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Research Article

# Analysis ofEndostatin Levels in Early Onset Preeclampsia, Late onset Preeclampsia and normal Pregnancy

## *Analisis Kadar EndostatinPadaPreeklamsiAwitandini, PreeklamsiAwitanLambatdanHamil Normal*

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**Abstract**

**Objective**: The purpose of this research is to analyzethe difference between the mean serum levels of endostatinin early onset preeclampsia (EOPE), late onset preeclampsia (LOPE) and normal pregnancy.

**Method:**This research design was an analytic cross-sectional observation on 80 pregnant women with EOPE, LOPE and normal pregnancy with matched gestational age in M. Djamil hospital, Reksodiwiryo hospital, Bhayangkara hospital, Rasidin Padang hospital, and Anak Air of Public Service, fromJuly to November 2015. Samples were selected based on consecutive sampling. Blood was collected intravenously, centrifuged and endostatinserum measured by ELISA method in the Laboratory of Biomedical Faculty of Medicine Andalas University. Normality test data with Shapiro-Wilk, unpaired t-test independent for mean difference, using SPSS.

**Result**: The mean levels of endostatinin the EOPE group and normal pregnancy group is 125±49.3 ng/ml and 90.8±28.3ng/ml ($p<0.05$), in the LOPE group and normal pregnancy group is 86.2±21.8 ng/ml and 78.3±19.5 ng/ml ($p>0.05$) and finally in the EOPE group and LOPE group is 125±49.3ng/mland 86.2±21.8 ng/ml ($p<0.05$).

**Conclusion**:The mean levels of endostatinin the PE group is higher than normal pregnancy. The mean levels of endostatinin the EOPE group is higher than LOPE group.

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**Key words:**endostatin, early onset severe preeclampsia, late onset severe preeclampsia

***Abstrak***

***Tujuan:****Tujuan penelitianiniadalahmenganalisiskadarendostatinantara preeklamsiawitandini (PEAD), preeklamsiawitanlambat (PEAL) danhamil normal.*

***Metode:*** *Desainpenelitianadalahcross sectionalterhadap 80 ibuhamil (PEAD, PEAL sertahamil normal denganusiakehamilansamadengankelompok PE-normal untukkelompok PEAD dan normal untukkelompok PEAL) di RSUPdr. M. Djamil, RS. Tingkat III dr. Reksodiwiryo, RS. Bhayangkara, RSUD. Dr.Rasidin, danPuskesmasAnak Air Padang, Juli-November 2015. Sampelberdasarkanconsecutive sampling. Darah intravenasampel, disentrifuge, didapatkan serum dan diukur dengan metode ELISA di LaboratoriumBiomedik FK- UNAND. Ujinormalitas data dengan Shapiro Wilk, bedareratadenganuji t tidakberpasangan, dandiolahmenggunakan SPSS.*

***Hasil:*** *Reratakadarendostatinkelompok PEAD denganhamil normal adalah 125±49,3 ng/ml dan 90,8±28,3ng/ml ($p<0,05$), kelompok PEAL denganhamil normal adalah 86,2±21,8 ng/ml dan 78,3±19,5ng/ml ($p>0,05$) dankelompok PEAD dengan PEAL adalah 125±49,3 ng/mldan 86,2±21,8ng/ml ($p<0,05$).*

***Kesimpulan****: Rerata kadar endostatin PE lebih tinggi dari pada hamil normal. Rerata kadar endostatin PEAD lebihtinggi dar ipada PEAL.*

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***Kata kunci****: Endostatin, Preeklams iAwitan Dini, Preeklamsi Awitan Lambat*

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## INTRODUCTION

Preeclampsia (PE) is one of the major problems in pregnancy that can cause maternal mortality being a factor in 10-15% of all maternal deaths worldwide.[1]The frequency of this condition varies from country to country.[2]

5-8% of pregnancies in USA are affected by this condition and it was the cause of 20% of the maternal deaths in Canada in 1999–2000.[3] High blood pressure caused 30% of all maternal deaths in Indonesia in 2010, and 3 - 10% of these involved preeclamsia.[4] About 45% of maternal deaths in West Sumatera are related to high blood pressure, preeclampsia and eclampsia. According to figures from Dr. M Djamil hospital preeclampsia is an increasing problem with 5.5% in 1998-2002,[5]8.3% in 2011, 11.5% in 2012 and 12.% in 2013.[6]

Preeclampsia can cause two different conditions depending on the stage of pregnancy. In the first 34 weeks of pregnancy EOPE is related to failure of trophoblast invasion and remodelling of spiral arteries in the uterus. LOPE after the 34 week mark, is caused by an increase in maternal blood vessel inflammation in a previously normal pregnancy or atherosis of a placenta that less elastic was previously ndeveloping normally.

The pathogenesis of PE begins when the angiogenic and antiangiogenic factors in the placenta are out of balance.[7,8]One antiangiogenic factor, endostatin, is a product of collagen XVIII with a C terminal and a molecular weight of 20 kDa. It causes endothelial cell apoptosis,[8] inhibits cell proliferation and migration. In some studies, the level of endostatin has been observed to be elevated in women with severe preeclampsia.[9,10] Other studies report no increase in levels in normal pregnancy indicating that the placenta is not contributing to serum concentration of endostatin. No clinically significant difference has been observed between preoxidised lipid levels in PE or normal pregnancy.[11] As a result of this controversy it was decided to conduct this study to establish the difference in mean levels of endostatin between EOPE and normal pregnancy, LOPE and normal pregnancy and between EOPE and LOPE.

## METHOD

This study design was an analytic cross-sectional observation in dr. M. Djamil hospital, dr. Reksodiwiryo hospital, Bhayangkara hospital, dr. Rasidin Padang hospital, and Anak Air ofcommunity health clinic, from July to November 2015.Ethical approval no. PE.27.2015 for the research was obtained from the ethical committee for research at dr. M. Djamil hospital.[12] The population studied was all women who were 20 weeks or more pregnant who were treated as outpatients or admitted to delivery wards in the these hospitals whether they had a normal pregnancy, EOPE or LOPE. Each PE sample was matched with a sample with normal pregnancy and similar gestational age.

Three ml blood samples were taken from the antecubital area by the technician in the delivery ward or midwifery clinic. These samples were placed in a vacutainer containing 2.5 ml ethylenediaminetetraacetic acid (EDTA) and centrafuged at 2000-3000 rpm. The serum was stored at -80 $^{0}$C in the Andalas University Biomedic laboratory in Padang. A 0,5ml sampel of the blood was placed in another vacutainerwithout EDTA for Hb and Leukocyte levels at the blood testing clinic.

Endostatin levels were measured using a Ray Bio Tech ELISA kit in the Andalas University Biomedic laboratory. Data obtained was analysed using the unpaired t test.

## RESULT

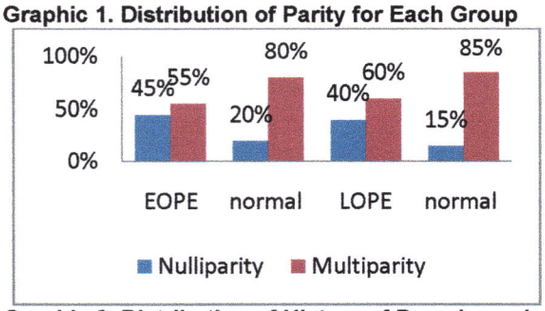
**Table1. Characteristics of the women in the <34 week gestation age sample**

| Characteristic | EOPE (Mean±SD) | Normal (Mean±SD) | P |
|---|---|---|---|
| Age (years) | 32.5±7.0 | 30.0±4.1 | 0,179 |
| BMI before pregnancy (kg/m$^2$) | 23.5±1.8 | 22.8±2.1 | 0,246 |
| Hb levels (gr/dl) | 11.3±1.5 | 10.8±0.9 | 0,220 |
| Leukocyte count (m$^3$) | 13373.6± 4829.8 | 8105.0± 1451.5 | 0,000 |

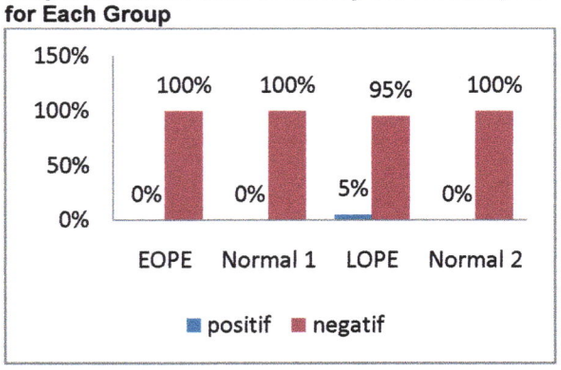
**Table 2. Characteristics of the women in the >34 week gestation age sample**

| Characteristic | LOPE | | Normal | | P |
|---|---|---|---|---|---|
| | Mean ±SD | Me (Min-Max) | Mean ±SD | Me (Min-Max) | |
| Age (years) | 31.2 ± 7.3 | | 30.0± 5.4 | | 0,559 |
| BMI before pregnancy (kg/m$^2$) | 25.3 ± 2.3 | | 23.1 ± 2.9 | | 0,012 |
| Hb levels (gr/dl) | 11.5± 1.6 | | 10.6± 1.1 | | 0,655 |
| Leukocyte count (m$^3$) | | 13305 (8400-24700) | | 8325 (5400-9900) | |

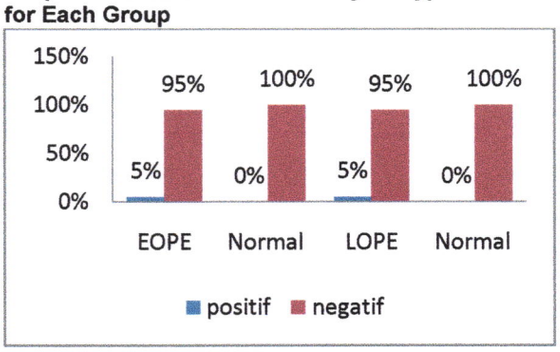
**Graphic 1. Distribution of Parity for Each Group**




**Graphic 2. Distribution of History of Preeclampsia for Each Group**




**Graphic 3. Distribution of History of Hypertension for Each Group**




**Graphic 4. Distribution of History ofDiabetes Mellitus for Each Group**

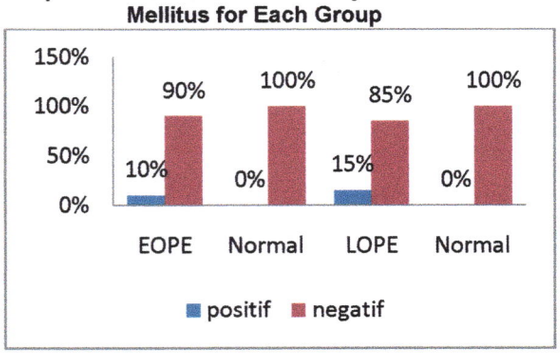



**Table3. Average Level of Endostatin for Each Group**

| Gestational Age | Endostatin Levels (ng/mL) Mean | SD | P |
|---|---|---|---|
| <34 weeks | | | |
| EOPE | 125.1 | 49.3 | 0,011* |
| Normal | 90.8 | 28.3 | |
| ≥34 weeks | | | |
| LOPE | 86.2 | 21.8 | 0,237 |
| Normal | 78.3 | 19.5 | |
| Severe PE | | | |
| EOPE | 125.1 | 49.3 | 0,003* |
| LOPE | 86.2 | 21.8 | |

(* difference is statistically significant)

## DISCUSSION

**Difference in average Endostatin levels between EOPE and normal pregnancy**

The average level of endostatin amongst EOPE subjects was 125.1 ng/ml compared to 90.8ng/ml for women with normal pregnancy. This difference of 34.3 ng/ml with a 95% confidence interval of 8.4-60.3ng/ml and p=0.011 is statistically significant (at the 95% level).

There is a significant increase in endostatin concentrations in PE patients compared with those with normal pregnancies from early pregnancy,[13] and a high level of endostatin at gestation age 16-20 weeks indicates a higher risk of developing PE.[14]

During pregnancy, extravilloustrophoblast (EVT) establishes uteroplacentalcirculation by invading the decidua and spiral arteries[15] beginning with the process of differentiation of the villi in the placenta, proliferation of cells invading the extracellular matrix (ECM), requiring a focal adhesion that is mediated by tyrosine kinase that is the focal adhesion kinase (FAK).16

The invasion of EVT is controlled by stimulation from pro danantimigration, *tumor growth factor-β 1-3 (TGFβ 1-3),tissue inhibitor metalloproteinase* (TIMP), and *plasminogen activator inhibitor (PAI-1* and 2). If an imbalance occurs between factors and the ECM like endostatin, it will contribute to a pathological disturbance of pregnancy such as preeclampsia.[15]

Endostatin is bound to several membrane protiens such as integrin α5β1, integrin αvβ3,[17] inhibiting the activation of cellular signaling components including FAK dan*extracellular regulated kinase*(ERK). Endostatin depresses*insulin growth factor*-II (IGF-II)- induces FAK Phosphorylation,/protein kinase B/mamalian target of rapamycin (mTOR),P70S6Kinase (S6K) and activation of ERK1/2.[15,17]

Endostatin inhibits FGF and VEGF, migration of human microvascular endothelial cells and influences the formation of blood vessels in the embryo.[18]Endostatin is also known to control migrasion and proliferation of cells like phospholipase C-γ (PLC-γ), PKB, p44/42, *mitogen activated protein kinase* (MAPK), p38 MAPK and *p21-activated kinase* (PAK) via the intracellular pathway.

One EOPE patient had the highest endostatin level (200,02ng/ml) measured. On average this group were *nulliparous with* gestation age was 29-30 weeks, had Hb levels of 15,3 gr/dl, a leukocyte count of 21800 m3, blood pressure 170/130 mmHg and proteinuria +2.

One mechanism that may influence the increase in high endostatinlevels in this group is immune maladaptation. In nulliparous EOPE mothers, there is inadequate production of blocking antibodies for the trophoblast to be protected. Furthermore there is a reduction in the expression of Human Leucocyte Antigen-G (HLA-G) in the cytotrophoblast resulting in a reduction in the protection of the trophoblast from destruction from natural killer (NK) and cytotoxic cytokines interleukin- 2 (IL-2) inhibiting the trophoblast invasion of the spiral arteries. For nulliparous particularly, conception that happens too quickly after the exposure to sperm does not allow sufficient timefor the mother to produce *blocking antibodies*.[19]

High maternal Hb levels will influence invivovascular resistance, Hb in red blood cells can

inhibit *endothelium dependent vasodilatation* so that an increase in hemoglobin concentration of 1 gm/dl can reduce the production of *Nitric Oxide* (NO).

It is known that NO plays a role in the migration of endotel cells induces *VascularEndothelial Growth Factor*(VEGF) important in increasing proliferation, migrasion and survival of endotel cells along with increasing the permeability of the capillaries. As embrio cells need oxygen as a source of energy for molecular processes, VEGF has an important role in the vasculogenesis and angiogenesis of the embryo. Damage to a single VEGF allele will result in abnormal blood vessels including those in the placenta resulting in embryo death at 10 to 12 days. 20

Endostatininterfers with the bonding process between VEGF and Kinase insert domain receptor (KDR/Flk 1/VEGFR2). KDR/Flk1/VEGFR2 is a VEGF receptor in endotel vascular cells. The binding of endostatinwith KDR/Flk1/VEGFR2 therefore will cause an increase in free VEGF that is then unable to function. The process of vasculogenesis and angiogenesis will be inhibited resulting in lack of blood vessels formation and blood flow..[9,20]

**Difference in Average Endostatin Levels Between LOPE and Normal Pregnancy**

The average value of endostatin for LOPE patients was 86,2ng/ml compared to 78,3ng/ml for normal pregnancies, a difference of 7,9 (95% Confidence interval range 5,38-21,10) The value of ρ=0,237 (ρ>0,05) indicating that this difference is not statistically significant.

*This lack of measurable difference could be a* result of several factors that influence the function of endostatin like the growth factor that stimulates the proliferation of cells ( fibroblast 2/ FGF2 or VEGF for example).[21]

This result differs from the findings of Mahmoud*and* Abdel Raouf (2006) who discovered significantly higher levels of endostatin and VEGF in PE patients compared to women with normal pregnancies or women who were not pregnant. The level of endostatin in patients with severe preeclampsia was higher than those withmild PE irregadless of time of onset.

Endostatin binds to several membrane proteins, such asfibronectin α5β1 receptor, and interacts with integrin α5β5 dan αvβ3. Endostatin also inhibits the binding sites endothelialcells and collagen type 1 ( interstitial collagen ).9

LOPE is influenced by extrinsic or maternal factors where specific conditions that increase the mass of the placenta such as diabeties or twins, or increases the suface area of the placenta like hypoxia from the mother or anemia will cause an excess of release of *synctitial knots*. Pregnant women with high maternal risk factors or inflamation reactions that are not in line with release of fragments of apoptosis trophoblastcause the inability of the maternal system to overcome the rise in apoptosis fragment numbers. Synctitial knots occur at 32 weeks. These two things in the end cause secondary necrosis in the blood that can trigger the clinical symptoms of LOPE.[19]

As this study was concerned with factors influencing the role of endostatin other than those related to the extrinsic or maternal factors discussed above, these women (anemia, diabetes, twins) were excluded from our sample. Hence the results from this study would not be expected to show the difference in average endostatin levels between LOPE and normal pregnancy observed in studies that included such patients.

***Difference in average Endostatin levels between* EOPE and LOPE**

The average endostatin level in EOPE was 125,09ng/ml compared to86,2ng/ml in LOPE; a difference of 38,9ng/ml (95% Confidence Interval 14.17-63.68). The result of the unpaired t test being ρ=0,003 (ρ<0,05) indicating that difference was statistically significant.

EOPE patients, but not LOPE patients, have

elevated endostatin levels compared to women with normal preganacies. Endostatin is an antagonis factor for angiogenesis that has a wide ranging influence on the inhibition of the angiogenesis process that has not been widely studied.[19]

The evidence suggests an imbalance between placenta angiogenic and antiangiogenic factors exists with PE that can endanger the vascular endotel and give rise to clinical symptoms in the mother. The rise in endostatin levels that occurs in the first trimester is related to the risk of developing PE.[13] Imbalance in the production of VEGF and endostatin is related to a number of conditions including systemic sclerosis, atherosclerosis as well as PEas it influences the cell proliferation, migration and apoptosis processes hence influencing morphogenesis dan maturation of blood vessels.[10]

Studies on mice reveal that there is a pathological change in the placenta with EOPE which has a detrimental effect on the fetus and its development that does not appear to occur in LOPE.[33] Hence it has been concluded that while EOPE is related perfusion of the uteroplacenta, LOPE is more often related to extrinsic factors like the larger size of the placenta or some systemic disease in the mother.[25]

Experimental studies have indicated that there is a positivecorrelation between endostatin levels and proteinuria value (p=0,663)in patients with chronic kidney disease. Endostatin is related toduration of hypertension and vascular index, myocardium and organdamage that targets the kidneys.[26] This is explained by the increasein circulation of the level endostatin increasing extracellularremodeling of the vascular network.[27]Endostatin will inhibit the functioning of VEGF-A, a factor in thedevelopment of the kidneys. VEGF-A protects the glomerulus capillarystructure and the endotel cell and the peritubular capillary damagerepair processes so inhibiting its function results in impaired renalfunction increasing the permiability of the basalis membranecausing leakage and subsequent proteinuria.[28]

EOPE subjects in this study had an average blood pressure of 167/111 mmHg compared to 174/113 mmHg in the LOPE group. It has previously been suggested that endostatin levels could be a marker for damage and remodeling of the extracellular matrix in a number of diseases. Long term hypertension induces remodeling of the cardiovascular extracellular matrix. In hypertension there is an induction of extracellular remodeling with the activation of metalloproteinase-2 and metalloproteinase-9 both of which play an important role in the degradation of collagen XVIII to endostatin.26

There was no difference in Hb levels observed between the groups in this study except there were some extreme values of Hb levels in EOPE.Phalapropkan (2008) concludes that women with high levels of Hb are at risk of developing of PE also. Hence it is thought that there are nother risk factors that influences Hb levels such as patterns of iron levels in the diet, iron supplements or levels of ferritin that have not yet been studied.

In the EOPE group the highest luekocyte count measured (21,800 $m^3$)and endostatin level 200 ng. One member of the LOPE group, produced a sample with the luekocyte count (24,700 $m^{3)}$ and endostatin level 84,3 ng.

Hypoxia in preeclampsia can activate leukocytes directly in intervillous space or can stimulate the production oflipoperoxideand proinflammatory cytokines by the placenta, which can in turn activate leukocytes as they move through the placental circulatiory system. InPE patients there occurs a modulation of neutrophil which increases the superoxide anion production to levels above NO which can cause endothelial damage .

The inflammation processes involving leukocytes requires the function of adhesion molecules; the most important of these in the recruitment of leukocytes are selectins, integrins of the immunoglobulin superfamily. Selectin is a single transmembranepolipeptide expressed in the circulatory cells; endothelium and blood cells. These molecules are avtivated after induction. Three types of selectin molecules are involved; L - selectin in leukocytes , E - selectin and P - selectin in the endothelial cells , these later particularly in platelets . Early PMN adhesion is mediated by E selectin that is required for the antiangiogenic activity of endostatin.[30]

Integrin, a transmembrane adhesion molecule is widely distributed in many cells. Endostatin binds to integrin αv on the surface of endothelial cells and influences the process of adhesion of leukocytes and *inhibits the process of angiogenesis.Endostatin binding to intergrinso an increase of endostatinis associated with functions by increase in number of leukocytes in the process of leukocyte adhesion and mediated by integrin.*[17]

The limits of this study is only to observe the average levels of endostatin as aantiangiogenic factor, hence it is not possible to judge the importance of the imbalance of the VEGF receptor, FAK or other angiogenic factors related to metabolism or function of endostatin in endotel cells. The bonding of endostatin with intergrin or selectin, both important markers in the adhesion process of leukocytes were not studied either.

***Conclusion***

The results of this study show that the average level of endostatin in PE is higher than in normal pregnancy. The average value of endostatin in EOPE is higher than in LOPE.

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