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REVIEW ARTICLE

RFLP-PCR ANALYSES TO CAPTURE POLYMRPHISM OF ALAD GENE ON INDIVIDUALS EXPOSED TO LEAD COMPOUND, MDA LEVEL AND HAEMOTOLOGICAL PROFILES

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ABSTRACT

Lead (Pb), is a toxic compound thus able to generate free radical and inhibit the δ-amino levulinic acid dehydratase activity, thereby affecting heme production. Due to its potential impact on human health, this study aimed to examine the polymorphism of the δ-ALAD gene in individual exposed to lead and to investigate its relationship with malondialdehydeand haematological profiles. Using a cross-sectional design, Venous blood were collected, genomic DNA was extracted and amplified using an RFLP PCR with the Msp-I restriction enzyme to for detect δ-ALADgenotype. Bloodleadconcentrations and haematological parameters were measured using atomic absorption spectroscopy (AAS) and a Haematology Analyzer, respectively, while MDA level were determined by TBARS method. One third of the subjects exhibited a polymorphic pattern for the δ-ALAD genewith band at 916 bp, 582 bp and 334 bp, whereas the remaining show inversely, a single at 916 bp. Individuals with the polymorphic genotype showed significantly higher blood-lead level, hemoglobin, erythrocyte and leucocyte counts compared to non-polymorphic individualsp<0.01). These findings suggest that the δ-ALAD gene polymorphism may impact δ-ALAD activity, increase oxidative stress as indicate by level, but decrease hemeproduction leading to anemia.

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INTRODUCTION

Lead, a toxic compound particularly to individuals working in such occupation like car repairer, welders, taxi drivers, policemen, plastic and pesticide-industries worker. This compound enters human body through inhalation of leaded-gasoline, vapors or leaded-basedpaints enters In the human body, lead binds to 40sulfhydryl groups (-SH), thereby inhibitingenzymatic activity. Chronic lead exposure of 40-60 ppm, (Charkiewicz, & Backstrand, 2020)) has been associated with a range of health problems including anemia, hypertension, renal failure, and decrease of immunity (Vogt, & Henderson, 2012; Pramod, *et al,*, 2017; Debnath *et al.,* & Manna, 2019). A study in Poland, with concerning of plant-waste and road transport, shows the utilization of coal asenergy source, caused a significant environmental pollution, (60 ppm). While studies on lead exposurealso demonstrated induction of oxidative stress by stimulating the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS),but depletingactivity of antioxidant enzyme. The resultant of this lipid peroxidation is damaging cell membranes, proteins, enzymes, and DNA, with malondialdehyde (MDA) serving as a reliable biomarker for such oxidative stress (Gupta, & Tiwari, 2012; Sirivarasai et al., 2015; Lanphear et al., 2018). Polymorphisms in the ALAD gene, is located on chromosome 9q34, and is indicatingif the sample population is susceptible to lead compound. At a DNA level, a polymorphism decreasesenzyme activity by 20–30% (Chuang et al., 2006); change Lysine amino acid to Asparagine and Guanine to Cytosine. This altered enzymatic function which is associated with decreased heme synthesis and increased in risk of anemia (Zheng, & Liang, 2011; Yang, & Sun, 2012; Puspitaningrum et al., 2018). Further, in Malaysia, India, and Vietnam showed polymorphism of δ-ALAD gene with allele ALAD-2 were 8.8%, 10.8%, and 4.3%consequtively.These people are more susceptible to anemia than healthy individuals; because the production of hem in this group is lower than healthy ones(Bijoor & Venkatesh, 2007). The current study was aimed to analyze the polymorphism of ALAD gene among the lead-exposed workers molecularly and its effect to hematological profile, and the MDA level among thew respondents

MATERIALS AND METHODS

Research design: This study applied a cross-sectional method on 30 male car repairmen from Purwokerto, Indonesia,with initial pool of 60 subjects. Inclusion criteria were male, aged between 25-55 years, with a minimum of 3 yearsworking

period/exposure time, and willing to participate voluntarily. Individuals with pre-existing anemia were excluded from the study. Ethical clearance was granted by the Medical Faculty of Gadjah Mada University, Yogyakarta, no KE/FK/294/EC.

Sample preparation: Following to the signing of informed consent, 10 mL of venousblood of each participant was drawn from the median cubital vein. The sample was then divided into three aliquots, 3 mL for DNA isolation, 3 mL for hematological profile analysis, and 4 mL for blood lead content and the MDA analyses, blood samples were centrifuged at 4000 rpm for 10 minutes to separates between the plasma (yellow color) from erythrocyte.

DNA Isolation and Genotyping

RFLP-PCR: RFLP- PCR was carried out under the following conditions: Forward primer 5'-AGA CAG ACA TTA GCT CAG TA-3; and reverse primer 5'-GGC AAA GAA CAG GTC CAT TC-3 applied to amplify the gene (Zheng, & Liang, 2011). PCR was carried out under the following conditions:for total of 30 cycles with initial denaturation 95°C for 5 minutes followed by denaturation at 95°C for 5 minutes, annealing at 55°C for 30 second, elongation at 72°C for 30 second, and final extension at 72°C for 5 minutes, and 26°C for 10 seconds. The PCR products were digested by using an Msp1 enzyme, and separated by electrophoresis on a 1.5% agarose gel stained by ethidium bromide. Visualization done under UV light, revealed distinct banding patterns (Fig 1)a wild-type (monomorphic) genotype. While the polymorphic genotype (GA) produced three bands at 916 bp, 582 bp, and 334 bp. The genotype AA located at 916 bp and 334 bp. The study on lead content in the blood was conducted using a standard of $PbSO_4$ solution (WHO, 2020); with various concentrations of 2 ppm, 5 ppm, 9 ppm, and 15 ppm consecutively. For this purpose, the AAS machine with a wavelength of 217.6 nm and 3.5 mÅelectrical current. The hematological profile was conducted by the Sysmex Haematology Analyzer (Puspitaningrum *et al*., 2018),meanwhile, the MDA level was analyzed using a TBA method, Cat Number: E-BC-K025-S(Elabscience, 2020).

Statistical analysis: Data are expressed as mean ± standard deviation (SD). Differences between the polymorphic and monomorphic groups were evaluated using an independent t-test, p-values < 0.05considered statistically significant.

RESULTS

Gene ALAD polymorphism

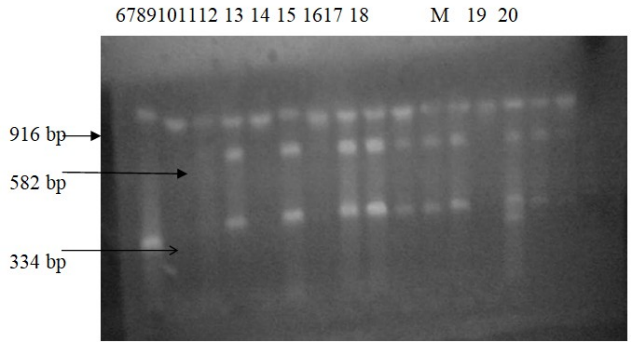

Figure 1. Visualization of the ALAD gene after digested by Msp-1 enzyme Remarks: No. 7, 10,12, 18,and 21 are genotypes of non polymorphic ALAD gene, with GG genotype (ALAD 1-1). M: Marker. No. 6, 9,11,13,14,15,16,17, and 20 arepolymorphic genotypes for the ALAD gene, with GA genotype (ALAD 1-2)

Gene ALAD Polymorphism: Figure 1 visualized ALAD gene fragmentations after being digested with monoMsp-1 enzyme. In this gel, lanes 7, 10, 12, 18, and 21 display a single band of 916 bp, corresponding to the monomorphic genotype (GG, or ALAD 1-1). In contrast, lanes 6, 9, 11, 13, 14, 15, 16, 17, and 20 show three distinct bands (916 bp, 582 bp, and 334 bp), indicating a polymorphic pattern (GA, or ALAD 1-2). Recent studies showed individuals with ALAD 2 allele tend to have higher blood-lead content than workers with ALAD 1 allele. From current molecular study, 33% respondent exhibited heterozygous genotype (GA,ALAD 1- 2), while the remaining were homozygous genotype (GG, ALAD 1-1). These results are partly consistent with Rujito et al., (2015); Puspitaningrum et al., (2017, 2018), more than half of their samples (94.7% and 51.7%)were homozygous for allele 1-1 and the rest were heterozygous(ALAD 1-2). They also reported that no workers had homozygous ALAD 2-2 allele. genotype. Overall, our findings suggest a strong correlation between ALAD polymorphism and blood-lead content, where heterozygous allele for ALAD 1-2 were characterized with higher leadcontent. ALAD G177C gene has two alleles (ALAD 1 and ALAD 2) with three phenotypes (ALAD 1-1, ALAD 1-2 and ALAD 2-2) (Sobhi *et al.,* 2019).ALAD 2 adsorb lead compound much easier than ALAD 1 alleleas represented in their blood lead content. 10 Respondentswith ALAD 1-2 genotypes, had three DNA bands at the size of 916 bp, 582 bp and 334 bp, whichindicates mutation. While, the rest were characterized ALAD 1-1 with monomorphic pattern at 916 bp (Figure 1) likewise the Rujito *et al*., (2015) where approximatelyonly5% respondent with ALAD 1-2 genotypes which causes a higher blood-lead content. Further, Puspitaningrum *et al*. (2017& 2018) showed less than half respondents had ALAD 1-2 genotypeand the rest had carrier for ALAD 1-1. These data imply that the workers exposed to lead compound and have ALAD 1-2 genotypes contained higher blood-lead than another genotype (ALAD 1-1).

Blood-lead content: Table 1 shows the level lead in both types ALAD 1-2 and ALAD 1-1,means all respondents are susceptible to lead compound which caused by the present of ALAD 1-2 gene.

Table 1. Blood-lead content of respondents with ALAD 1-2 and 1-1 gene

Group	Blood lead level (ppm)	p-value
Polymorphism	0.69 ± 0.045	0.001
Monomorphism	0.39 ± 0.083	

Car's paints contain 4% Pb-oxide, 14% Fe Pb-oxide, 21% Pb-phosphate, 3% Fe Pb-sulphate, and 2% MnPb-oxide in very small particles, the painters, therefore, inhaled and adsorb these compounds every day. Following to inhalation, the lead compound goes to the lungs through respiratorytract and adsorb by the blood (Vitayavirasuk *et al.,* 2005; Dapdour *et al*., 2016). In addition, motor vehicles might also increase lead compound to surrounding air and caused increasement of tetraethyl lead (TEL) which m in the human body, is converted into Pb triethyl, and enters the cell to bind with –SH group. Finally, it will inhibitenzymes activities, including δ -ALAD (Grecka *et al*., 2017). This study noted an increase of lead in the blood due to the presence of polymorphic gene, which is in line with Wan*et.l.,* (2014)people with polymorphic pattern for the ALAD gene, contained higher lead compound in their blood.

Malondyaldehide (MDA) level: Table 2.shows MDA level of polymorphic gene pattern respondents higher than monomorphic.

Table 2. The differences of MDA level between polymorphism and moonomorpjiic

Group	MDA level(µmol/L)	p-value
Polymorphism	1.71 ± 0.30	0.000
Monomorphic	1.19 ± 0.33	

Normal individual has MDA level ≤1 µmol/L, but this studynoted that all workers exposed to lead compound have higher MDA than normal people. In humanbody, lead induces oxidative stress but decrease activities of the following enzymes like SOD, GPx and CAT. Consequently, resulted in stopping the production of lipid peroxidation and damages on the MDA, a goodindicator of lipid peroxidation and oxidative stress (Ibrahim *et al*., 2012; Mohamed *et al*., 2016; Charkiewicz&Backstrand, 2020). Similarly, the MDA level increases when is disturbed lead detoxification process in the liver by inhibiting activity of ofGlutathion-S Transferase (GST) enzyme which resulted in a failure of detoxification processes. In this situation liver cannot eliminate lead which will be accumulated in the liver (Hunaiti&Soud, 2000; Bijoor& Venkatesh, 2007); further, the lead triggers lipid peroxidation and induces damage ofliver cell membrane through increasement MDA the blood. A similar result reveal from a study in Turkey, where 25 students worked part time as car's mechanics for 8 hours/day and total of 5 weeks, noted an increasement of MDA level. This, increased blood-lead content above the normal people. Meanwhile, in Poland lead-blood content elevated up to 1.32 ± 0.2 µmol / L, and Pb-blood levels increase between 0.43 ± 0.05 ppm (Oktem *et al*., 2004). Haematological analyses in this study noted that Hb level (Table 3), erytrocyte (Table 4) and leukocyte (Table 5) of the workers with polymorphic pattern in ALAD gene performed differently from those monomorphic gene pattern ($p<0.05$).

Hemoglobin level (g/dL): Table 3. shows the Hemoglobin (Hb) level of therespondent with polymorphism. It is noted that, this group has lower Hb than monomorphic respondents and so parallel with Chung *et.al.,* (2006), the polymorphic pattern decreases the activity and expression of the enzyme by 20-30%.

Table 3. Hemoglobin level

Group	Hb level(g/dL)	p-value
Polymorphism	10.46 ± 0.92	0.001
Monomorphism	13.35 ± 0.54	

Lead compound which enters human body, inhibits activity of δ –ALAD enzyme and decreases the hemoglobin level. Such δ –ALAD enzyme called cytosolictogether with ferrochelatase catalyze Fe substance to form hem.A comparative study done by Nakhaee *et al*., (2019) showed an average of Hb in individuals with high blood-lead content are lower than health people (12.6 g/dL and 15.2 g/dL respectively). This result indicated that individuals expose to lead compound in their blood are at risk to have a lower Hb level than the healthy individuals. According to (Yang *et al*., 2012; Puspitaningrum., 2017), the decrease and inhibition of ALAD's enzyme in people with polymorphic pattern causes a lower hemoglobin than monomorphic individuals.

The Total Erythrocyte number: Table 4., shows erythrocyte level of polymorphic pattern is lower than monomorphic respondents. This figure is a represent of lead compound which bind to the erythrocytes by 90% and further, causes destabilization on the cell membrane/ membrane fluidity and finally increases hemolysis. As stated by (Kianoush *et al*., 2013; Balali-Mood *et al*., 2021), lead compound is considered as a hemolytic agent because of its capability in destructing erythrocytes through formation of lipid peroxides in cell membranes.

Table 4. Erythrocyte level in polymorphic and monomorphic respondents

Group	Erythrocyte count (x 10^6 /µL)	p-value
Polymorphism	3.05± 0.31	0.001
Non-polymorphism	4.37± 0.16	

According to Omobowale *et al*. (2014), decreasing the erythrocytes causes the damage of renal proximal tubules, a place to produced erythrocytes, by inhibition of 5-nucleotidase pyrimidine enzyme activity and further decrease erythrocytes and retention of ribosomal DNA. Here, the erythrocytes are found as immature forms such as the blue spot erythrocytes and the reticulocytes; when the number of immature erythrocytes increases the mature erythrocytes decreases (Oktem *et al*., 2004; Dongre *et al*., 2011). When the erythropoietin formation is disturbed it may cause the decrease in the function of cell progenitor as well as the erythrocytes.

Table 5. Total leucocyte in polymorphic and monomorphic individuals

Group	Total Leukocyte count (x 10^3 /µL)	p-value
Polymorphism	11.908 ±1.124	0.001
Non-polymorphism	7.256 ± 0.760	

Total Number of Leucocyte: Lead compound will breakdown membrane lipidand chemotactic movement of polymorphonuclear cells (neutrophils) and finally active in phagocytosis (Farkhondeh *et al*., Moin, 2014). Increase in neutrophils numbers, triggerslead radical's (Pb tri ethyl) compounds and granulocyte colony-stimulating factor (G-CSF) cytokines in bone marrow. This study noted that bone marrow producesmore immature neutrophil than mature and increase phagocytosis. Similar results reported that the presence of lead compound in mice increases number of leukocyte and altering the structure of monocyte to be more reactive which enclose cytoplasmto form basophilic aggregate and vacuole. Indicating aninflammatory response to chronic condition (Saeed, 2015; Ragini *et al*., 2011). The next process is activation of humoral and cellular specific immune systems by B and T lymphocytes besides by activation of a helper cell / T cell. The T cell, later help the B cell to produce antibodies and so increases lymphocytes (Ercal *et al*., 2000; Fenga, et. al., 2017; Mishra,& Rani (2003), examined the level of lead compound in workers in Taiwan industry, noted the blood-lead content of this group ranging between 0.75-1.28 ppm. At this level, the total number of T cell memory (CD 4+ and CD8+) cell and lymphocyte increased. Furthermore, study toward the US residents (Sarasua *et al*., 2012), reported that people who live surrounded industrial areas with air and soil lead pollution of > 0.15 ppm, also increased the B lymphocytes and antibodies (IgA, IgG, and IgM). Finally, it resulted in These processes result in the increase of leukocyte.

Clinical implication: This study noted that exposure of lead compound reveals a polymorphism in their ALAD gene leads to anemia. Considering to this, early precaution and prevention should be considered.

Limitation of this study: This study unfortunately, did not examine the level of ALAD gene and so unable to detect whether the expression of that enzyme was either decrease or stable in a normal level.

CONCLUSION

Respondents with the polymorphic pattern on δ-ALAD gene means that their blood-lead content and the MDA were higher than those monomorphic of individuals. Statistically, they are also showed a significant difference in some parameters. The polymorphic group had a lower hemoglobin, erythrocyte, and leukocyte than monomorphic.Lead compound did not only cause polymorphism on their δ-ALAD gene, but alsoaffects MDA level and changes haematological profile and anemia.

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