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Superoxide Dismutase Levels and Polymorphism (*Ala16val*) In Tuberculosis Patients with Diabetes Mellitus in Medan City

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Abstract

BACKGROUND: Infectious diseases and metabolic disorders would result in oxidative stress in cells. Superoxide dismutase (SOD) is an antioxidant present inside cells that acts against oxidative stress. SOD gene polymorphism can affect the activity and levels of SOD.

AIM: This study aimed to analyse SOD levels and polymorphism of gene (ala16val) that regulated SOD in tuberculosis patients with diabetes mellitus in Medan city.

METHODS: A total of 40 tuberculosis patients with diabetes mellitus and 40 healthy subjects participated in the study. The levels of SOD were measured using enzyme-linked immunosorbent assay (ELISA). Analysis of SOD gene polymorphism (ala16val) was done using polymerase chain reaction-restriction fragment lengths polymorphisms (PCR-RFLP) with BsaW1 as the restriction enzyme. The statistical significance was determined using the Mann Whitney test, Fisher's exact test, and Kruskal Wallis test ($p < 0.05$).

RESULTS: The SOD levels of tuberculosis patients with diabetes mellitus were lower than those of the healthy subjects (102.474 ± 36.07 U/L vs 294.543 ± 58.75 U/L, $p < 0.05$). Patients of tuberculosis with diabetes mellitus tend to have more value/Val genotypes than the healthy group (57.5% vs 50%, $p > 0.05$). There was no association between SOD levels and SOD gene polymorphism (ala16val) in tuberculosis patients with diabetes mellitus.

CONCLUSION: In this study, there was an association between the levels of SOD and tuberculosis patients with diabetes mellitus, but not for the SOD gene polymorphism (ala16val). The SOD gene polymorphism (ala16val) was not the key role to influence the SOD levels in tuberculosis patients with diabetes mellitus in Medan city.

Introduction

Tuberculosis is an infectious disease which has been known to result in the highest mortality rate among bacterial infections in the world. Since 1992, the World Health Organization (WHO) has announced that tuberculosis infection is a global emergency. In Indonesia, tuberculosis infection is one of the major public health problems. Indonesia is one of five countries in the world which have the highest number of tuberculosis patients along with India, China, South Africa, and Nigeria [1].

To date, experts suggest that there is a disorder in the immune system of tuberculosis patients [2]. The cell's defence towards tuberculosis infection activates the immune system and causes oxidation-reduction (redox) imbalance. Antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and others, can decrease this effect. However, previous studies showed that the activity or levels of SOD decreased in tuberculosis patients [3], [4].

In tuberculosis patients, the decrease of antioxidant levels can be worse in the presence of comorbidity conditions, such as metabolic disorder namely diabetes mellitus (DM). DM is an important risk factor for tuberculosis [5]. Eight of ten countries

with the highest incidence of DM are also the countries with the highest number of tuberculosis cases [6]. Several epidemiological studies have suggested the need to see the interaction between tuberculosis infections and DM [7].

DM is a chronic metabolic disorder that occurs when the pancreas is unable to produce insulin or when there is an occurrence of cell failure in using insulin effectively. This condition leads to metabolic disorders characterised by elevated blood glucose levels or hyperglycemia. Various complications of DM are related to increased oxidation of blood glucose. There is an abnormality in immunity and cell-mediated phagocyte function. The process will produce reactive oxygen species (ROS) which will increase oxidants and lipid peroxides [8]. A previous study showed an increase in lipid peroxides compounds, such as malondialdehyde in hyperglycemic conditions [9].

In DM, increased oxidants and lipid peroxides correlated with the decrease of antioxidant levels [10]. Previous studies exhibited a reduction of antioxidant levels in diabetic patients [11], [12]. SOD synthesis is regulated by specific SOD gene sequences. Single nucleotide polymorphism (SNP) or substitution of one nucleotide base in the SOD gene contributes to the expression of SOD. Differences in gene sequences determine the changes in gene expression and affect disease development [13]. SNP in the SOD gene was associated with the total antioxidant capacity in DM [14]. A study on diabetic patients by Flekac et al., (2008) found that SOD1 and SOD2 polymorphisms affect SOD activity [15].

Therefore, this study aimed to analyse the superoxide dismutase levels and SOD gene polymorphism (ala16val) in tuberculosis patients with diabetes mellitus in Medan city.

Methods

Subjects

This study was a cross-sectional design. Forty patients diagnosed with tuberculosis and diabetes mellitus were recruited at the health care facilities for pulmonary disease in Medan city. The diagnoses were made based on the criteria of Indonesian Lung Doctor Association [16] and the criteria of Indonesian Endocrinology Society [17]. The healthy subjects were recruited from a gym place in Medan city. The healthy subjects should not have a familial history of diabetes mellitus. Subjects who took vitamin supplements, who had infections such as HIV, hepatitis, or other malignant diseases, and who drank alcohol were excluded from this study.

Ethics

Each subject gave written informed consent after receiving a brief description of the purposes and benefits of the study. The procedure has been approved by the ethics committee of the Faculty of Medicine, Universitas Sumatera Utara (No. 327/2017) following the Second Declaration of Helsinki. The study was conducted from July 2017 to June 2018.

Sample collection

Blood sample (5 ml) was collected in plain and EDTA tube from the median cubital vein. The blood sample was centrifuged at 3000 rpm for 10 minutes. The blood glucose level was measured within 2 hours after being taken. The serum was stored at -80°C for further measurement of SOD levels.

DNA was extracted from the whole blood using the genomic DNA extraction kit (Promega, USA). The DNA template was then stored at a temperature of -80°C for the next stage of amputation. All process was carried out at the Integrated Laboratory of the Faculty of Medicine, Universitas Sumatera Utara.

Measurement of Blood Glucose Levels

The blood glucose levels (BGLs) were directly measured by the glucose oxidase-peroxidase (GOD-PAP) enzymatic colourimetric method and read with a spectrophotometer at a wavelength of 500 nm.

Measurement of Superoxide Dismutase (SOD) Levels

The SOD levels were measured using an enzyme-linked immunosorbent assay (ELISA) method with a commercial kit (Lab science, USA). The detection range was 3 to 900 U/L with a sensitivity of 1.52 U/L.

Amplification of SOD Gene

The DNA was amplified with the polymerase chain reaction (PCR) method. The amplification was performed using Forward primer 5'-CAG CCC AGC CTG CGG AGA CGG-3 'and Reverse primer 5'-CTT GGC CAA CGC CTC CTG GTA CTT-3'. Before performing the amplification process, the PCR solution (25 µl) was made by mixing master mix (12.5 µl), primers (1 µl each), DNA template (2 µl), and nuclease-free water (8.5 µl) (Promega, USA).

The amplification process started with initial denaturation of DNA at 95°C for 5 minutes, followed by (95°C for 45 s (melting), 54°C for 30 s (annealing), and 72°C for 30 s) for 30 cycles of amplification, and a final extension at 72°C for 5 minutes [18]. PCR

products were analyzed on 2% agarose gel electrophoresis and staining with ethidium bromide.

Restriction Fragment Length Polymorphism (RFLP) of SOD gene polymorphism (ala16val)

The PCR reaction mixture (10 µl) was digested using restriction fragment length polymorphism (RFLP) method by the 0.2 µl of restriction endonuclease BsaW1 enzyme at 60°C in 10 minutes. The RFLP products were analysed on 4% agarose gel electrophoresis and staining with ethidium bromide.

Statistical Analysis

Statistical analysis was performed using SPSS version 22. Comparison of the mean of SOD levels in both groups was carried out using the Mann Whitney test. The Fisher's exact test was performed to assess the association of genotype and alleles in SOD gene polymorphism (ala16val) between both groups. In the group of patients, differences of the SOD levels on genotype variants were analysed using the Kruskal Wallis test whereas Hardy-Weinberg equilibrium (HWE) in both groups was analysed using the Chi-Square test.

Results

This study involved 40 tuberculosis patients with diabetes mellitus and 40 healthy subjects. The characteristics of the subject in both groups are shown in Table 1.

Table 1: Characteristics of studied groups

Characteristics	Patient (N = 40)	Healthy subject (N = 40)
Age (years), mean ± SD	53.26 ± 8.77	45.2 ± 10.90
Gender (male/female), N (%)	26 (65.0)/14 (35.0)	21 (52.5)/19 (47.5)
Duration of disease (years) mean ± SD	3.61 ± 1.38	-
Smokers N (%)	18 (45.0)	15 (37.5)
Blood glucose levels ad random (mg/dl) mean ± SD	295.51 ± 57.85	113 ± 19.8

The mean age of subjects in the patient group was 53.26 ± 8.77 years old, whereas the mean of subjects in the healthy group was 45.20 ± 10.90 years old. Based on gender, more male subjects were found than female subjects in the patient group (65% vs 35%). Similarly, there were more male subjects that female subjects in the healthy group (52.5% vs 47.5%). The mean duration of disease for 3.61 ± 1.38 years. 45% of the subjects in the patient group were smokers while 37.5% of the subjects in the healthy group were smokers. The mean BGLs in the patient group was 295.51 ± 57.85 mg/dl compared to 113 ± 19.8 mg/dl in the healthy group.

The mean levels of superoxide dismutase (SOD) both in patients and healthy control groups can be seen in Table 2.

Table 2: The mean levels of SOD in patient and control groups

	Patients group		Control group		p-value
	Mean	SD	Mean	SD	
SOD (U/L)	102.474	36.07	294.543	58.75	0.010

The SOD levels in the patient group were lower than the healthy group (102.474 ± 36.07 vs 294.543 ± 58.75), respectively. The Mann-Whitney test showed a significant difference between both groups ($p < 0.05$).

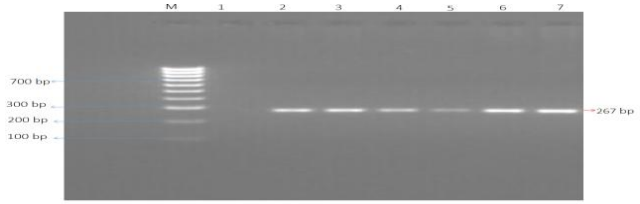

Figure 1: The PCR product of the SOD gene on 2% agarose gel electrophoresis. Lane M: DNA Ladder 100 bp; Lane 1: Negative Control; Lane 2, 3, 4, 5, 6, 7: PCR product of SOD gene at 267 bp

The PCR and the PCR-RFLP product of the SOD gene polymorphism (ala16val) can be seen in Figure 1 and Figure 2.

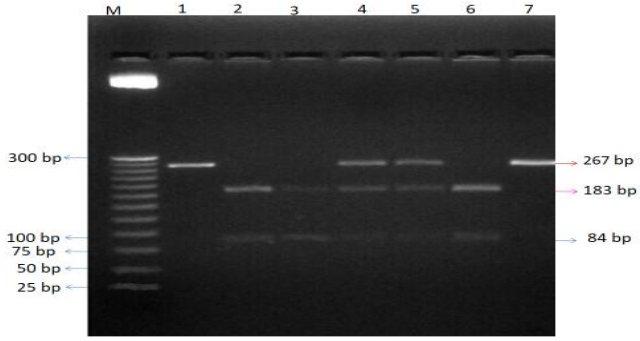

Figure 2: The PCR-RFLP product of the SOD gene Ala16val polymorphism on 4% agarose gel electrophoresis. Lane M: DNA Ladder 25 bp; Lane 1, 7: homozygous Ala/Ala without any cutting of PCR product; Lane 2, 3, 6: homozygous mutant Val/Val had two bands at 184 bp and 84 bp; Lane 4, 5: heterozygous Ala/Val had three bands at 267, 184 bp and 84 bp

The PCR product of the SOD gene was at 267 bp (Figure 1). After the digestion with BsaW1 restriction enzyme, the PCR product showed three different patterns. The ala/ala homozygous wild-type produced a band at 267 bp (without any cutting of PCR product). The ala/Val heterozygous showed three bands at 267 bp, 183 bp, and 84 bp. Whereas, the val/val homozygous mutant had two bands at 183 bp and 84 bp (Figure 2).

Frequencies of genotype and allele of SOD gene polymorphism (ala16val) in patients and healthy control groups can be seen in Table 3.

The ala/ala frequency in the patients and

healthy subjects were similar (5%). The ala/val frequency in the healthy group was higher than the patient group, but the val/val frequency in the patient group was higher than the healthy group. However, there was no statistical difference found in the genotypes and alleles between patients and healthy subjects ($p > 0.05$).

Table 3: Comparison in genotype and allele frequencies of SOD gene Ala16val polymorphism between the patients and healthy

Genotype	Patient (N = 40)	Healthy subject (N = 40)	P
Ala/ala	2 (5%)	2 (5%)	
Ala/val	15 (37.5%)	18 (45%)	0.863
Val/val	23 (57.5%)	20 (50%)	
Allele	Patient	Healthy subject	P
Ala	19 (23.75%)	22 (27.5%)	
Val	61 (76.25%)	58 (72.50%)	0.580

The association between levels of SOD and genotypes of SOD gene polymorphism (ala16val) in tuberculosis patients with diabetes mellitus shows in Table 4.

Table 4: Levels of SOD according to the genotype of SOD gene polymorphism (ala16val) in patients

	Genotype	Mean	SD	P
SOD levels (U/L)	Ala/ala (n = 2)	116.1092	6.68	0.051
	Ala/val (n = 15)	87.3318	12.47	
	Val/val (n = 23)	111.1639	24.20	

The SOD levels in the val/val genotype were lower than the ala/ala wild-type genotype. However, based on the Kruskal Wallis test, the association between genotypes of the SOD gene and SOD levels was not significant ($p > 0.05$).

Discussion

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb). The occurrence of oxidative stress in tuberculosis patients will cause an increase in ROS which is formed during the phagocytic actions on Mtb [18]. In metabolic disorders such as diabetes mellitus, the condition of hyperglycemia will trigger oxidative stress [19]. These conditions require antioxidant activities. Antioxidants are compounds which have complex and comprehensive functions against oxidative stress conditions [20].

The body can produce antioxidant compounds named endogenous antioxidants. SOD is one of the most important endogenous antioxidants and classified as the main antioxidant in homotetrameric enzyme group due to the ability in preventing cells from free radicals and oxidative stress [21]. SOD was first identified in 1938 by Mann and Keilin. SOD works as an antioxidant by preventing the initiation stage in the chain reactions of free radicals formation from ROS. SOD enzymes can degrade ROS superoxide anions into oxygen and hydrogen peroxide. However, in the condition of infection and metabolic disorders, the number of ROS significantly increases, resulting in more NADH and FADH2 electron donors enter the electron transport chain. The increase of ROS will increase the rate of lipid peroxidation which contributes to the production of free radicals. Oxidative modification, in turn, may inactivate SOD and decrease SOD levels [22], [23].

The present study found that serum superoxide dismutase (SOD) levels of tuberculosis patients with diabetes mellitus were significantly lower than the healthy subjects. Several previous studies have found that SOD levels were significantly lower in tuberculosis patients than the control group. The percentage of SOD inhibition in tuberculosis patients was lower than that of the healthy subjects [3], [4]. Other previous studies also showed that the SOD levels in the group of diabetic patients were lower than the group of healthy subjects [24], [25]. To the best of our knowledge, no study of SOD levels in tuberculosis patients with diabetes mellitus has been reported.

In the present study, the genotype frequency of SOD gene polymorphism (ala16val) in tuberculosis patients with diabetes mellitus and the healthy subjects was also compared. The result showed that val/val genotype as the mutant genotype was higher in the patient group than the healthy subject group although the difference was not statistically significant. This research was the first study which reported this finding. Several previous studies have only investigated the association between SOD gene polymorphisms (ala16val) and diabetes mellitus patients, but not SOD gene polymorphism (ala16val) in tuberculosis patients with diabetes mellitus. Past studies reported that val/val genotype was the most common in diabetic patients, and there was an association between SNP in SOD gene with diabetes mellitus [15], [26], [27].

SOD gene is located at the twenty-five point of the long arm of chromosome six which has five exons and four introns. The expression of SOD gene sequence influences SOD activity. Single nucleotide polymorphism (SNP) (i.e. the substitution of one of the nucleotide base in the SOD gene) contributes to the different expression of SOD. SOD gene polymorphism (ala16val) is a commonly known polymorphism. In this type of polymorphism, a single-nucleotide is substituted from C to T resulting in a change of the amino acid from ala/ala (GCT) to valine/val (GTT) at the 16th residue (ala16val) of the gene sequence. As a result, the SOD gene polymorphism (ala16val) has three genotypes, namely ala/ala genotype as wildtype genotype, and ala/val genotype and val/val genotype as mutant genotypes [13].

It has been reported that val/val genotype is associated with lower SOD activity than the ala/ala genotype. The substitution from ala to val reduces

SOD activity and synthesis by 30 – 40% and decreases the transport efficiency of SOD into the mitochondria. This change is considered as an important pathophysiological mechanism that makes people with val/val genotype have a risk factor for certain type of diseases. Furthermore, the val/val genotype of the SOD gene has been reported to be associated with a lower SOD efficiency against oxidative stress [28].

However, the present study did not find a significant association between the levels of SOD and genotypes of SOD gene polymorphism (ala16val) in tuberculosis patients with diabetes mellitus. Environmental factors such as nutrition intake and lifestyle were not assessed in this study. The activity and level of SOD can be influenced by environmental factors [29]. The SOD levels in this population might be more influenced by environmental factors, so there was no relationship between the level of SOD and the genotypes of SOD gene polymorphism (ala16val).

The other possibility of this finding might be due to the relatively small number of samples.

The distribution of SOD gene polymorphism (ala16val) in both groups was compared according to the Hardy – Weinberg Equilibrium (HWE) using the chi-square goodness-of-fit test. The results showed that SOD gene polymorphisms (ala16val) fulfilled the HWE. As the large population in Medan city is dominated by migrants, people commonly practice cross-ethnicity marriage. This condition results in a consistency of Hardy-Weinberg law in the population [30].

The present study also found that men were more likely to suffer from DM than women. This finding is consistent with a study in North India and North-west Nigeria [31]. Although gender is not a risk factor for infectious diseases including tuberculosis infection, it may be a risk factor for DM [32], [33].

In the present study, there was an association between SOD levels and tuberculosis patients with diabetes mellitus, but not for SOD gene polymorphism (ala16val). Moreover, SOD levels were not associated with val/val genotype in SOD gene polymorphism (ala16val) in the group of tuberculosis patients with diabetes mellitus in Medan city. These findings suggest that SOD gene polymorphism (ala16val) is not an important factor in influencing SOD levels in tuberculosis patients with diabetes mellitus in Medan city. Further study should be conducted using a large sample size, and the environmental factors of the research subjects should be analyzed.

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