Research Report

The Changes of H_2O_2 Level and Glutathione/Glutathione dioxide Ratio with the Administration of N-Acetylcystein, Vitamin C, and Vitamin E towards in Vitro Eclampsia Human Umbilical Vein Endothelial Cell Model

Perubahan Kadar H_2O_2 dan Rasio Glutathione/Glutathione dioksida antara penambahan N-Acetilsistein, Vitamin C dan Vitamin E ke model HUVEC's Eklampsia in Vitro

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Abstract

Objective: To identify the optimum dosage to lower H_2O_2 and to increase the highest ratio of Glutathione/Glutathione dioxide in the media of eclampsia HUVEC's model with the administration of NAC, Vitamin C, and Vitamin E.

Method: We compare the concentration of H_2O_2 and ratio of glutathione (GSH) glutathione dioxide (GSSG) in eclampsia HUVEC's model (as control group), and administration of three different dose of N-Acetyl Cystein (NAC), vitamin C, vitamin E and combination of them.

Result: The addition of NAC, vitamin C and vitamin E to eclampsia HUVEC's model can reduce the H_2O_2 level and increase GSH/GSSG ratio ($p < 0.05$). The optimal dose for NAC administration is 2 μM, while greater dose hamper the result. Combination of three antioxidants showed the best result compare to single antioxidant.

Conclusion: The lowest level of H_2O_2 and the highest ratio of GSH/GSSG is achieved with the administration of the combination of NAC 2 μM, Vitamin C 100 μM and Vitamin E 100 μM.

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Keywords: HUVECs, eclampsia plasma, antioxidant

Abstrak

***Tujuan**: Untuk mengetahui dosis yang paling optimal menurunkan kadar H_2O_2 dan meningkatkan rasio GSH/GSSG paling tinggi pada media model HUVECs eklampsia yang ditambah dengan NAC, vitamin C dan vitamin E.*

***Metode**: Penelitian eksperimental laboratorik (in-vitro), menggunakan kultur HUVEC's yang dipapar plasma Eklampsia (Model HUVEC's Eklampsia) sebagai kontrol (K). Model HUVEC's Eklampsia ditambah dengan antioksidan NAC 2, 4, dan 8 μM (KN2, KN4 dan KN8), vitamin C dan vitamin E dan kombinasi ketiganya, dibandingkan konsentrasi H_2O_2 dan rasio GSH/GSSG.*

***Hasil**: Penambahan NAC, vitamin C dan vitamin E dapat menurunkan kadar H_2O_2 dan meningkatkan rasio GSH/GSSG pada model eklampsia HUVEC secara bermakna ($p < 0,05$). Pemberian NAC 2 μM lebih baik dibandingkan dengan dosis 4 μM atau 8 μM. Kombinasi tiga antioksidan memberikan hasil lebih baik dibandingkan antioksidan tunggal.*

***Kesimpulan**: Kadar H_2O_2 paling rendah dan rasio GSH/GSSG paling tinggi pada pemberian kombinasi NAC 2 μM , vitamin C 100 μM dan vitamin E 100 μM.*

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***Kata kunci**: HUVECs, eklampsia plasma, antioksidan*

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INTRODUCTION

Preeclampsia is a specific pathological condition in a pregnat woman.[1] In this condition we will find endothelium dysfunction as the central disorder. Preeclampsia's clinical manifestations are hypertension and proteinuri, which shows two major disorders in the vascular system, vasoconstriction and the increase of vascular permeability.[2]

In the blood test of individuals with preeclampsia, high level of oxidant [hydroxyl (OH), hydrogen peroxide (H_2O_2), super oxide (O)] and low level of antioxidant [Glutathione (GSH), Vitamin C, Vitamin E] are present. The antioxidant is low because it is used to absorb high oxidant. It causes the level of oxidant to rise above that of the antioxidant, a condition referred to as oxidative stress. Due to the deficiency of the antioxidant, the pregnant woman who suffers from preeclampsia cannot control the high oxidative stress that may destroy the endothelium cell of maternal blood vessel and causes endothelium dysfunction.[3] The preeclampsia patients had high levels of free radicals. The free radicals were transferred from the placenta which experienced ischemia-reperfussion. These free radicals had the nature to destroy, so that their production had to be controlled by enzymes or vitamins which served as antioxidants.[4]

The normal cellular physiological process requires an oxidation-reduction system of GSH, which is important in keeping intracellular homeostasis. This system uses GSH as the substrate in a reaction that involves glutathione peroxide (GPx) to detoxify peroxides such as H_2O_2 and lipid peroxide. This reaction results in oxidated GSH (GSSG). Oxidized glutathione will be changed further into GSH by glutathione reductase in a reaction that needs hexose monophos-

phate and NADPH. The changes of oxidized glutathione into GSH is controlled by glutathione reductase The ratio of GSH toward GSSG is kept high to minimalize intracellular disulfide accumulation.[5] In the pregnancy with preeclampsia, NADPH decreases, so there is a lack of GSH, too.[6] The administration of external compound with the same activity as GSH can improve the synthesis, such as N-acetylcystein (NAC).[7]

Vitamin C and E are known as important antioxidants in the preeclampsia healing. Vitamin C is a liptin antioxidant, working in cytosol and in extracellular medium. Since it is not synthezised in the body, there should be enough diet to prevent oxidative stress.[8] The level of vitamin C is detected as low in individuals with preeclampsia. Vitamin E is the primary antioxidant soluble in fat, working in the cell membranes to prevent lipid peroxidation. Vitamin E can also be found in lipoprotein particles and the level is higher than lipid. In some researches, vitamin E can be found in women with preeclampsia.[9]

This research used Human Umbilical Endothelial Cells (HUVEC's) exposed by preeclampsia pregnancy plasma, then NAC or Vitamin E were administered, showed that resistance to the activation of *Nuclear Factor-kappa Beta* (NF-κβ) and expression of *Intercellular Cells Adhesion Molecule-1* (ICAM-1).[7] A research by Candra (2007) who used HUVEC's exposed by severe preeclampsia pregnancy plasma and eclampsia as eclampsia model, demonstrated increase level of H_2O_2 oxidant and the low ratio of GSH/GSSG compared to normal pregnancy plasma. It illustrated of that the plasma of severe preeclampsia pregnancy and addition cytoxic eclampsia can, and cause oxidative stress in HUVEC's endothelium cell. The level of oxidative cell in HUVEC's endothelium cell which is drawn as cell experiences necrosis and apoptosis.[10]

This research is to identify the optimum dosages to lower H_2O_2 and to increase the highest ratio of GSH/GSSG in the media of eclampsia HUVEC's model with the administration of NAC, Vitamin C and Vitamin E.

METHOD

The research was conducted in the Biomedical laboratory of Medical Faculty of Brawijaya University is an experimental research using HUVEC's. HUVEC's was taken from umbilical of babies born by section secarea with inclusion criteria healthy mothers whose hemoglobin ≥ 10 in normal pregnancy. Endothelial cell of umbilical vein was cultured up to monolayer about 3 - 4 days (HUVEC's). After that HUVEC's which was monolayer exposed with 2% eclampsia pregnancy plasma as the model of eclampsia in vitro HUVEC's model [controlled (K)]. In group 1; The administration of NAC 2, 4, 8 μM in the eclampsia HUVEC's model, Group 2; The administration of NAC 2 μM, NAC 2 μM + Vitamin C 100 μM and Vitamin E 100 μM in the eclampsia HUVEC's model, Group 3; The adminsitration of NAC 4 μM, NAC 4 μM + Vitamin C 100 μM and Vitamin E 100 μM in the eclampsia HUVEC's model, Group 4; The administration of NAC 8 μM, NAC 8 μM + Vitamin C 100 μM and Vitamin E 100 μM in the eclampsia HUVEC's model. Incubation was conducted for 24 hours, then we evaluate the level of H_2O_2 and ratio of GSH/GSSG. The examination of H_2O_2 used the method from NWLSS™ Hydrogen Pe-roxide Assay, while the examination of GSH and GSSG used the method of Micro-Glutathione Assay, Anal. Biochem.[11,12] Statistical analysis used SPSS 14, discrimination test using One Way Anova with realibility of 95%, then to identify which exposure indicated the difference, BNT test with 95% realibility was conducted.

RESULT

There were 4 experimental groups, and in each group there was some treatment compared to the controlled group.

Table 1. Average level ± SD of H_2O_2 and ratio of GSH/GSSG with the administration of NAC 2, 4, 8 μM, vitamin C 100 μM, Vitamin E 100 μM in eclampsia HUVEC's model compared to the controlled group.

Treatment	Parameter			
	H_2O_2 μM/ml (x,Æ ± SD)	p Anova	GSH/GSSG (x,Æ ± SD)	p Anova
K	8.59±0.37	p=0.000	0.09 ±0.003	p=0.000
KN2	4.69±0.59		2.682±0.074	
KN4	7.06±0.14		1.334±0.078	
KN8	7.47±0.62		1.064±0.157	
K	8.59±0.37	p=0.000	0.09 ±0.003	p=0.000
KN2	4.69±0.59		2.682±0.074	
KN2C	4.33±0.09		4.362±0.328	
KN2E	4.24±0.14		3.046±0.304	
KN2CE	3.77±0.49		5.649±0.163	
K	8.59±0.37	p=0.000	0.09 ±0.003	p=0.044
KN4	7.06±0.14		1.334±0.078	
KN4C	5.62±0.22		2.642±0.524	
KN4E	5.11±0.39		3.168±0.697	
KN4CE	4.90±0.27		3.997±0.302	
K	8.59±0.37	p=0.000	0.09 ±0.003	p=0.000
KN8	7.47±0.62		1.064±0.157	
KN8C	6.44±0.10		0.744±0.169	
KN8E	7.06±0.26		1.064±0.146	
KN8CE	7.06±0.25		1.407±0.079	

K = Controlled: HUVEC's Eclampsia Model
Group 1 = K *KN2: K+NAC 2 μM*
KN4: K+NAC 4 μM
KN8: K+NAC 8 μM
Group 2 = K *KN2: K+NAC 2 μM*
KN2C: K+NAC 2 μM+Vitamin C 100 μM
KN2E: K+NAC 2 μM+Vitamin E 100 μM
KN2CE: K+NAC 2 μM+Vitamin C+Vitamin E 100 μM
Group 3 = K *KN4: K+NAC 4 μM*
KN4C:K+NAC 4 μM+Vitamin C 100 μM
KN4E: K+NAC 4 μM+Vitamin E 100 μM
KN4CE: K+NAC 4 μM+Vitamin C+Vitamin E 100 μM
Group 4 = K *KN8: K+NAC 8 μM*
KN8C: K+NAC 8 μM+Vitamin C 100 μM
KN8E: K+NAC 8 μM+Vitamin E 100 μM
KN8CE: K+NAC 8 μM+Vitamin C+Vitamin E 100 μM

The level of H_2O_2 in KN2 was significantly lower than the controlled group (p=0.000), while in KN4 and KN8 they were insignificantly lower than the controlled group with p of 0.050 and 0.163, respectively. In KN2, it was significantly lower than KN4 and KN8 with p of 0.008 and 0.003, respectively, but KN4 was insignificantly lower than KN8 (p=0.464). Ratio of GSH/GSSG to the KN2, KN4 and KN8 were significantly higher than the controlled group (p=0.000), in KN2 it was also significantly higher than KN4 and KN8 with (p=0.000), while KN4 and KN8 these were the same (p=0.093).

The level of H_2O_2 was significantly lower in KN2C compared to the controlled group (p=0.000), so was the level in KN2E with KN2CE (p=0.000). In KN2C it was insignificantly higher than in KN2E (p=0.855), it was also insignificantly higher in KN2C than KN2CE (p=0.328) and in KN2E it was significantly higher than KN2CE (p=0.420). The ratio of GSH/GSSG was significantly higher in KN2C than the controlled group (p=0.000), as well as in KN2E and KN2CE (p=0.000). The difference can also be seen in KN2C and KN2CE compared to KN2 (p=0.000), while KN2E was insignificantly higher in KN2 (p=0.188). In KN2C it was significantly higher compared to KN2E (p=0.002), KN2CE compared to KN2C (p=0.001), and between KN2CE and KN2E (p=0.000).

The level of H_2O_2 was significantly lower in KN4 compared to the controlled group (p=0.004), and it was also significantly lower in KN4C, KN4E and KN4CE compared to the controlled group with the same value of p (p=0.000). In KN4C it was significantly lower than KN4 (p=0.006). KN4C was insignificantly higher compared to KN4E and KN4CE with p=0.224, 0.114 respectively, and in KN4E it was insignificantly higher than KN4CE (p=0.631). The ratio of GSH/GSSG was insignificantly higher in KN4 compared to the controlled group (p=0.058), while in KN4C, KN4E and KN4CE compared to the controlled group (p=0.000). In KN4C, KN4E and KN4CE it was also significantly higher compared to KN4 with p of 0.029, 0.003, 0.001, respectively.
Meanwhile, in KN-4E it was insignificantly higher compared to KN4C (p=0.180), KN4CE compared to KN4C (p=0.076), and KN4CE compared to KN4E (p=0.602).

The level of H_2O_2 was insignificantly lower in KN8CE compared to the controlled group (p=0.058), and it was also insignificantly lower in KN8C and KN8E with p (0.053, 0.054), respectively. In KN8C it was insignificantly lower than the one in KN8E and KN8CE with p (0.770, 0.340), respectively. It was also insignificantly lower in KN8E compared to KN-8CE (p=0.499). The ratio of GSH/GSSG was significantly higher in KN8C, KN8E and KN8CE compared to the controlled group with p (p=0.004, 0.000, 0.000), respectively. In KN8C and KN8E it was insignificantly lower than the one in KN8 with p (p=0.107, p=0.802) respectively, and NAC 8 μM + Vitamin C 100 μM + Vitamin E 100 μM (p=0.089). In KN8C it was insignificantly lower than the one in KN8E and KN8CE with p (p=0.161, 0.004) respectively, KN8E compared to KN8CE (p=0.058).

Correlation between the level of H_2O_2 and the ratio of GSH/GSSG:

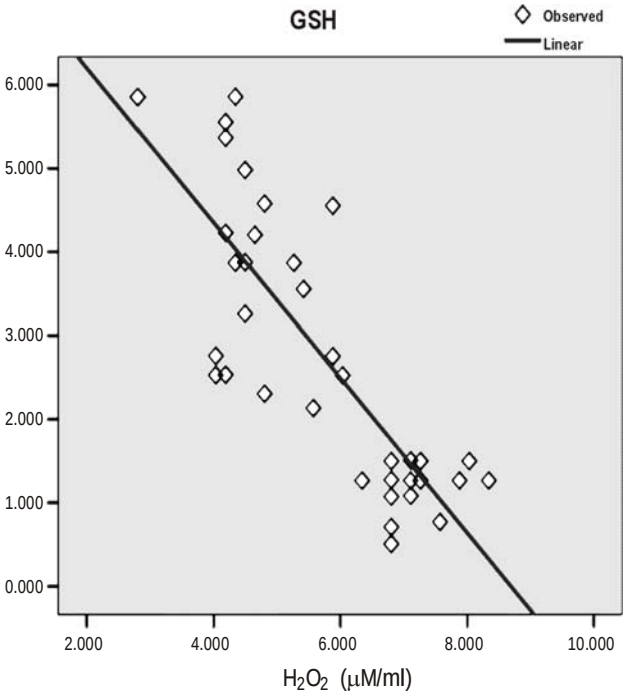

Figure 1. Regression analysis between the level of H_2O_2 and the ratio of GSH/GSSG.

The correlation between the level of H_2O_2 and the ratio of GSH/GSSG in HUVECs culture, indicates reciprocal correlation, meaning that the higher H_2O_2 level the lower the GSH/GSSG ratio.

DISCUSSION

Hydrogen peroxide is a biochemical radical formed by hydroxyl radical which has a high reactivity with the presence of metal transition ion. The stable characteristic of H_2O_2 its high diffusion power, whether in the cells and among the cells in the free radical reaction. Hydrogen peroxide has bond weakness in O-O. This weakness makes H_2O_2 change to free radical in acid and base atmosphere, and even simultaneously. Hot hydrogen peroxide may be decayed to form two free radicals.[13]

In previous study, H_2O_2 level is significantly higher in HUVEC's being exposed to 2% of eclampsia pregnancy plasma (eclampsia HUVECs model) compared to the one exposed to normal pregnancy plasma.[10] That research had proved that eclampsia pregnancy plasma contains oxidative stress substances that may induce HUVEC's to produce H_2O_2. Beside oxidative stress substances in eclampsia pregnancy plasma, there are also inflammation substances such as cytokine and syncytiotrophoblast microfragments that assumed to induced inflammation reaction from HUVEC's endothelium cell.[14] It can be explained that endothelium always produces O_2o, if HUVEC's endothelium is exposed to plasma that contains oxidative stress and inflammation substances. It is assumed that HUVEC's produces higher O_2o and because there is H^+ atom it

will produce higher H_2O_2. The level of GSH/GSSG ratio is significantly lower in eclampsia HUVEC's model compared to the one being exposed to the Normal pregnancy plasma. It is assumed that in the eclampsia HUVEC's model, the resulting GSH is used to absorb H_2O_2 radical resulted.

N-Acetylcysteine can increase the intracellular cysteine for the synthesis of GSH and to prevent from GSH oxidation. N-Acetylcysteine is a compound that contributes cysteine for the synthesis GSH, because it has an active group of -SH. Glutathione is an antioxidant that plays an important role in protecting the cells from free radical attacks. All living cells have a self defense mechanism toward free radical attacks.[5] Endothelium tends to be exposed by oxidative stress because there is always a contact with leukocyte. In the cell circulation, if leukocyte being activated it will produce Reactive Oxygen Species that may cause oxidative stress condition. Endothelium in the blood vessel is the primary target if there is an oxidative stress.[4]

In group 1, we found that the lowest significant level of H_2O_2 was achieve with the administration of KN2 compared to the administration of KN4 and KN8 in eclampsia HUVEC's model. The dosage of NAC 2 μM in KN2 is an optimal dose to decrease the level of H_2O_2. In KN2, most cysteine turns into cystine and when it enters the cell, cystine through the system of $X\text{-}_c$ (transport system that does not depend on the level of Na+) turns into cysteine that needs NADPH. Cysteine is used as a precursor to form GSH. It is assumed that when cystine enters the cell, all turn into cysteine and that formed GSH is used to absorb H_2O_2 radical, while the formed GSSG can be changed back into GSH, because may be NADPH level is still enough turn back GSSG into GSH, and cystine turns into cysteine.[5,15] From the research it was proved that the ratio of GSH/GSSG has the highest level and H_2O_2 is the lowest in the dosage of NAC 2 μM.

The higher the dosage of NAC, the higher H_2O_2 level was and the lower GSH/GSSG ratio was. It illustrates that the higher NAC level, it may has a toxic effect to the cell. This condition is suited to the clinical study that giving NAC dosage of 1.2 gram/day or more may results in oxidative stress.[15] In the administration of NAC 4 and 8 μM, the level of H_2O_2 was higher than NAC 2 μM. In administration of NAC 4 μM and NAC 8 μM dosage, most cysteine turns to cystine. When cystine enter the cell, it shall turn into cysteine that needs H^+ atom from NADPH, while in eclampsia pregnancy plasma being exposed to HUVEC's, NA-DPH will decrease and is assumed to cause the lack of NADPH in HUVEC's. Not all cystine turns to cysteine as precursor in the forming of GSH. Cystine is a form of an oxidized cysteine, so there will be an accumulation of oxidant cystine. It is assumed that it will increase the oxidative stress in HUVEC's cell. The oxidated GSH cannot be changed back into GSH because of the lack of NADPH, so there will be an accumulation of oxidant GSSG.

Vitamin C is an antioxidant that can stop the propagation of peroxidation process by giving its H^+ atom. Vitamin C also helps to bring back the oxidized Vitamin E and GSH into non radical Vitamin E and GSH,[13] while the administration of Vitamin C and Vitamin E to mothers who suffer from preeclampsia shall be of no use. The administration of Vitamin E and C will lower oxidative stress oxidatif if they are given in the beginning of pregnancy, and will result in lower preeclampsia.[9]

In group 2, the level of H_2O_2 was lower in KN2C, KN2E and KN2CE compared to the one in KN2 although it was insignificant. Glutathione, Vitamin C and Vitamin E give their H^+ atom when absorbing H_2O_2 free radical that will form GSSG, Vitamin C and Vitamin E radicals. Vitamin C and Vitamin E can give their H^+ atom to absorb GSSG radical and change it back to GSH. Glutathione can bring back Vitamin C radical and Vitamin E radical into non radical Vitamin C and Vitamin E. Vitamin C can give its H^+ atom to absorb Vitamin E radical. Vitamin C can interact directly with plasma membrane that gives electrons to the Vitamin E radical in oxidoreduktase activity to the trans-plasma membrane so that it can lower the free radical better than only using NAC 2 μM.[13]

The same also happened in group B, where, the level of H_2O_2 was lower in KN4C, KN4E and KN-4CE than KN4. It is assumed that the combination of NAC 4 μM antioxidant + Vitamin C 100 μM + Vitamin E 100 μM can cooperate in absorbing formed H_2O_2 radical when the antioxidant works to absorb free radical, so that it is more effective in lowering the level of H_2O_2.

The administration of the combination of NAC 4 μM and Vitamin C 100 μM, Vitamin E 100 μM will increase the level of GSH/GSSG ratio. The highest ratio of GSH/GSSG is in the combination of NAC 4 μM + Vitamin C 100 μM + Vitamin E 100 μM. It can be explained that the administration of combined antioxidant is more effective than single antioxidant administration, the more effective one is the administration of 3 antioxidant combinations consist of NAC 4 μM + Vitamin C 100 μM + Vitamin E, because the three antioxidant work in synergy to win the free radical.

In group 4, KN8CE can lower the level of H_2O_2, but the decrease was insignificant with the administration of KN8. It is assumed that the oxidative stress condition caused by Eclampsia plasma and NAC 8 μM, Vitamin C may have prooxidant and antioxidant properties. The level of GSH/GSSG ratio was lower than the one administrated with NAC 8 μM. It is assumed that Vitamin C 100 μM and Vitamin E 100 μM have more prooxidant properties. The higher level of H_2O_2 was found in the administration of Vitamin C 100 μM and Vitamin E 100 μM. It is assumed that when Vitamin C and Vitamin E absorb free radical then Vitamin C and Vitamin E radicals are formed and cannot be turned back into non radical Vitamin C and Vitamin E, so that they have more prooxidant properties.

In this research, from the controlled group 1, 2, 3 and 4, indicates that the higher the level of H_2O_2, the lower the GSH/GSSG ratio (figure 1). It can be explained that GSH can absorb H_2O_2 oxidant into GSSG and if H_2O_2 is higher, the existing GSH is not enough to absorb H_2O_2, and the consequence is that GSH/GSSG gets lower.

CONCLUSION

The addition of NAC, vitamin C and vitamin E to eclampsia HUVEC's model can reduce the H_2O_2 level and increase GSH/GSSG ratio ($p < 0.05$). The optimal dose for NAC administration is 2 μM, while greater dose hamper the result. Combination of three antioxidants showed the best result compare to single antioxidant.

The lowest level of H_2O_2 and the highest ratio of GSH/GSSG is achieved with the administration of the combination of NAC 2 μM, Vitamin C 100 μM and Vitamin E 100 μM.

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