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Effect of Cadmium Exposure in Polymorphisms Gene NOS3, Blood Cadmium Level, Nitric Oxide Level, Blood Pressure and Antioxidant Enzymes

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Abstract. Cadmium is a heavy metal widely used in human life, especially in the electroplating industry and a mixture of textile materials. Cadmium that enters the body binds to the metallothioneins protein. It can increase the formation of free radical compounds, thereby inhibiting enzyme activity such as nitric oxide synthase3. This gene regulates the expression of endothelial nitric oxide which produce nitric oxide. Nitric oxide role in regulated blood pressure as vasodilator with Angiotensin II as vasoconstriction. The susceptibility to Cd exposure will increase if the polymorphisms gene is found in population. The objective of this studies were to know the effect of cadmium to gene NOS3 polymorphisms on NO, systolic and diastolic blood pressure and antioxidant enzyme in Cd-exposed individual. The genotype individual were analyzed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLP) with MboI restriction enzyme followed by descriptive analyzed. Parameter recorded were blood Cd, NO level, SOD, systolic and diastolic. Data were analyzed by independent t-test. These result showed that 20% of 40 individual cases subject were detected as polymorphisms individual of NOS3 gene, with GA genotype. Their fragment DNA located on 206 bp, 119 bp and 87 bp, but non polymorphisms individual is only located on 206 bp. The result show cadmium could influence polymorphisms NOS3 gene and decrease NO production followed by increasing of blood pressure both systolic and diastolic. Cadmium also decrease antioxidant enzyme SOD and GPx level.

Keywords: Cadmium; systolic; diastolic; SOD; GPx

1. Background

Cadmium is a heavy metal which belongs to class II B in the periodic system. This metal will readily react with ligands containing elements of O, S and N. In the body Cadmium is toxic agent, because it reacts with ligands that are essential for normal body function. [9] living cells there are various ligands, such as: OH⁻, -COO⁻, -OPO₃H⁻, -C=O, -SH, -S-S-, -NH₂ and NH which can form complex bonds with metals such as Cadmium. Cd exposure also causes increased free radicals in the body and causes oxidative stress characterized by decreased levels of Super Oxide dismutase (SOD) and Glutathione peroxidase (GPx) (1-2). Cadmium in the body accumulates in the kidneys and the liver mainly binds to metallothionein. The interaction between Cd and metallothionein, will inhibit enzymes activity and one of them is endothelial enzyme Nitric Oxide Synthase (eNOS) (3). The enzyme eNOS is an enzyme that produces nitric oxide (NO) which functions in the regulation of blood pressure. The inhibition of eNOS activity cause the production of Nitric Oxide decreases result in decreasing elasticity of blood vessels. This will lead to increase vascular angiotensin II activity resulting in continuous vasoconstriction of blood vessels. This event causes elevated blood pressure called hypertension (4,5).

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Association of blood cadmium with hypertension found in the Korean general population (6,5).

The susceptibility of exposure heavy metals in human will increase when gene polymorphisms are present (7). Chuang *et al.* (2006) states that gene polymorphism is an important factor affecting human susceptibility to the toxic substances such as Cd. Polymorphisms at DNA level can cause decrease in enzyme activity by 20-30%. Nitric oxide synthase3 gene polymorphism (G894T) is found in exon 7, which converts glutamate amino acid into asparagine at the position of amino acid 298 (8). G894T polymorphism causes eNOS enzyme activity to decrease, resulting in reduced NO production. Hernayanti *et al.*, 2012 found that NOS₃ polymorphisms in carrier polymorphism NOS₃ gene individuals had lower NO levels in individuals without NOS3 gene polymorphism. Low NO levels will activate the matrix of metalloproteinase-9 and pro-inflammatory cytokines (Tumor Necrosis Factor α) and increased Matrix Metalloprotein-9 (MMP-9) but decreased their inhibitor namely Tissue Inhibitor Metallo Proteinase (TIMP), so hypertension develops into atherosclerosis (9-10). Welding workshop workers are one group of individuals who are at risk of exposure to Cd and are at risk of developing

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hypertension, especially for individuals carrying NOS3 gene polymorphism. Early detection of hypertensive events due to Cd exposure, especially on the carrier of NOS3 gene polymorphism, through NOS3 molecular analysis and NO, SOD and GPx levels, systolic and diastolic blood pressure can help prevent early prevention of hypertensive disease from atherosclerosis.

Research by early detection of cadmium pollution, especially from the welding process, one of the source of cadmium exposure will contribute to reduce carbon emissions in the community. In welding process in addition to the Cd particles, carbon monoxide (CO) gas is also produced. CO gas is one of the pollutants in the air resulting from incomplete carbon combustion in gasoline. This will cause carbon emissions in the air to increase. Reduction of carbon emissions due to Cd and CO in the air means supporting a low-carbon program in community development so that people live more prosperously

1. Material and Method

Research Design

The research design used an observational analytical design with cross sectional approach (11). The research was conducted from June to September 2016 and the research located in welding workshop Purwokerto and Sokaraja. Target population is welding laborers, while the population is affordable in this study are workshop workers in Purwokerto and surrounding areas amounted to 40 people. Sample size due to population less than 100 people, then all eligible populations of inclusion and exclusion criteria were studied. Inclusion criteria: respondents who were willing to sign Informed Consent, were not taking hypertension drugs and exclusion criteria were respondents who were taking hypertension drugs and respondents who not present at the time of the research. The independent variable is a Cd exposure. The dependent variables are the change Cd level in blood as well as changes in systolic blood pressure and diastolic welding laborers. The parameters observed were systolic and diastolic blood pressure, Cd blood level, NO level, SOD and GPx activities.

Determination of Cd level

Respondents were taken blood in the vein section of mediana cubiti with the syringe of 5 mL. 5 ml of blood is divided into 3 parts, 3 mL for DNA isolation, 2 mL for Cd and NO examination. Cadmium level detected using ACGIH method with AAS machine at wavelength 228.6 nm and strong currents of 3.5mA⁰.

Isolation of DNA

Isolation of DNA are performed with Guanidin method. (Hingorani,1999). Erithrocyte in blood sampel is lysed using lysing buffer. Then placed in refrigerator for 20 minutes and centrifuse 750 g at 8000 rpm for 10 minutes. The sampel was

preparation with Guanidin, SE buffer, chloroform, NaCl 4 M and isopropanol and stored at 40°C.

Determination of polymorphism NOS3 gene

Amp⁴ication gene with PCR (Hingorani *et al.*,1999). Forward primer : 5'-CAT GAG CGT CAG CCC CAG AAC-3' and Reverse primer : 5'-AGT CAA TCC CTT TGG TGC TCA C-3', result product located at 206 bp. PCR conditions include a 5-minute denaturation of 94°C, 35 cycles consisting of 94°C denaturation 1 minute, annealing 60°C 1 minute, elongation 72°C 1 minute and final elongation 72°C, 7 minutes. PCR product was digested by MboI endonuclease restriction enzyme. The results of the d¹²stion were analyzed by electrophoresis using agarose gel and visualized with etidium bromide under UV light. The electrophoresis result were : GG was not cut off, TT was cut into 2 fragments along 119 and 87 bp, GA ¹¹3 fragments along 206 bp, 119 bp and 87 bp.

Determination of Nitric Oxide level

Determination of Nitric Oxide level by Griess Method (12). Measured simple absorbance value at wavelength 520-560 nm with microplate reader. Created regression curve Y: NO standard and X: NO concentration. Made the correlation regression curve based on the concentration of standard solution.

Measurement of blood pressure by digital tensimeter equipment

Installed this instrument approximately 2 cm below the left hand, then tightened. The tensimeter is turned on by pressing the on button. After 2 minutes exit the number indicates systolic pressure (upper number) and diastolic pressure (bottom number). The Reading results are expressed with mmHg units.

Determination of SOD activity using RanSOD (13)

Sample of SOD read on spectrophotometer with wavelength 505 nm. Standard curve graphs are created to determine SOD activity and results are expressed in U/L.

Determination of GPx activity

Determination of GPx activity (14). Sample were read on Elisa Reader with wavelength 450 nm. Standard curve graphs are created to determine GPx activity and results are expressed in pg / mL units.

Data analysis

The molecular analysis of NOS3 gene polymorphism was analyzed descriptively, after the digestion results were visualized with etidium bromide under UV light. Meanwhile, to know the effect of NOS₃ gene polymorphism on blood Cd, NO level, GPx and SOD activity using Chi square test.

2. esult and Disccusion

The results of NOS3 gene analysis of 40 case-subjects after digestion with MBoI enzyme were found that 8 individuals with NOS3 polymorphism

or about 20% NOS₃ gene polymorphism with GA genotype, while 32 or 80% were found without polymorphism with GG genotype. Individual DNA bands with polymorphism are cut into 3 fragments along 206 bp, 119 bp and 87 bp (heterozygote mutants), whereas without polymorphism lies at 206 bp (10) (Figure 1).

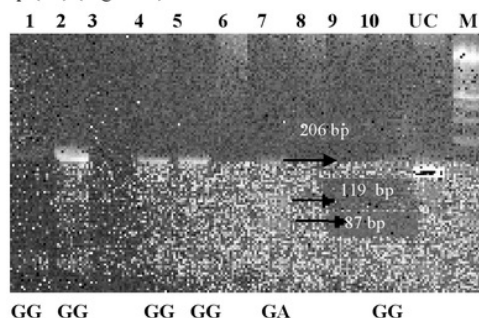


Figure1. Gene that codes NOS₃ digested by restriction MBOI enzyme on case subjects. No1,2,4,5,10 (GG) = Genotype individual without polymorphism NOS₃. DNA band not digesting lies at 206 bp. No7 (GA) = Genotype individual with polymorphism NOS₃ gene. DNA band cutting to 3 fragment lies at 206 bp, 119bp and 87 bp.

The result of measurement of blood Cd level from 40 worker of welding labores ranged from 0,2-2,32 ppm with the mean level equal to $0,86 \pm 0,65$ ppm (normal threshold value $< 0,1$ ppm). In individual carriers of NOS₃ gene polymorphisms, blood levels of Cd were found to be higher than non- polymorphic individuals. Average Cd level in individuals with NOS₃ gene polymorphism was found to be 2.06 ± 0.143 ppm, whereas in non polymorphic individuals it was found to be lower at 0.56 ± 0.24 ppm. Increased blood levels of Cd occur both in individual carriers of NOS₃ gene polymorphism as well as in non-polymorphic individuals.

The result of statistical test of systolic blood pressure on individual carrier of NOS₃ gene polymorphism is significant different compare with non polymorphism individual (t count = 4.35 and t table $\alpha 1\% = 2,704$). Individual systolic pressure of NOS₃ polymorphism carrier is higher than non polymorphism individual that is $157,75 \pm 3,42$ mmHg, whereas non polymorphism individual is $139,69 \pm 11,32$ mmHg.

The result of statistical test of diastolic blood pressure also showed very significant difference between individual carrier polymorphism with non polymorphism individual (t count = 4.93 and t table $\alpha 1\% = 2,704$). Individual diastolic blood pressure of polymorphism carrier is higher that is $95,37 \pm 7.89$ mmHg while non polymorphism individual is $85,12 \pm 4,17$ mmHg. The mechanism of hypertension to Cd exposure is due to the cadmium that enters the body following blood circulation and is circulated throughout the body by bonding with the amino acid metallothionein (Mt) to form Cd + Mt (cadmium + metallothionein) complex. The Cd +

Mt bond is stable and will inhibit the endothelial activity of nitric oxide synthase (eNOS). The inhibition of eNOS activity cause the production of Nitric Oxide decrease result in decreasing elasticity of blood vessels. This leads to increase vascular angiotensin II activity, resulting in continuously vasoconstriction of blood vessels. These events cause elevated blood pressure or hypertension (4-5) 2 times from normal will decrease NO levels by 5% and increase systolic blood pressure by 0.75 mmHg and diastolic by 1.077 mmHg (15).

Based on the grouping of hypertension category, the result of group normotension 3 people (7,5%), group of pre hypertension 9 people (22,5%), group of hypertension level 1 counted 22 people (55%) and group of hypertension level 2 counted 6 people (15 %). Individual carriers of NOS₃ gene polymorphisms with grade 2 hypertension were 5 (20%) while those with hypertension level 1 were 3 (7.5%). In non polymorphism group, the result of group of normotensi 3 people (7,5%), group of pre hypertension 9 people (22,5%), group of hypertension level 1 were 17 people (42,5%) and found no group of hypertension level 2.

The results of statistical tests of NO levels in individual carriers of NOS₃ gene polymorphism differed significantly with individuals without polymorphism (t count = -5.28 and t table $\alpha 1\% = 2.704$). Nitric oxide concentration in individual carrier of NOS₃ gene polymorphism was lower than non polymorphism of 27.4 ± 1.76 $\mu\text{mol} / \text{L}$, whereas in non polymorphism individuals were 37.85 ± 5.39 $\mu\text{mol} / \text{L}$.

Cd exposure in individual carriers of NOS₃ gene polymorphism is more severe hypertension than that of non-polymorphism individuals. This is in accordance with the opinion of Chuang et al., 2006 which states that a person's susceptibility to exposure to toxic compounds will increase when a gene polymorphism is found, as it will cause a 20% -30% decrease in enzyme function, compared to individuals without gene polymorphism.

The result of statistical test of SOD level showed very significant difference between individual carrier of NOS₃ gene polymorphism with non polymorphism individual (t count = 4.24 and t table $\alpha 1\% = 2,704$). SOD levels in individual carriers of the NOS₃ gene polymorphism were lower at 112.87 ± 4.72 U / L compared with non polymorphic individuals with SOD levels of 137.85 ± 5.39 U / L.

Cd bonds with methallotionein in the liver and kidneys increase the production of free radicals in the body such as superoxide anions, hydroxyl radicals and hydrogen peroxide. These free radicals will lead to lipid peroxidation reaction that characterized by the elevated of mallondialdehyde (MDA) level and cause oxydative stress characterized by decreasing activity of the antioxidant enzymes SOD, GPx and catalase (16).

The function of SOD is to capture O_2^- and convert it to hydrogen peroxide (H_2O_2). But if O_2^- formation is excessive, SOD are not able to neutralize O_2^- and convert it into H_2O_2 . The SOD enzyme located in the cytoplasm and extra cellular require ion co-factors Cu and Zn, whereas SOD located in mitochondrial requires the Mn co-factor. Cadmium is antagonistic to the metal ion cofactors. Cadmium will substitute the Cu, Zn and Mn, on the other hand the function of these metals are to arrange SOD, so that the SOD enzymes in the cytoplasm, extracellular fluid and mitochondrial become inactive (17-18).

The result of statistical analysis for GPx activity showed a very significant difference between individual carrier of NOS3 gene polymorphism compared with non polymorphism individual (t count = 8.46 and t table α 1% = 2,704). GPx activity in individual carriers of NOS3 gene polymorphism was lower at $45.33 \pm 3.57 \mu\text{mol/L}$, whereas the individuals with polymorphisms were $92.62 \pm 15.33 \mu\text{mol/L}$.

Cadmium could substitute Selenium (Se) which is a cofactor for GPx activity, so the GPx enzyme becomes inactive. The GPx function is to continue the SOD function to convert H_2O_2 to H_2O and O_2 . As a result there is accumulation of free radicals of O_2^- and H_2O_2 . Cd bonds with methallotionein on the liver and kidneys will decrease glutathione levels that are substrates for GPx. Glutathione also functions in the body's antioxidant defenses. Lack of glutathione production will cause damage to the liver and kidneys getting worse and will increase the toxicity of Cd (Johri, et al, 2010).

3. Conclusion

This result show that the exposure of Cd influence in NOS3 gene. Polymorphism of NOS3 gene in welding labour was found 20% and in non polymorphism individual 80%. Cd level, systolic and diastolic blood pressure in individual with NOS3 gene polymorphism is higher, and they have NO, SOD and GPx levels are lower than that of non polymorphism individual. The incidence of hypertension in individual carriers of NOS3 gene polymorphisms is found as many as 5 people (20%) with hypertension level 2, while 3 people (7.5%) with hypertension level 1. In non polymorphism individuals is found normotension 3 people (7.5%), pre hypertension 9 people (22.5%), hypertension level 1 as many as 17 people (42.5%) and hypertension level 2 is not found.

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