. After that it is given flavonoid genistein of cowpea extract of eye drop 50 μM of 4 times/day.
- Group V : 15 right cornea of rats + NaOH 1 M application at the cornea central during 60 seconds. After that it is given flavonoid genistein of cowpea extract of eye drop 100 μM of 4 times/day.
- Each day the rats were given feed suitable with the needs up to enucleation at the hour of 6^{th}, 24^{th}, 48^{th}, 96^{th}, and 168^{th}, while for negative control group it is done enucleation at day 4^{th}

The cornea samples taking for observation of MMP-9 and VEGF observation

- Line between the highest point (A and C) at the anterior stroma surface and cornea back surface (B and D) were used as references of defect edge area.(Figure 1)
- The defect edge area is selected randomly (A-B or C-D), MMP-9 and VEGF expressions were examined at all cornea thickness, with 1 mm in width.

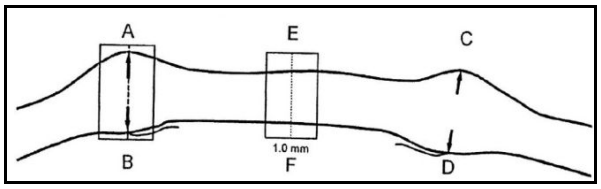

Figure 1. Location of cornea samples taking

Examination of cornea immunohistochemical

Including the process of:

1. Making block paraffin
2. De-paraffinization process
3. Examination of MMP-9 and VEGF expression
4. Calculation of MMP-9 and VEGF expression

Statistical analysis

The data were analyzed by two way anova to prove the difference between control, dosage of cowpea extract giving to change the MMP-9 and VEGF expression based on treatment time at the rats cornea. The statistical test can be said as significant if $p<0.05$. The sample homogeneity test using Lavene statistic and sample normality using Kolmogorof-Smirnov. If the data do not fulfill the normality and variance homogeneity assumption then it can be done by Friedman test. If it is obtained difference, it is continued by post hoc test (multiple comparison, Tukey). To know the

relationship and the influence of cowpea extract dosage to the MMP-9 and VEGF expression, we used the correlation-regression test. The calculation process used computer software of SPSS 16 for Windows.

Results

Genistein in the cowpea extract has influence to decrease the MMP-9 and VEGF expression. It can be seen in table 1 presented the mean of MMP-9 expression in each treatment group based on the observation duration. While in table 2 presented the VEGF expression.

Table 1. Mean of MMP-9 Expression for Each Treatment Group Based on Observation Time

Duration of observation	Positive control	25 µM	50 µM	100 µM
6 hour	16,00 ± 4,36[a]	15,33 ± 3,06[a]	19,00 ± 1,00[a]	16,33 ± 2,08[a]
24 hour	18,00 ± 5,20[b]	16,00 ± 2,65[b]	15,33 ± 3,79[b]	15,33 ± 3,21[b]
48 hour	25,00 ± 1,73*	17,33 ± 3,21[c]	14,00 ± 3,61*	10,67 ± 1,15*
96 hour	29,00 ± 2,65*	15,00 ± 3,46*	11,33 ± 1,53*	10,67 ± 1,53*
168 hour	30,00 ± 2,65*	15,67 ± 2,52*	11,00 ± 2,65*	9,67 ± 2,08*

Explanation: same letters show not significant difference (p>0.05)*) p<0.05 = significant difference with the control

Table 2. Mean of VEGF Expression for Each Treatment Group Based on Observation Time

Duration of Observation	Positive control	25 ìM	50 ìM	100ìM
6 hour	13,33±4,93[abcd]	14,33±3,21[bcde]	12,00±2,00[abc]	17,67±3,21[cdef]
24 hour	13,00±3,60[abcd]	12,67±2,51[abcd]	11,00±1,00[abc]	15,00±4,00[bcde]
48 hour	18,67±2,08[cdef]	11,00±1,00[abc]	9,33±0,58[ab]	9,33±2,08[ab]
96 hour	21,67±2,51[ef]	20,00±1,00[def]	5,67±1,52[a]	6,00±1,00[a]
168 hour	25,33±3,78[f]	20,00±1,00[def]	6,3±2,89[a]	6,33±1,53[a]

Explanation: same letters show not significant difference (p>0.05) *) p<0.05 = significant difference with the control

Based on the expression results of MMP-9 and VEGF at the four treatment groups showed the influence of each cowpea dosage, where the higher dosage then the longer observation time, then the lower MMP-9 and VEGF expression of cornea. So, it can be concluded that the giving of cowpea extract has influence to the MMP-9 and VEGF expression decrease. Figure 2 showed the MMP-9 expression at the four treatment groups, and figure 3 showed VEGF expression at the four treatment groups.

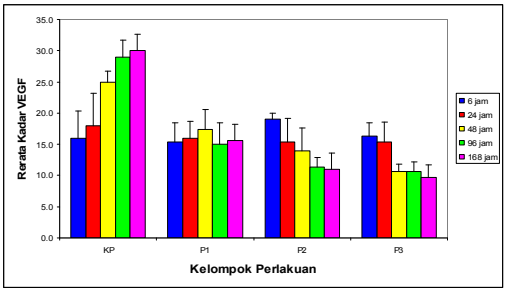

Figure 2. Chart of MMP–9 Expression on Four Treatment Group

- KP : positive control (without cowpea extract)
- P1 : 1st control with 25 µM cowpea extract
- P2 : 2nd control with 50 µM cowpea extract
- P3 : 3rd control with 100 µM cowpea extract

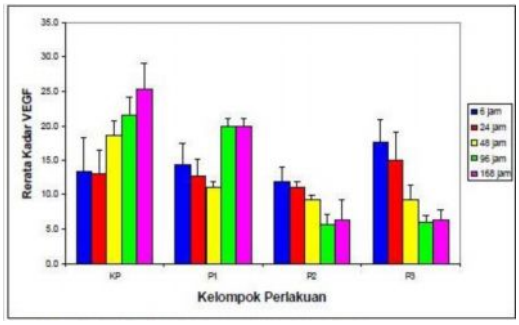

Figure 3. Chart of VEGF Expression on Four Treatment Group

- KP : positive control (without cowpea extract)
- P1 : 1st control with 25 μM isoflavonoid genistein cowpea extract
- P2 : 2nd control with 50 μM isoflavonoid genistein cowpea extract
- P3 : 3rd control with 100 μM isoflavonoid genistein cowpea extract

To know the influence of various dosage of genistein in the cowpea extract of eye drop topical at the inflammatory model cornea to the MMP-9 and VEGF expression, it is tested by two way ANOVA. Before the test is done, it needs fulfillment for some data assumptions, that is the data should have normal distribution and homogenous variance. Based on the data normality test using Kolmogorov-Smirnov can be concluded that the variance of MMP-9 and VEGF expression data were homogenous.

Then they were analyzed to know the MMP-9 and VEGF expression data difference based on the four treatment groups (KP, P1, P2, and P3) and the observation time, using two way ANOVA (analysis of variance). Based on the observation results of MMP-9 and VEGF expression difference showed the influence of each cowpea extract dosage, where the higher dosage the longer observation, will be followed by the lower MMP-9 and VEGF expression. Then, based on the descriptive evaluation, according to the MMP-9 and VEGF expression mean, it can be said that the cowpea extract giving influence the MMP-9 and VEGF expression decrease if compared with the MMP-9 and VEGF expression at the positive control group.

Based on ANOVA results, it showed the significance value for treatment groups of cowpea extract dosage to the MMP-9 and VEGF expressions is 0.000 ($p<0.05$) and to the VEGF is 0.000 ($p<0.05$), so it can be concluded that there is difference of MMP-9 and VEGF expression based on the cowpea extract dosage. For the observation time showed significance value of MMP-9 expression is 0.990 ($p>0.05$) and VEGF expression is 0.140 ($p>0.05$), so it can be concluded that there was no difference of MMP-9 and VEGF expression based on the observation time.

For interaction between treatment groups of cowpea extract dosage variation and the observation time showed significance to the MMP-9 expression is 0.000 ($p>0.5$) and to the VEGF expression is 0.000 ($P>0.05$), so it can be concluded that there is MMP-9 and VEGF expression difference based on interaction between cowpea extract dosage with the observation time.

The MMP-9 and VEGF expression differences as the influence of the cowpea extract giving also can be seen in the figure 4 and 5.

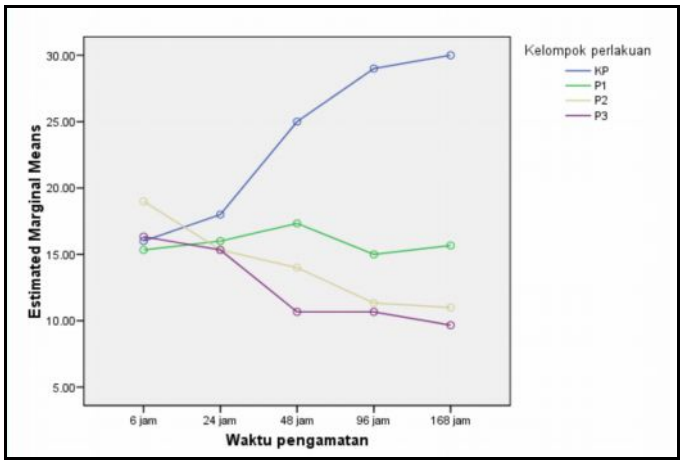

Figure 4. MMP-9 expression graph based on the treatment dosage and treatment time

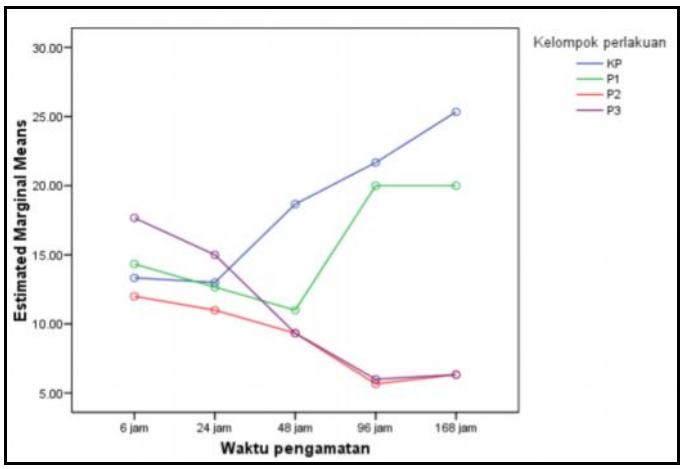

Figure 5. VEGF expression graph based on the treatment dosage and treatment time

Then to know the relationship magnitude of cowpea extract in dosage of 25, 50 and 100µM eye drop topical at the model rat cornea with MMP-9 and VEGF expression, then it was used correlation test .

Table 3. Conclusion of MMP-9 expression correlation test

Explanation	r	P	Conclusion
MMP-9 expression with observation time	0.004	0.487	No significant correlation
MMP-9 expression with dosage treatment group variation of cowpea extract	-0.606	0.000	There was significant correlation

Based on analysis above (table 3) it can be known that the dosage treatment group of cowpea extract (r =-0.606, p =0.000) has significant correlation ($p<0.05$, Ho was rejected) with MMP-9 expression, with negative correlation direction (because the correlation coefficient had negative value). It means the dosage increase of cowpea will decrease the MMP-9 expression and vice versa. While for the observation time (r =0.004, p =0.487) did not have significant correlation ($p<0.05$, Ho was rejected) with MMP-9 expression. It mean the increase or decrease of MMP-9 expression did not correlate with the observation time.

Based on analysis results above (table 4), it can be known that the treatment group of cowpea extract concentration (r =-0.493, p =0.000) had significant correlation ($p<0.05$, Ho was rejected)

with cornea VEGF expression, with negative correlation direction (because correlation coefficient had negative value). It meant the concentration increase will decrease the cornea VEGF expression and vice versa. While the observation time (r = 0.057, p =0.333) did not have significant correlation (p<0.05, Ho was rejected) with cornea VEGF expression. It meant the increase or decrease of VEGF expression did not correlate with the observation time.

Table 4. Conclusion of VEGF expression correlation test

Explanation	r	P	Conclusion
VEGF expression with observation time	0.057	0.333	No significant correlation
VEGF expression with dosage treatment group variation of cowpea extract	-0.493	0.000	There was significant correlation

How much influence of treatment group variation of cowpea extract and the observation time to the MMP-9 and VEGF expression, can be known by using the regression analysis. Based on the test by using linear regression showed that there was significant influence (p = 0.000<0.05, Ho was rejected) from the dosage treatment of cowpea extract to the MMP-9 expression, while the observation time (p =0.967>0.05) did not influence significantly to the MMP-9 expression. Beside that at the regression test results also showed the determinant coefficient value R^2 of 36.7% that stated that the influence level of the observation time and extract dosage of cowpea to the MMP-9 expression, in the form of percentage, and the remain percentage (1-R square) of 63.3% of the MMP-9 expression variance was influenced by the other factor beside the observation time and cowpea extract dosage.

Discussion

Inflammatory induction

In the research, we used inflammatory model cornea by chemical cauterization to stimulate the angiogenesis. Cogan stated that the cornea neovascularization pathogenesis is cornea stroma edema after trauma. The new blood vessel will emerge earlier and more intensive at the most edema cornea area.[5] At the process involves the MMP-9 to the occurrence of cornea neovacularization. At the inflammatory condition, it will occurs neutrophile invasion that will stimulate the MMP-9 expression at the cornea. And the MMP- 9 will induce the neovacularization occurrence.[27]

Knighton *et al* stated that lesion or inflammation is hypoxia condition that relates with angiogenesis. The hypercellular condition at the inflammatory acute phase caused the hypoxia environment that become the angiogenesis trigger. Growth factor that is secreted at the condition is VEGF that will be strengthened by cytokine such as interleukin that is known also able to stimulate the VEGF production. Some researches also showed that when the inflammatory cascade occurs, enzyme cyclooxigenase 2 (COX 2) activation occurred. Beside at the inflammatory process, COX 2 also improve at the neoplasm angiogenesis. COX 2 stimulate the VEGF stimulation by the inflammatory cell that initiate the angiogenesis.[28]

Inflammatory induction at cornea can be done with some procedures, such as the using of AgNO3, NaOH, and application of bFGF plate at the cornea stroma.[29-30] At the research, inflammatory induction done by using NaOH 1 M by placing the filter paper that has been immersed in the NaOH 1 M at the cornea tips for 60 seconds. The induction success is marked with the emergence of whitish color at the induction place. The preliminary study showed that NaOH 1M induction give inflammatory response in the form of whitish color at the induction place, and followed with hyperemia and blefarospam. At the research, we used NaOH because the simple method and easier to be used compared with the bFGF plate, and more potent compared with AgNO3. The inflammatory response begin to appear at the 3-6 hours after inflammatory induction with NaOH 1 M, while if using AgNO3, the response emerges after 24 hours.[7,30-31]

The decrease of matrix metalloproteinase-9 (MMP-9) and VEGF expression after giving of cowpea extract

The angiogenesis mediator that is under research was MMP-9. Where MMP-9 has role in process of migration and tubulogenesis in the angiogenesis. MMP-9 has role in degrading the extracellular matrix (ECM) of endothelial cell of blood vessel basal cell. Also has role in damaging the cornea epithelial integrity, the release of angiogenic factor, cytokine receptor and molecule adhesion.[4-6] At the research to the rabbit cornea, it was done keratotomy and chemical trauma, at the keratotomy condition, it is obtained MMP-9 has important roles in re-epithelialization process of basal membrane. But at the chemical condition, it is obtained the MMP-9 over expression that will cause basal membrane degradation so there was re-epithelializatioin failure and cornea neovascularization occurs.[25-26,31] In Zhang research, it was stated that inhibition to the MMP-9 expression will accelerate the cornea lesion healing.[31]

While growth factor that selected in the research was VEGF. According to the research by Amano *et al*, VEGF is key mediator in the cornea angiogenesis process.[32] VEGF was produced by inflammatory cell such as macrophage, monocyte that was induced by interleukin such as IL-1, IL-8 through inflammatory cascade. The VEGF inhibition able to decrease cornea neovascularization of 50%. Soumyajid stated that VEGF secretion was influenced by clylooksigenase 2 (COX 2) at the prostate neoplasm cell culture where angiogenesis at the prostate neoplasm caused by inflammation process.[28]

To know the MMP-9 and VEGF expression amount at normal cornea in the research given negative control group. Today there is no research that showed VEGF expression amount at the normal cornea but only VEGF-1/ft-1 expression done by Sigh *et al*. The negative control group also has role as comparator for MMP-9 and VEGF expression parameters between dosage treatment to determine the effective dosage (ED) of cowpea extract. The MMP-9 and VEGF expression at the negative control different significantly to the positive control.

MMP-9 at the positive control began to increase at the 6th hour and will increase steadily at the first day up to the fourth day. According to Azar *et al*, MMP-9 will be expressed 6 hour after cornea injuring, experience the peak in the 1st-3rd day and not detected again at the 7th day or more.[5] And according to Amano *et al*, mRNA VEGF expression is little at the normal cornea and increase ten times after cornea lesion.[32] The expression can be seen after 6 hours after lesion, experience the peak at the 1st – 3rd day and can not be detected again at the 7th day or more.

Based on the two way anova test to the MMP-9 expression at the four group obtained $p<0.05$. It showed the influence of each extract dosage of cowpea, where the higher extract dosage of cowpea the longer observation, will be followed by the lower MMP-9 expression. Suitable with the research by Gupta *et al*, Banerjee *et al*, Pomfrey *et al*, Lee *et al*, Kumar *et al*, Teruko *et al*, and Liu *et al*, stated that genistein inhibit the MMP-9 expresion either directly or through inhibition of growth factor that is the upregualtor of MMP-9. Genistein also decreases the MMP-9 expression as the proangiogenic factor through inhibition of protein tyrosine kinase (PTK) activity.[10-11,16-18,33]

The test results of two way anova to VEGF expression showed that the higher dosage and the longer genistein giving will be followed by the lower cornea VEGF expression ($p<0.05$). The genistein giving dosage 100 µM decreased the VEGF and MMP-9 expression the lowest compared with the dosage of 25µM and 50µM. The results suitable with the research by Wei *et al* and Li *et al* where the genistein giving decreased the VEGF expression up to day 21st after NAOH 1 M induction.[30,34] For interaction between treatment group of genistein variation and the observation time showed there were VEGF and MMP-9 expression differences based on interaction between genistein dosage variation and observation time. It is suitable with the research by Wei *et al* and Li *et al* where the giving of genistein at the rabbit cornea decreased the VEGF expression up to day 21st after NaOH 1 M induction.[30,34]

Correlation of Matrix Metalloproteinase-9 (MMP-9) and VEGF Expression Decrease By Giving Various Cowpea Extract Dosage

Meanwhile, based on the correlation test it is known that the dosage treatment group of cowpea extract dosage had significant correlation ($p<0.05$) with MMP-9 expression, with negative correlation direction. It means the increase of cowpea extract dosage will decrease the MMP-9 expression. It is suitable with the research by Gupta *et al*, Banerjee *et al*, and Joussen *et al* stated that the genistein dosage increase able to decrease the MMP-9 expression at the rabbit cornea either directly or through inhibition of VEGF as upregulator.[16-17,29] At the research of Touny stated that dosage genistein of 10-20μM able to decrease the growth and proliferation of prostate cancer cell, at the little dosage <10μM genistein stimulate the estrogen-sensitive cell lines.[35]

In the some research, it was stated that effective dosage of genistein in decreasing the MMP-9 expression and other ocular angiogenesis mediator was 50μM. Hdeib concluded that genistein 50μM is inhibitor of MMP-9 secretion that was potent to mammae cancer cell.[36] Joussen *et al* give genistein 0.5 mg/ml in the form of eye drop at the rabbit cornea and found that at the dosage genistein able to inhibit the MMP-9 expression at the rabbit cornea.[29]

The genistein effectiveness peak of dosage 50μM was at 72 hour and after that the effect decreased.[35] It explained why there was MMP-9 expression after 96th hour and 168th hour, where the genistein activity was not balanced because of LPS induction of induced NF-KB and by tyrosine kinase protein activation and induction of TNFα and IL-1β that occurred at first 4th -24th hour and the peak at the 72nd hour.[9,37-38] It was explained why there was MMP-9 expression decrease after 72 hours.

Based on the regression test also showed that the cowpea extract dosage influence to the MMP-9 expression up to 36.7%. While 63.3% of MMP-9 expression variance was influenced by other factor beside cowpea extract dosage. In some research it was stated that the genistein mechanism in decreasing the MMP-9 expression in the inflammation process by inhibiting directly to the MMP-9 expression or through inhibition of tyrosine kinase protein activation.[11,13-18]

While other factors that was possible to influence the MMP-9 expression was the VEGF expression decrease so it will be occurred the cellular proliferation decrease, angiogenesis decrease, and VEGF also the upregulator of MMP-9.[11,14,18,27] In the research of Hasan, it was found the influence of genistein in the cowpea extract to the VEGF expression decrease of 24.4%.[40] Other factor that influences the MMP-9 expression possibly was the presence of antiangiogenesis molecule such as thrombospondin, angiostatin, and endostatin.[5,9,37-38]

In the research it was known that MMP-9 expression mean at the negative control was 3.6667. So it could be known that the effective dosage (ED) of the methanol extract dosage of cowpea reach the MMP-9 expression mean of 3.6667 needed dosage of 89.9456. It was suitable with the Joussen *et al* research that stated the antiangiogenesis effect of genistein in vitro with half maximal effect at the 150μM.[29]

Based on the correlation test to the VEGF expression, it was obtained the results of dosage treatment group and genistein observation time had significant relation ($p<0.05$), with cornea VEGF expression, with negative correlation direction. From the test, it can be concluded that the genistein dosage improvement will decrease the cornea VEGF expression.[29,41] The results suitable with Guo *et al* and Joussen *et al* that concluded the increasing genistein isoflavonoid concentration able to decrease the VEGF expression at the rabbit cornea. According to Guo *et al* that concentration of genistein isoflvaone of 10-50μM decrease the accumulation of PC-3 nuclear inducible factor-1 alpha (HIF-1α). HIF-1α is the most important transcription factor that regulates the VEGF to the hypoxia.[41]

In the research, the genistein effective dosage was different at each research time because at the multiple comparison test obtained significant differences. According to Guo *et al*, concentration of genistein isoflavone 5-50μM able to decrease the VEGF expression and expression of H1Fα

stimulated VEGF.[41] According to Wei *et al*, genistein concentration 30g/L or 30μM, the decreasing effect of VEGF expression at the rat cornea that was induced by NaOH 1 M. Beside that, concentration of genistein 30μM decreased the cornea neovascularization at the rabbit.[30,32] According to Li *et al*, inhibition onset of COX 2 expression by concentration of genistein 25μM occurs up to 24^{th} – 48^{th} hour then the effect will be stable after 72 hours while the VEGF expression increased at the 48 hours.[42]

Some researches stated the genistein isoflavonoid mechanism in decreasing the VEGF expression in the inflammatory process. At the initial stage of inflammation, genistein isoflavonoid inhibit the COX 2 enzyme. The inhibition of COX 2 enzyme able to decrease the VEGF expression in the cornea angiogenesis.[28] At the inflammation condition, there was hypercelular condition of the tissue. According to Amano *et al*, the hypercellular condition caused the tissue become hypoxia environment. The hypoxia environment stimulate the expression increase of hypoxia-inducible factor-1 alpha that is the most important transcription factor in regulating VEGF to the hypoxia.[32] According to Guo *et al*, isoflavonoid genistein decreased the VEGF basal expression and hypoxia inducible factor-1 alpha at the prostate cancer cell.[41] Beside that, isoflavonoid decreased the mRNA VEGF expression, basal VEGF at the prostate cancer cell, ovarium and mammae.[34,41] At the study in vitro, isoflavonoid genistein decreased the hypoxia stimulated VEGF expression at the HUVEC and PC-3 cells.[43]

From data r2 can be concluded the cowpea concentration conclusion influence significantly to the cornea VEGF expression up to 24.4%. While 75.6% of cornea VEGF expression variance was influenced by other factors beside the observation time and cowpea extract concentration. Other factor that influence the VEGF expression possibly was the antiangiogenesis molecule presence such as thombospodin (TSP-1), PEDF, endostatin.[25,44]

Endostatin is antiangiogenesis that works through various ways, such as stopping the gelatinase (MMP-2 and MMP-9), inhibiting bFGF and VEGF, and inhibits the TNFα activation.[5,10,25] According to Kumar in his research stated that endostatin stops some metalloproteinase, that is gelatinase group (MMP-2 and MMP-9) of 12% from the MMP-9 expression.[10]

Thrombospondin is antiangiogenesis that expressed at the bowman membrane, endothelial cell, and descement membrane, thrombosondin expression increases at the inflammatory condition. Azar and Shakiba stated that thrombospondin works as the antiangiogenesis by inhibiting MMP-9 expression at the endothelial cell.[5,25]

Angiostatin works as antiangiogenesis by inhibiting endothelial cell proliferation, migration and tubular formation. According to Qazi, at the murine model, angiostatin inhibits angiogenesis by inhibiting the expression of MMPs (MMP-2, MMP-3, MMP-7, MMP-9, and MMP-12) and bFGF.[25,26]

The research limit such as, the lack research time where it should be observed for 21 days up to the neovascularization occurred completely.

Conclusion:

Based on the research, it can be concluded that there was MMP-9 and VEGF decrease after giving the cowpea extract (*Vigna unguiculata*) at the inflammatory model cornea. And there was correlation of MMP-9 and VEGF expression decrease by giving the cowpea extract of eye drop topical with concentration of 25, 50, and 100μM, but the giving time does not influence.

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