INTRODUCTION

One of the major causes of maternal deaths is hypertension during pregnancy. Based on population census data in 2010, 3.2% of maternal mortality was caused by hypertension during pregnancy [1]. One of the hypertension complications during pregnancy is preeclampsia. The incidence rate of preeclampsia in developed countries is range from 2% to 8% [2]. A research conducted by Cho et al showed that the incidence rate of preeclampsia in South Korea is 3.1% [3]. In Indonesia, a research conducted by Warouw and team in RSUP Dr. R. D Kandau Manado showed that the incidence rate of preeclampsia was 6%; meanwhile, a research conducted by Djannah in RSU PKU Muhammadiyah Yogyakarta concludes that the incidence rate of preeclampsia was 16.1% [4,5].

Clinically, preeclampsia is signed by a rise in blood pressure and proteinuria occurs after 20 weeks of pregnancy [5]. Genetically, in Indian woman, preeclampsia is associated with gene polymorphism, and there was significant association between vascular endothelial growth factor (VEGF-C) 405G and VEGF-C [6]. The exact cause of preeclampsia is not known yet until now but is likely to involve several factors such as antiphospholipid antibody syndrome, kidney disease, diabetes mellitus, obesity, systemic erythematosus, and nulliparity [7]. The imbalance of angiogenesis factor such as VEGF and renin-angiotensin system (RAS), and antiangiogenesis factor like sFlt-1 is expected as the cause of placenta failure, angiogenesis, and vasculogenesis [8,9]. In normal pregnancy, there is a correlation expression of (pro)renin receptor ([P]RR) with VEGF mRNA [10]. A research conducted by Kanda et al on primary human retinal microvascular endothelial cells (HRMECs) concludes that there is a relation between the activity of [P]RR and angiogenesis on retinopathy diabetic [11]. The raise of prorenin concentration will increase the level of VEGF mRNA. That is, why is it believed that there is an involvement of [P]RR in VEGF regulation mechanism on pathogenesis preeclampsia so that this research is conducted to analyze the expression relation between (P)RR and VEGF on placenta of the third-semenat pregnant woman with preeclampsia.

METHODS

This research was an observational study with control–case study design. It was conducted at the Laboratory of Molecular Biology for Oxidative Stress, Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia. It was conducted on January–April 2017. Samples used in this study were collected from the placenta tissues of woman with the third-trimester pregnancy divided into two groups: Normal and preeclampsia (biological material stored). There were 34 tissue’s samples of normal pregnancy and 34 of preeclampsia pregnancy. This research has already approved by Health Studies, Faculty of Medicine Ethics Committee, Universitas Indonesia, number 104/UN2.F1/ETIK/2017.

The total RNA isolation process uses total RNA mini kit (Geneaid). The concentration and purity of RNA were measured using Varioskan. The average concentration of RNA total samples was 175.88 (14.025–588.7) µg/mL, while the average purity index was 1.874 (1.6–2.1).

The analysis of relative expression of (P)RR and VEGF mRNA was conducted with reverse transcriptase-PCR technique, using SensiFast™ SYBR No-ROX one-step kit real-time polymerase chain reaction (qRT-PCR) kit as same as Dongare et al [12]. For analysis of (P)RR gene, the primer used was forward primer 5’-GAT GGT GAA GGG AGT GAA CAA-3’ and reverse primer 5’-TGG AAT TTG CAA CAC TGT CAA G-3’. For analysis of VEGF gene, the primer used was forward primer 5’-CTG AGT GGT GAA GGG AGT GAA CAA-3’ and reverse primer 5’-CTT TTG TCT GCA TTC AC-3’. For reference gene, this research used 18s rRNA gene with primer: Forward
VEGF mRNA expression increased 2.83 times higher than normal placenta. On placenta tissue with preeclampsia complication, expression of relative mRNA VEGF between preeclampsia placenta and normal placenta. Based on Mann–Whitney test, there was a significant difference of median level of two groups at mRNA (P)RR.

In preeclampsia, the relative expression of mRNA VEGF was 2.83 times higher than the normal samples (Fig. 2).

The protein level of VEGF and (P)RR

Based on the result of protein analysis by applying Sandwich ELISA technique, we found out that there was a substantial difference of VEGF protein average between normal group and preeclampsia group (p=0.0001) (Fig. 3). The VEGF protein expression on preeclampsia group was lower than the normal group. The same case also occurred with (P)RR protein concentration. On preeclampsia group, the (P) RR protein concentration average was lower than normal group (p=0.0094) (Fig. 4).

Result data of VEGF protein (Fig. 3.) showed that there was a significant difference between preeclampsia and normal placenta, p=0.0001. VEGF protein expression in preeclampsia placentas was lower than in normal placentas.

RESULTS

VEGF and (P)RR mRNA relative expression

The research concluded that there was a distinguished relative expression of relative mRNA VEGF between preeclampsia placenta and normal placenta. On placenta tissue with preeclampsia complication, VEGF mRNA expression increased 2.83 times higher than normal placenta (Fig. 1). Based on Mann–Whitney test, there was a substantial difference of mRNA VEGF median expression between preeclampsia group and normal ones (p=0.02).

Based on the analysis result, it was found out that mRNA (P)RR expression on preeclampsia placenta was 1.7 times higher than normal placenta. Based on Mann–Whitney test, there was a significant difference of median level of two groups at mRNA (P)RR.

Normality test of all data was conducted with Kolmogorov–Smirnov test. If the data were normal, it would be presented in average and deviation standard. However, if the data were not normal, it would be presented in median and in range of minimum-maximum. Statistical analysis was incomplete, then the Mann–Whitney test applied. Statistical analysis for the correlation between two variables applied by Pearson test. The statistical significance limit applied was p<0.05. All statistical analyses were processed using computer with social science (SPSS) software for Windows version 20.

RESULTS

VEGF and (P)RR mRNA relative expression

The research concluded that there was a distinguished relative expression of relative mRNA VEGF between preeclampsia placenta and normal placenta. On placenta tissue with preeclampsia complication, VEGF mRNA expression increased 2.83 times higher than normal placenta. Based on Mann–Whitney test, there was a significant difference of mRNA VEGF median expression between preeclampsia group and normal ones (p=0.02).

Based on the analysis result, it was found out that mRNA (P)RR expression on preeclampsia placenta was 1.7 times higher than normal placenta. Based on Mann–Whitney test, there was a significant difference of median level of two groups at mRNA (P)RR.

In preeclampsia, the relative expression of mRNA VEGF was 2.83 times higher than the normal samples (Fig. 2).

The protein level of VEGF and (P)RR

Based on the result of protein analysis by applying Sandwich ELISA technique, we found out that there was a substantial difference of VEGF protein average between normal group and preeclampsia group (p=0.0001) (Fig. 3). The VEGF protein expression on preeclampsia group was lower than the normal group. The same case also occurred with (P)RR protein concentration. On preeclampsia group, the (P) RR protein concentration average was lower than normal group (p=0.0094) (Fig. 4).

Result data of VEGF protein (Fig. 3.) showed that there was a significant difference between preeclampsia and normal placenta, p=0.0001. VEGF protein expression in preeclampsia placentas was lower than in normal placentas.
that VEGF played an important role in vasculogenesis and angiogenesis found at cytotrophoblast and syncytiotrophoblast (STB) [16]. It showed on normal trophoblast cell culture, VEGF mRNA expression could be and proliferate in vasculogenesis and angiogenesis process [15]. Flt-1 and KDR/Flk-1. When VEGF protein was bound to its receptor, it could initiate the transduction signal on the endothelial cell to migrate and proliferate in vasculogenesis and angiogenesis [14]. Protein VEGF has two receptors, Flt-1 receptor which is a tyrosine kinase receptor, it might cause one of the symptoms was hypertension; the decrease of VEGF protein be activated, followed by AKT. Activated AKT would phosphorylate eNOS autophosphorylation of tyrosine794 residue (Tyr794). Then, PI3K will increase on endothelial NO synthase (eNOS) expression on the cell that cultured and incubated with VEGF. It happened through the activation of PI3K and ERK phosphorylation [26]. When VEGF was bound to Flt-1 receptor which is a tyrosine kinase receptor, it might cause autophosphorylation of tyrosine794 residues (Tyr794). Then, PI3K will be activated, followed by AKT. Activated AKT would phosphorylate eNOS cause the NO production to increase. The same effect also happened when VEGF was bound to KDR/Flk-1 receptor [27]. On preeclampsia, one of the symptoms was hypertension; the decrease of VEGF protein expression could cause vasoconstriction due to the repression of NO expression. NO was main endothelial relaxation modular. It acts as paracrine and autocrine to protect cardiovascular homeostasis, tonus vascular muscle, and also microvascular permeability.

**Correlation between expression of (P)RR and VEGF**

Based on Pearson correlation test result, it was concluded that there was no correlation between VEGF mRNA and (P)RR mRNA in normal or preeclampsia group. However, there was a substantial correlation of (P)RR and VEGF protein expression on normal and group with preeclampsia syndrome. On normal group, there was a positive strong correlation between (P)RR protein expression and VEGF protein expression (p=0.005; R=0.441). On preeclampsia group, there was also a positive correlation of medium relation between (P)RR and VEGF protein expression (p=0.012; R=0.401).

**DISCUSSION**

Preeclampsia is a serious threat for pregnant women, even for health workers. Preeclampsia could cause significant effect on maternal morbidity and mortality. Besides, it could also cause prenatal, preterm birth, and other fetal growth problems [13].

Continuous implantation process with completed vasculogenesis and angiogenesis is the factors that create a good place for the fetus grows. Woman with VEGF gene polymorphism is often experience repeated abortion as the VEGF protein was the main growth factor in vasculogenesis and angiogenesis [14]. Protein VEGF has two receptors, Flt-1 and KDR/Flk-1. When VEGF protein was bound to its receptor, it could initiate the transduction signal on the endothelial cell to migrate and proliferate in vasculogenesis and angiogenesis process [15].

On normal trophoblast cell culture, VEGF mRNA expression could be found at cytotrophoblast and syncytiotrophoblast (STB) [16]. It showed that VEGF played an important role in vasculogenesis and angiogenesis process. Based on the analysis of VEGF mRNA expression in this research, it was concluded that there were substantial differences of VEGF mRNA expression between preeclampsia and normal group. The VEGF mRNA expression on preeclampsia group increased 2.83 times higher than normal group. The same conclusion was also stated by Chung et al. that there was an increase of VEGF mRNA expression 2.8 times higher than preeclampsia [17]. Escudero et al. also concluded that there was an increase of VEGF mRNA expression on women with preeclampsia syndrome in Chile compared to the normal group [18]. On the other hand, Andraweera et al. who conducted research about group pregnant women in Adelaide Australia concluded differently that the expression of VEGF mRNA of placenta on preeclampsia was lower than the normal group [19].

In this research, protein concentration of VEGF placenta on preeclampsia group was lower than the normal one. The low concentration of VEGF protein on preeclampsia was expected related to the failure of angiogenesis. A research conducted by Gannon et al., about Arabic Tunisian women, found that VEGF plasma concentration on preeclampsia was lower than normal pregnancy [20]. Livingston et al. who conducted a research of pregnant women in Ohio concluded that there was restriction of VEGF serum concentration on serious preeclampsia [21]. Maynard et al., in his research, stated that free VEGF on serum decreased on preeclampsia pregnancy. The increase of sFlt-1 that was alternatively spliced from Flt-1 caused the VEGF bound to Flt-1 decreases and ultimately might cause problems in signal transduction in vasculogenesis and angiogenesis process. It was also supported by the antiangiogenesis effect 48 h post-labor [22].

Another function of VEGF is for growth and proliferation of glomerulus and peritubular endothelial cell [23]. VEGF inhibitor could prevent neovascularization on tumor cell, while treatment of VEGF antagonist on cancer patient would cause proteinuria and hypertension, similarly as the symptoms in preeclampsia [22,23]. Pregnant mice that were induced with sFlt-1 would show glomerular enlargement with capillary occlusion due to hypertrophy on glomerulus capillary endothelial cell. There were also protein resorption problems on podocytes [22]. These factors were the cause of hypertension and proteinuria on preeclampsia [24,25].

VEGF will influence the expression of nitrite oxide (NO). There was an increase on endothelial NO synthase (eNOS) expression on the cell that cultured and incubated with VEGF. It happened through the activation of PI3K and ERK phosphorylation [26]. When VEGF was bound to Flt-1 receptor which is a tyrosine kinase receptor, it might cause autophosphorylation of tyrosine794 residues (Tyr794). Then, PI3K will be activated, followed by AKT. Activated AKT would phosphorylate eNOS cause the NO production to increase. The same effect also happened when VEGF was bound to KDR/Flk-1 receptor [27]. On preeclampsia, one of the symptoms was hypertension; the decrease of VEGF protein expression could cause vasoconstriction due to the repression of NO expression. NO was main endothelial relaxation modular. It acts as paracrine and autocrine to protect cardiovascular homeostasis, tonus vascular muscle, and also microvascular permeability.

RAS or known as RAS plays a major role in pathogenesis preeclampsia. In early pregnancy, prorenin, (P)RR, and angiotensin II type 1 receptor were found on extravillous trophoblast (EVT). It explains the effect of RAS components in trophoblast migration [10]. In normal pregnancy, there were an increase of RAS components which were renin, angiotensinogen, angiotensin I, and aldosterone [28,29]. It does not happen to an increase of RAS components in trophoblast migration. When the ligand of (P)RR and VEGF protein expression on normal and group with preeclampsia was not followed by hypertension in normal pregnancy [28]. Maynard et al., in his research, stated that free VEGF on serum decreased on preeclampsia pregnancy. The increase of sFlt-1 that was alternatively spliced from Flt-1 caused the VEGF bound to Flt-1 decreases and ultimately might cause problems in signal transduction in vasculogenesis and angiogenesis process. It was also supported by the antiangiogenesis effect 48 h post-labor [22].
bound to (P)RR plays a role in increasing neovascularization [30]. This role was supported by the existence of (P)RR at STB and EVT, and also (P)RR mRNA expression in normal pregnancy was higher at the early pregnancy than at the term pregnancy.

In this research, the (P)RR mRNA expression in preeclampsia pregnancy was higher than the normal group. On the other hand, the (P)RR protein expression was in preeclampsia was lower than in normal group. It shows that there were post-transcription arrangements in (P)RR expression. To fulfill its needs, cell tries to increase the expression of (P)RR mRNA in aiming that the protein expression would also increase. On the other hand, Thomason et al. analyzed the (P)RR protein expression in pregnant mice discovered that there was an elevation of (P)RR protein expression in preeclampsia, but there was no correlation between them [30].

(Pro)renin receptor existence is very important in placentation process. It is believed that there is a correlation between (P)RR expression and VEGF expression. A research conducted by Kanda et al., on HMVECs concluded that there was a correlation between the activity of (P)RR and angiogenesis on diabetic retinopathy. The elevation of protein concentration would increase the level of VEGF mRNA expression [11,31]. Binding of prorenin with (P)RR would stimulate ERK phosphorylation, also it will trigger an action without involving angiogenin 1. The involvement of (P)RR was proved by the inactivated (P)RR that would also inactivate ERK. Inhibitor (P)RR could suppress the expression of VEGF on pregnant diabetic mice. The resistance of VEGF expression occurred through the suppression of ERK½ phosphorylation [32,33]. This research also shows the relations between (P)RR protein expression and VEGF protein expression. There was positive correlation between normal group and preeclampsia with medium relation between (P)RR protein expression and VEGF protein expression. It proves that there was an involvement of (P)RR in VEGF expression arrangement on preeclampsia.

CONCLUSION

VEGF protein expression decreased in line with the decreasing of (P)RR protein expression on the placenta of the third-trimester pregnant women with preeclampsia. There was a correlation between the expression of (P)RR and VEGF.

ACKNOWLEDGMENTS

We wish to express our gratitude to the Directorate of Research and Public Service University of Indonesia for the Research Grant. This research was funded by grants of PUPT-DIKTI in 2017 and PITTA in 2016. Therefore, we would like to extend our gratitude to Kemenristek Dikti and Universitas Indonesia. In addition, there is no conflict of interest in this research.

AUTHORS’ CONTRIBUTION

Author contributions were as follows: Nelly Marissa measured mRNA relative expression and ELISA of markers, Sri Widia A Jusman data analysis and correction, Yuditia Purwosunu collect the samples, and Ani Retno Priyanti searched foundation as person in charge and manuscript correction.

REFERENCES

1. WHO. Maternal and Reproductive Health. Geneva; WHO: 2015. Available from: http://www.who.int/gho/maternal_health/en/.
2. Chen CW, Jaffe IZ, Karumanchi SA. Pre-eclampsia and cardiovascular disease. J Cardiovasc Res 2014;101:579-86.
3. Cho GJ, Park JH, Shin SA, Oh MJ, Seo HS. Metabolic syndrome in the non-pregnant state is associated with the development of preeclampsia. Int J Cardiol 2016;203:982-6.
4. Warouw PC, Suparman E, Wagey FW. Characteristics of preeclampsia in RSUP PROF. Dr. R. D. Kandau Manado. J E Clinic 2016;4:1-7.
5. Dhajjnah SN, Arianti IS. Epidemiology of preeclampsia/eclampsia incidenes in PKU Muhammadiyah general hospital in Yogyakarta during 2007-2009. Bull Penelit Sist Kesehat 2010;13:378-85.
6. Andraweera PH, Dekker GA, Laurence JA, Robert CT. Placental expression of VEGF family mRNA in adverse pregnancy outcomes. Placenta 2012;33:467-72.
7. Uzan J, Carbonnel M, Piconne O, Asmar R, Ayoubi JM. Pre-eclampsia: Pathophysiology, diagnosis, and management. J Vasc Heal Risk Manag 2011;7:467-74.
8. Baumfeld Y, Novack L, Wiznitzer A, Sheiner E, Henkin Y, Sherf M, et al. Pre-conception dyslipidemia is associated with development of preeclampsia and gestational diabetes mellitus. PLoS One 2015;10:e0139164.
9. Rodriguez M, Moreno J, Hashun J. RAS in pregnancy and preeclampsia and eclampsia. Int J Hypertens 2012;2012:1-6.
10. Zárate A, Saucedo R, Valencia J, Manuel L, Hernández M. Early disturbed placentical ischemia and hypoxia creates immune alteration and vascular disorder causing preeclampsia. Arch Med Res 2010;41:219-24.
11. Pringle KG, Tadros MA, Callister RJ, Lumbers ER. The expression and localization of the human placentical prorenin/renin-angiotensin system throughout pregnancy: Roles in trophoblast invasion and angiogenesis? J Placenta 2011;32:956-62.
12. Dongare S, Rajendran S, Sendhil Kumari S, Gupta SK, Mathur S, Saxena R, et al. Genistein alleviates high glucose induced toxicity and angiogenesis in cultured human RPE cells. Int J Pharm Sci 2015;7:294-8.
13. Kanda A, Noda K, Saito W, Ishida S. (Pro)renin receptor is associated with angiogenic activity in proliferative diabetic retinopathy. Diabetologia 2012;55:3104-13.
14. Sun Y, Chen M, Mao B, Cheng X, Zhang X, Xu C. Association between vascular endothelial growth factor polymorphism and recurrent pregnancy loss: A systematic review and meta-analysis. Eur J Obstet Gynecol Reprod Biol 2017;211:169-76.
15. Gavala GS, Liangos M, Trachana SP, Bagrati T, Arapinis C, Liasos C, et al. Angiogenesis-related pathways in the pathogenesis of ovarian cancer. Int J Mol Sci 2013;14:15885-909.
16. Shorea VH, Wang C. Vascular their endothelial growth factor, human placenta trophoblast growth factor and receptors in isolated. Placenta 1997;18:657-65.
17. Chung J, Song Y, Wang Y, Magness R, Zheng J. Differential expression of VEGF, EG-VEGF, and VEGF receptors in human placentas from normal and pre-eclamptic pregnancies. J Clin Endocrinol Metab 2004;85:2484-90.
18. Escudero C, Celis C, Saaz T, San Martin S, Valenzuela FJ, Aguayo C, et al. Increased placental angiogenesis in late and early onset preeclampsia is associated with differential activation of vascular endothelial growth factor receptor 2. Placenta 2014;35:207-15.
19. Azaliani AF, Zainul-Rashid MR, Chandramaya SF, Faroksi WI, Nurwadah A, Wong YP, et al. Vascular endothelial growth factor expression in placenta of hypertensive disorder in pregnancy. Indian J Pathol Microbiol 2017;60:515-20.
20. Gannoun M, Bourrell S, Raguenza N, Zitouni H, Nouvellon E, Maleh W. Placental Growth factor and vascular endothelial growth factor receptor-2 in human. Cytokine 2016;79:1-6.
21. Livingston J, Chin R, Haddad B, Mc Kinney E, Ahokas R, Sibai B. Reductions of vascular endothelial growth factor and placental growth factor concentrations in severe preeclampsia. Am J Obs Gynecol 2000;183:14-1.
22. Maynard SE, Min J, Merchant J, Lin KH, Li J, Mondal S, et al. Excess placental soluble fms-like hypertension, and proteinuria in. J Clin Invest 2003;58:564-81. Available from: http://www.jci.org/articles/ view/17189.
23. Schrijvers BF, Flyvbjerg A, De Vriese AS. The role of vascular endothelial growth factor (VEGF) in renal pathophysiology. Kidney Int 2004;65:2003-17.
24. Björndahl M, Cao R, Eriksson A, Cao Y. Blockage of VEGF-induced angiogenesis by preventing VEGF secretion. Circ Res 2004;94:1443-50.
25. Roberts JM, Rajakumar A. Preeclampsia and soluble fms-like tyrosine kinase 1. J Clin Endocrinol Metab 2009;94:2252-4.
26. Lin MI, Sessa WC. Vascular endothelial growth factor signaling to endothelial nitric oxide synthase: More than a fleeting moment. Circ Res 2007;101:579-86.
27. Andraweera PH, Dekker GA, Laurence JA, Robert CT. Placental expression of VEGF family mRNA in adverse pregnancy outcomes. Placenta 2012;33:467-72.
28. Uzan J, Carbonnel M, Piconne O, Asmar R, Ayoubi JM. Pre-eclampsia: Pathophysiology, diagnosis, and management. J Vasc Heal Risk Manag 2011;7:467-74.
29. Baumfeld Y, Novack L, Wiznitzer A, Sheiner E, Henkin Y, Sherf M, et al. Pre-conception dyslipidemia is associated with development of preeclampsia and gestational diabetes mellitus. PLoS One 2015;10:e0139164.
30. Rodriguez M, Moreno J, Hashun J. RAS in pregnancy and preeclampsia and eclampsia. Int J Hypertens 2012;2012:1-6.
31. Zárate A, Saucedo R, Valencia J, Manuel L, Hernández M. Early disturbed placentical ischemia and hypoxia creates immune alteration and vascular disorder causing preeclampsia. Arch Med Res 2010;41:219-24.
of preeclampsia. Ren Physiol 2012;53715:614-25.
29. Uraoka M, Ikeda K, Nakagawa Y, Koide M. Prorenin induces ERK activation in endothelial cells to enhance neovascularization independently of the renin–angiotensin system. Biochem Biophys Res Commun 2009;390:1202-7.
30. Thomason J, Reyes M, Allen SR, Jones RO, Beeram MR, Kuehl TJ, et al. Elevation of (Pro)Renin and (Pro)renin receptor in preeclampsia. Am J Hypertens 2015;28:1277-84.
31. Narita T, Ichihara A, Matsuoka K, Takai Y, Bokuda K, Morimoto S, et al. Placental (pro) renin receptor expression and plasma soluble (pro) renin receptor levels in preeclampsia. J Placenta 2016;37:72-8.
32. Kanda A, Ishida S. The vitreous renin-angiotensin system is mediated by soluble (pro)renin receptor in diabetic retinopathy: A new implication of the receptor-associated prorenin system. Taiwan J Ophthalmol 2013;3:51-3.
33. Satofuka S, Ichihara A, Nagai N, Ozawa Y, Fukamizu A, Tsubota K, et al. (Pro)renin receptor–mediated signal transduction and tissue renin-angiotensin system contribute to diabetes-induced retinal inflammation. Diabetes 2009;58:1625-33.