EFFECTS OF COWPEA (*VIGNA UNGUICULATA)* EXTRACT IN VEGF EXPRESSION OF CORNEAL INFLAMMATION RAT MODEL (*RATTUS NOVERGICUS* STRAIN *WISTAR)*

Yuliono Trika Nur Hasan*, Nanda Wahyu Anandita*, Husnul Khotimah**

ABSTRACT

Purpose *: to evaluate the effects of Methanolic extract of Cowpea (Vigna unguiculata) administration on vascular endothelial growth factor (VEGF) expression in rats with corneal-alkali induced inflammation.*

Methods *: This was an interventional experimental study. Sixty three rats were randomly selected, 3 rats as control, 60 rats were treated with 1 M NaOH (as inflammation model) and administered with 25 ìM,50 ìM and 100 ìM of genistein in Cowpea (Vignau ngucuilata) extract four times daily. Alkali burn by 1 M NaOH infiltration using filter paper applicated on the center cornea of right eye for 60 seconds. Aquabidest as positive control and Cowpea (Vigna ungucuilata) extract treatment perfused immediately after alkali burn. VEGF observed at 6, 24, 48, 96 and 168 hours by immunohistochemical method.*

Results *: Each dose of Cowpea (Vigna unguiculata) extract administration significantly decreased the VEGF expression (p = 0,00). Differences of VEGF expression based on administration time were not significant (p=0,033). Interaction between time of administration and dose of administration had influenced the VEGF expression (R Square* (r^2) *=24,7 %). The Linear regression between VEGF expression and dose resulted in estimated effective dose (ED) with Y = 16.486– 0.079 X(Y = VEGF expression , X = dose of administration). Considering VEGF expression in normal cornea was 12, we found the effective dose of methanolic Vigna unguiculata extract was 61.94 ìM.*

Conclusion *:Methanolic extract of Cowpea(Vigna unguiculata) decreased the VEGF expression on alkali burn corneal inflammation in rats but no differences in VEGF expression based on time administration. Dose of 50 ìM genistein in Methanolic extract of Cowpea (Vigna unguiculata) was close to effective dose 61.94 ìM.*

Keywords : *VEGF expression, alkali burn, Cowpea (Vigna unguiculata) extract, cornea.*

INTRODUCTION

Corneal inflammation is an important clinical sign and is often found in cases of chemical trauma, herpetic keratitis, corneal graft rejection and other conditions. Inflammatory cell infiltration,edema and neovascularization associated with reversible corneal opacity and visual lost. *(Gagen D. 2011)*

During inflammation, secretion of chemokines and cytokines occur and stimulate growth factor to the wound healing process. In addition to wound healing, growth factors play an important role in the process of corneal angiogenesis. (*Gagen D, 2011)*

Corneal neovascularization is the growth of blood vessels from the limbus towards the central cornea caused by the loss of immune privileged and balance pro- and anti-angiogenic disturbed. This condition characterized by new blood vessels in the cornea that causes a decrease in clarity and interfere with vision. Corneal neovascularization is a leading cause of vision loss and blindness from corneal disease all over the world. In the United States approximately 4% of the population occurs corneal neovascularization and 1.4 million patients are at risk ofcornea neovascularization every years.*(Wei, HZ, Zhu, 2009)*

Pathological conditions such as inflammation, infection, degenerative and trauma can induce corneal neovascularization. Several growth factors and proteinase enzymes involved in corneal neovascularization. Angiogenic factors

* *Ophthalmology Department of Brawijaya Medical Faculty Malang/ Saiful Anwar General Hospital*

*,** *Pharmacology Department of Brawijaya Medical Faculty*

released from corneal epithelial cells and stromal cells and infiltration of immune cells such as macrophages, leukocytes, monocytes. Angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (bFGF), tumor necrosing factor (TNFá) excessive secreted in angiogenesis process. *(Wei, HZ, Zhu, Z.L. 2009, Fotsis, T. Pepper, H. Adelcreutz, G. Felischmann, T. Hase, R. Montesano, L. Scheigeger., 1993, Polkowski K. 2000)*

Some studies suggest that VEGF plays an important role other than growth factor in cornea neovascularization.[4] VEGF concentration was significantly more in the cornea than normal cornealneovascularization. Excessive expression of VEGF causes angiogenesis activation stages that include proteolysis activity, endothelial cell proliferation, migration and tubulogenesis *(Azar, D.T. 2006, Fotsis, T. Pepper, H. Adelcreutz, G. Felischmann, T. Hase, R. Montesano, L. Scheigeger., 1993)* In addition, VEGF directly

induces endothelial cell proliferation in angiogenesis. Inhibition of VEGF can prevent corneal neovascularization compared to 30-50% inhibition of other mediators of angiogenesis and maintain clarity kornea *(Wei, HZ, Zhu, Z.L. 2009, Azar, D.T. 2006)* Cowpea is a source of phytoestrogens and widely used isoflavone component, which is the main component genistein and daidzein *(Amano S, Rohan R, Kuroki M, Tolentin M, Adamis A. 1998, Guo L., Hussain A. A., Limb G. A. and Marshall J. 1999)* Genistein (4H-1-benzopyran-4-1,5,7- Dihydroxy-hydroxiphenyl 3-4) is the family of Leguminosae isoflavonoids which is a synthetic intermediate in other isoflavonoids.*(Agarwal, R. 15 October 2000, Takeshi. U. 2006*) According to Yuaris*et al* (2012), cowpea (KT 6 variant) growing in Nusa Penida Bali contains high isoflavonoids. This is likely due to the different soil nutrients. Some studies show that genistein decreased VEGF expression in cultured HUVEC, and carcinoma. Moreover genistein decrease the synthesis of cycloksigenase enzymes 2 (COX 2) that would induce the expression of VEGF in inflammation. To determine the effect of isoflavonoid genistein methanoic extract of cowpea on the expression of VEGF in the process of corneal angiogenesis, we used NaOH 1 M topical to induce corneal inflammation in the study.

METHODS

This study is an experimental method with a double-blind randomized between groups of treatment with the control group. Experimental animals Rattus novergicus wistar strain were used and randomly selected with the following inclusion criteria: (1) Sex male rats, (2) adult with age between 10 -12 weeks, (3) weight between 150-250 g rats and (4) healthy rats (active, pure white fur, health eyes). Exclusion criteria were died sample during treatment, corneal infection. Sixty right cornea rats were divided into four treatment groups, the positive control group given distilled water four times a day and the treatment group were given extracts of cowpea isoflavonoid genistein concentration of 25 ìM, 50 and Data were analyzed using two way ANOVA test and correlation-regression. The process of computation is performed with the aid of computer software SPSS 16 for windows.

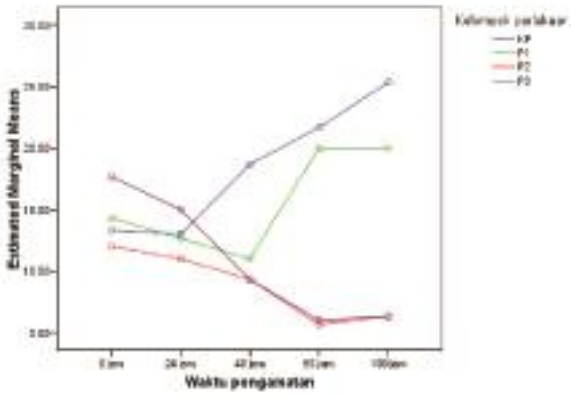
RESULTS

Table 1 and Figure 1 presents the mean and the interaction of VEGF expression by treatment group based on time observation. The mean expression of VEGF in the negative control in this study was 12. On the positive control tends to increase from 24 hours to 168 hours. At a concentration of 25 ìM VEGF expression at 6 hours and 24 hours is almost the same as a positive control and then decreased at 48 to 168 hours. At a concentration of 50 ìM decreased VEGF expression began at 6 hours and then declined later settled in 96 hours and 168 hours. At a concentration of 100 ìM, VEGF expression is greater than the positive control for up to 24 hours and then begin to decline at 48 hours and settled at 96 hours and 168 hours

Table 1. The mean expression of VEGF each group of Treatment Based on Time Observations

Time	Positive Control	Concen-tration 25 µM	Concen-tration 50 µM	Concen tration 100 µM
6 hr	13,33 ± 4,93^{abcd}	14,33 ± 3,21^{bcde}	12,00 ± 2,00^{abc}	17,67 ± 3,21^{cdef}
24 hr	13,00 ± 3,60^{abcd}	12,67 ± 2,51^{abcd}	11,00 ± 1,00^{abc}	15,00 ± 4,00^{bcde}
48 hr	18,67 ± 2,08^{cdef}	11,00 ± 1,00^{abc}	9,33 ± 0,58^{ab}	9,33 ± 2,08^{ab}
96 hr	21,67 ± 2,51^{ef}	20,00 ± 1,00^{def}	5,67 ± 1,52^{a}	6,00 ± 1,00^{a}
168 hr	25,33 ± 3,78^{f}	20,00 ± 1,00^{def}	6,3 ± 2,89^{a}	6,33 ± 1,53^{a}

Table 2 shows the test results ANOA Two way between groups. Based on the results of the ANOVA showed that there were differences in the expression of VEGF by variations in cowpea extract concentration (p = 0.00) and there was no difference in VEGF expression by long-time observation 0140 (p> 0.05). For the interaction between treatment groups extract concentration variation cowpea and time of observation obtained p = 0.000 (p>0.05), and concluded that there are differences in the expression of VEGF by the interaction between variations in the concentration of extract of cowpea and observation time.



Variabel	F	Sig. (p)
Time observation	1.840	0.140
Groups	43.034	0.000
Interaction group and time	11.693	0.000

Based on the results of linear regression analysis showed that there was a significant effect (p = 0.000 <0.05) of treatment cowpea extract concentration on the expression of VEGF cornea, while the length of time of observation (p = 0623>0.05) had no significant effect on the expression of VEGF cornea. The results are the following regression equation Y = 16.880-0.079 x with Y = corneal VEGF expression and X = concentration of extract Based on the above calculation can be seen that the Effective dossage (ED) from cowpea extract concentration to achieve an average corneal VEGF expression by 12, it takes a concentration of 61.94iM.

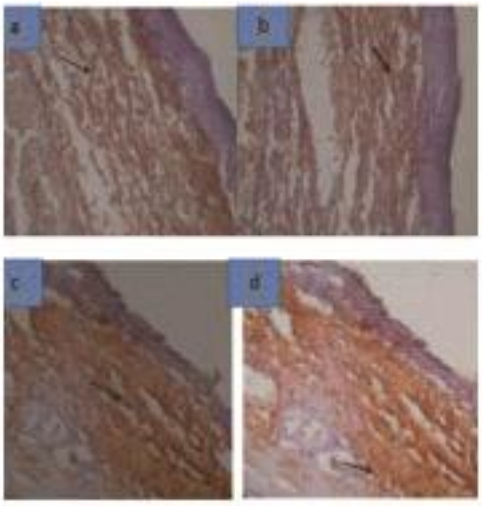


Figure 2. VEGF expression at 6 h after induction with (a) as positive contro, (b) cowpea extract 0,25µM, (c) cowpea extract 0,50 µM, (d) cowpea extract 100 µM

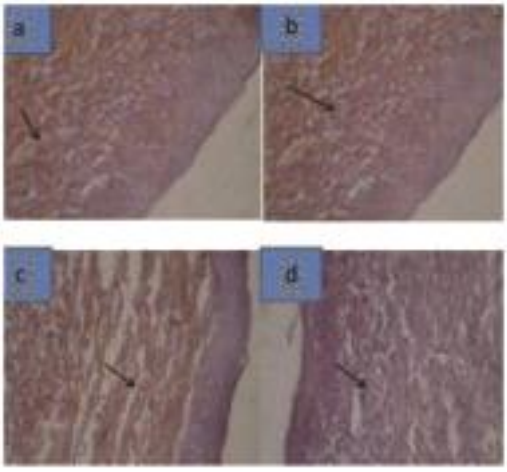
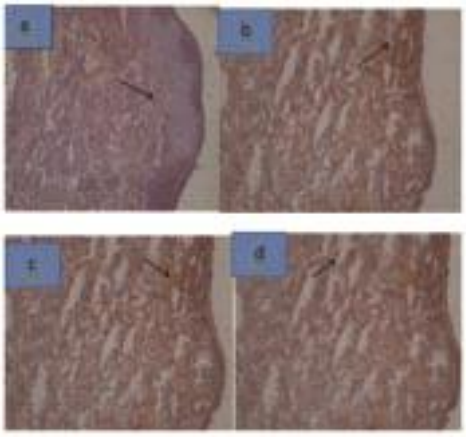


Figure 2. VEGF expression at 24 h after induction with (a) as positive contro, (b) cowpea extract 0,25μM, (c) cowpea extract 0,50 μM, (d) cowpea extract 100 μM



DISCUSSION

Growth factors selected in this study were VEGF. According to Amano et al (1998), VEGF is a key mediator in the process of corneal angiogenesis kornea.*(Amano S, Rohan R, Kuroki M, Tolentin M, Adamis A. 1998)* VEGF produced by inflammatory cells such as macrophages, monocytes induced by interleukin such as IL-1, IL-8 through a cascade inflamasi.*(Gagen D., 2011, ,Amano S, Rohan R, Kuroki M, Tolentin M, Adamis A. 1998)*. Inhibition of VEGF can reduce corneal neovascularization by 50%. Soumyajid (2010) concludes that the secretion of VEGF by cyclooksigenase 2 enzyme (COX 2) in cell cultures of prostate neoplasia where angiogenesis in prostate neoplasia were caused by the inflamation.*(Soumyajit, M.Srirangam, R. 2010)* were significantly different compared to the positive control group. Decreased expression of VEGF concentration of 25 ìM significantly different than the concentration of 50 ìM and 100 ìM. The data are consistent with the Guo et al (2006) that the isoflavone genistein concentrations of 10- 50 ìM decrease the accumulation of PC-3 nuclear inducible factor-1 alpha (HIF-1á). HIF-1á is an important transcription factor that regulates VEGF against hipoxia.*(Guo L., Hussain A. A., Limb G. A. and Marshall J. 1999)* Increased expression of VEGF after 98 and 168 hours may be caused by the activity of isoflavonoid genistein to decrease VEGF expression is not balanced by the increased expression of VEGF. Increased expression of VEGF-related induction of COX 2 overload occurs in 72 hours. Isoflavonoid genistein supressed COX 2 expression through NF-kB within 72 hours after that the effect decreased. According to Li et al (2011), the onset of inhibition of COX 2 expression by genistein concentration of 25 ìM occur until 24-48 hours later the effect persists after 72 hours, while increased VEGF expression at 48 hours. This makes the expression of VEGF tended to increase after 72 hours at[14,15]VEGF expression in the positive control begins to increase significantly at the sixth hour and peaked at 48 hours. According to Amano et al (1998), VEGF expression began to increase on the first day and peaked on third day, while Wei et al (2009) stated that VEGF expression of rabbit cornea induced increases NaOH and settled on the third day.*(Wei H, ZiLan, 2009)*

In the treatment group concentration of 25 ìM, decreased expression of VEGF were significantly different compared to the positive control group. Decreased expression of VEGF concentration of 25 ìM significantly different than the concentration of 50 ìM and 100 ìM. The data are consistent with the Guo et al (2006) that the isoflavone genistein concentrations of 10-50 ìM decrease the accumulation of PC-3 nuclear inducible factor-1 alpha (HIF-1á). HIF-1á is an important transcription factor that regulates VEGF against hipoxia. *(Guo L., Hussain A. A., Limb G. A. and Marshall J. 1999)*

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occurs in 72 hours. Isoflavonoid genistein supressed COX 2 expression through NF-kB within 72 hours after that the effect decreased. According to Li et al (2011), the onset of inhibition of COX 2 expression by genistein concentration of 25 ìM occur until 24-48 hours later the effect persists after 72 hours, while increased VEGF expression at 48 hours. This makes the expression of VEGF tended to increase after 72 hours at a concentration of 25 ìM.

In the treatment group concentration of 50 ìM, decreased expression of VEGF begin at 6 hours until 96 hours after the activity settled up to 128 hours (p = 0.00). Based on the study by Li et al (2002), a concentration of 50 ìM genistein decrease the expression of VEGF and VEGFR within 48 hours in cell culture prostatic neoplasms.This effect was slightly reduced after 36 hours and was reduced to half after 72 hours. There is no scientific explanation for the paradoxical effects ini.*(Li et al, 2011)* Yu et al (2008) showed that genistein concentration of 50 ìM decrease the expression of VEGFprotein and VEGF mRNA expression in breast cancer cells 24-72 hours after administration and the effect then decreased.(*Lin Y,Yue. L. 2007)*

At a concentration of 100 ìM, VEGF expression increased at 6 hours and 24 hours. Decreased expression of VEGF decreased at 24 hours and settled in the next hour (p = 0.00). High VEGF expression at 6 hours and 24 hours were susceptible of toxicity effects. After the second day will decrease due to the effects of the extract genistein achieved onset.*(Joussen, Rohrscheneider, Reichling, Kirchoff, Kruse, 2000, Luo H. 2008)* Expression of VEGF that persists after 48 hours in accordance with the research joussen (2007) on ovarian cancer cell cultures, a concentration of 100 ìM genistein has a similar effect to the concentration of genistein 50 ìM in reducing expression VEGF.*(Kim.H, Wonil.K. Hyo.L.J, 2010)*

In this study effective dossage (ED) of the isoflavonoid genistein concentration cowpea extract was 61.94 ìM. This concentration is slightly larger than the study by Guo et al (2006) at 50 ìM in HUVEC, Li et al (2002) at 50 ìM in prostate cancer cells, Pepper et al (2004) of 30 ìM on BME, Yu et al (2008) at 50 ìM in cancer mamma.13, 14.16 but the result is smaller than the study conducted by Jaussen et al (2010) and Wei et al (2009) using a concentration of 5 mg / ml and 10 mg / ml in mouse models with chemical cauterization.*(Joussen, Rohrscheneider, Reichling, Kirchoff, Kruse, 2000)*

From the regression (r2) cowpea extract considerable influence on the expression of VEGF cornea up to 24.4%. While 75.6% of corneal VEGF expression variability is influenced by factors other than time observation and cowpea extract concentration. Other factors that influence the expression of VEGF is probably the molecule antiangiogenesis as thombospodin (TSP-1), PEDF,endostatin.*(Gagen D., 2011, Azar, D.T. 2006)*

CONCLUSION

Isoflavonoid genistein of extracts of cowpea (*Vigna unguiculata*) topical ophthalmic concentration of 25 ìM, 50 ìM and 100 ìM decreased the expression of corneal VEGFinduced by NaOH 1 M in rat. Effective dose (ED) extracts of cowpea research was 61.94 ìM

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