

## Correlation of Interleukin-10, Superoxide Dismutase (SOD), and Malondialdehyde (MDA) Levels with HbA1c in Pediatric Type 1 Diabetes Mellitus

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### ABSTRACT

Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by pancreatic  $\beta$ -cell destruction and considered to be correlated with oxidative stress. This study aimed to investigate the association of oxidative stress [superoxide dismutase (SOD) and malondialdehyde (MDA) levels], inflammation [interleukin 10 (IL-10)], and glycemic control (HbA1c) in pediatric T1DM patients. This study included 25 T1DM subjects and 25 healthy control subjects and was designed as a cross-sectional study. SOD, MDA, and IL-10 levels were measured by ELISA. We obtained that that IL-10 and SOD levels were significantly decreased in the T1DM group, but MDA and HbA1c levels were significantly elevated in the T1DM group. IL-10 levels were positively correlated with SOD levels and negatively correlated with MDA and HbA1c. SOD levels were negatively correlated with HbA1c levels. MDA was positively correlated with HbA1c levels. IL-10 and SOD levels were significantly decreased, but MDA and HbA1c levels were significantly elevated in the T1DM group.

**Keywords:** Type 1 diabetes mellitus, superoxide dismutase, malondialdehyde, interleukin 10, HbA1c

### INTRODUCTION

Type 1 diabetes mellitus (T1DM) is an autoimmune disease caused by pancreatic  $\beta$ -cell destruction as the result of several interacting factors, including genetic vulnerability and environmental exposure [1]. Ninety percent of T1DM cases occur in children and adolescents. Its incidence varies worldwide, the highest being in the Finnish population (40 per 100 000 people), and the lowest in the Chinese population (0.1 per 100 000 people per year) [2]. Data gathered from the Coordination Framework Unit of Pediatric Endocrinology, Indonesian Pediatric Association indicated a prevalence of T1DM was 731 cases in 2012 [3]. Other data from Saiful Anwar General Hospital, Malang showed that there were 35 T1DM patients ranging in age from 1 to 18 years (2005-2013) [4].

Destruction of pancreatic  $\beta$  cells is mediated by T-cell hyperreactivity, which in turn induces the release of autoantibodies. Impaired immunoregulation causes T-

cell autoreactivity, then induces islet cell inflammation, and finally causes pancreatic  $\beta$ -cell destruction [3, 5].

A previous study showed that oxidative stress plays an important role in diminished insulin action and secretion. Oxidative stress in diabetic patients might be caused by decreased antioxidant enzyme activity and elevated reactive oxygen species (ROS) levels, which in turn cause further lipid peroxidation and glycation [6]. A previous study showed that decreased activity of several enzymes, such as superoxide dismutase (SOD), peroxidase (Px), ceruloplasmin (Cp), and glutathione peroxidase (GSH-Px), as well as elevated glutathione disulfide (GSSG), were present in erythrocytes and tissues from diabetic patients [7].

Interleukin 10 (IL-10) is an anti-inflammatory cytokine with the ability to downregulate inflammatory cytokine production and thus inhibit T-cell activation. IL-10 is a potent inhibitor of Th1 cytokines, such as IL-2 and IFN- $\gamma$ . In T1DM, proinflammatory cytokines are

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How to cite:

Astari LF, Cahyono HA, Widjajanto E (2017) Correlation of Interleukin-10, Superoxide Dismutase (SOD) and Malondialdehyde (MDA) Levels with HbA1c in Pediatric Type 1 Diabetes Mellitus. J. Trop. Life. Science 7 (3): 286 – 292.



elevated and anti-inflammatory cytokines are decreased as compared with the nondiabetic individuals [8]. Glycemic control is related to both microvascular and macrovascular complications. Good glycemic control could increase the quality of life of T1DM patients. High HbA1c levels reflect poor glycemic control [9].

To date, a study on the association of oxidative stress and inflammation status with glycemic control in T1DM patients in Indonesia has not been conducted. This study aimed to investigate the association of IL-10, SOD, and malondialdehyde (MDA) levels with HbA1c levels in pediatric T1DM patients.

## MATERIALS AND METHODS

### Study design

A cross-sectional (observational, analytic) study was conducted to compare the levels of IL-10, SOD, and MDA with those of HbA1c in pediatric T1DM patients. All procedures were approved by the Research Ethics Committee of Saiful Anwar General Hospital, Malang.

### Subjects

A total of 50 subjects were included in this research and divided equally into two groups (control and T1DM group). The inclusion criteria for subjects were as follows: diagnosed as T1DM, aged between 1 and 18 years old, and allowed by his/her parents to participate (informed consent). The exclusion criteria for subjects were the following: T1DM patients who had other diseases,

such as other autoimmune diseases, liver, and renal impairment, and anemia and consumption of antioxidants

within 3 weeks before the study. To establish good matching of subjects and controls, the inclusion criteria for controls were age between 1 and 18 years and allowed by his/her parents to participate (informed consent). The exclusion criteria for controls were T1DM patients; diagnosed with the autoimmune disease (such as SLE), severe infection, liver and/or renal impairment, or anemia; and consumption of antioxidants within 3 weeks before the study. All subjects (control and T1DM group) were taken from the Pediatric Ward, Saiful Anwar General Hospital, Malang.

### Measurement of SOD

SOD was measured by a competitive ELISA method as previously described. Briefly, blood samples to which EDTA was added were centrifuged at 1,000 rpm for 15 minutes. A 50 µL volume of sample or standard was added to each well of a microtiter plate and an equal volume of biotinylated antibody was added and incubated for 45 min at 37°C. After washing, 100 µL HRP conjugate was added to each well and then incubated for 30 min at 37°C. After washing, 90 µL tetramethylbenzidine (TMB) substrate was added to each well and then incubated for 15 minutes at 37°C. Finally, 50 µL stop solution was added and 30 minutes later, the absorbance was read in a microplate reader at 450 nm. SOD measurements were conducted at the Physiology Laboratory, Medical Faculty of Brawijaya University, Malang.

Table 1. Subject characteristics

Characteristics	Control (n = 25) Mean ± SD	DM (n = 25) Mean ± SD
Sex:		
Male	9	10
Female	16	15
Age	10.04 ± 3.43	11.48 ± 2.60
%IBW	90.52 ± 5.15	93.34 ± 8.83
Erythrocyte count (/µL)	5.12 ± 0.53	5.22 ± 0.54
Leucocyte count (/µL)	8,747 ± 929.79	8,920.40 ± 715.74
Thrombocyte count (/µL)	305.46 ± 61.94	340.36 ± 32.26
Urea level (mg/dL)	22.68 ± 2.88	23.58 ± 3.79
Creatinine (mg/dL)	0.66 ± 0.15	0.66 ± 0.15
SGOT (U/L)	30.56 ± 3.32	30.88 ± 3.49
SGPT (U/L)	31.32 ± 3.35	31.30 ± 3.27

Note: There were no significant differences in subject characteristics (independent samples *t*-test, *p* > 0.05), indicating that the control and T1DM groups were appropriately matched. IBW: Ideal Body Weight.



### Measurement of MDA

The method of malondialdehyde measurement was based on the reaction of chromogenic reagents N-methyl-2-phenylindole (NMPI) with MDA at 45°C as previously described.<sup>10</sup> The procedure began by adding 10 mL probucol to each well. After 200 µL sample and 600 µL R1 reagent (NMPI in acetonitrile) were added, the sample was mixed by using a vortex. A 150- µL volume of hydrochloric acid was added to each well, mixed again by using a vortex and incubated for 60 min at 45°C. After incubation, the sample was centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed and the absorbance read at 586 nm. MDA measurements were conducted at the Physiology Laboratory, Medical Faculty of Brawijaya University, Malang.

### Measurement of IL-10

IL-10 levels were measured by ELISA as described by the manufacturer.<sup>10</sup> A 200-µL volume of assay diluent was added to each well and incubated for 1 h. After that, 100 µL standards was added to control and sample wells, which were covered with an adhesive strip and then incubated for 2 h at room temperature. After four washes with wash buffer (400 µL), 100 µL antibody was added to each well and incubated for 1 h. After incubation, 100 µL avidin-horseradish peroxidase (HRP)-conjugated IL-10 was added to each well. The microplate was then covered with an adhesive strip, incubated for 30 min at room temperature, and rewashed. A 100-µL volume of tetramethylbenzidine (TMB) substrate solution was added to each well, incubated for 30 min at room temperature, and 100 µL stop solution was added. Optical density was measured at 450 nm by using a microplate reader. Absorbance values were used to plot a standard curve and to calculate the IL-10 level in each sample. IL-10 measurements were conducted at the Physiology Laboratory, Medical Faculty of Brawijaya University, Malang.

### Measurement of HbA1c

Hemoglobin A1c (HbA1c) levels were measured in whole blood samples to which EDTA was added. A 5-µL blood sample was mixed with 1.5 mL diluent solutions before analysis. The level of HbA1c in the sample was measured by using a Bio-Rad D-10TM.<sup>10</sup> HbA1c measurements were conducted at the Clinical Pathology Laboratory, Saiful Anwar General Hospital, Malang.

## RESULTS AND DISCUSSION

### Subject characteristics

In this research, subjects were T1DM patients who

routinely attended the Endocrinology Department of Saiful Anwar General Hospital for outpatient care during the research period. Table 1 shows subject characteristics of the two groups.

### SOD and MDA levels

Results showed that both SOD and MDA levels were significantly different between T1DM and control groups (independent samples t-test,  $p < 0.05$ ). SOD levels in the T1DM group were significantly lower as compared with control group. Conversely, MDA levels in the T1DM group were significantly higher as compared with the control group.

### IL-10 levels

Results showed that the IL-10 level was significantly different between the T1DM and control groups (independent samples t-test,  $p < 0.05$ ). IL-10 levels in the T1DM group were significantly higher as compared with the control group.

### HbA1c levels

Results showed that HbA1c levels were significantly higher as compared with the control group (independent samples t-test,  $p < 0.05$ ).

### Correlation and path analysis of SOD, MDA, and IL-10 with HbA1c levels

Results showed that all variables were significantly correlated with each other. IL-10 was positively correlated with SOD levels ( $p < 0.05$ ,  $r = 0.853$ ) and negatively correlated with MDA levels ( $p < 0.05$ ,  $r = -0.866$ ). IL-10 was also negatively correlated with HbA1c levels ( $p < 0.05$ ,  $r = -0.813$ ). SOD levels were negatively correlated with HbA1c levels ( $p < 0.05$ ,  $r = -0.762$ ). MDA levels were positively correlated with HbA1c levels ( $p < 0.05$ ,  $r = 0.973$ ) (Table 2).

Table 2. Levels of SOD, MDA, IL-10, and HbA1c between T1DM and control groups

Variable	Control ( $n = 25$ )	T1DM ( $n = 25$ )
	Mean $\pm$ SD	Mean $\pm$ SD
SOD (µmol/L)	384.08 $\pm$ 49.60	158.06 $\pm$ 56.87*
MDA (µmol/L)	0.40 $\pm$ 0.12	1.69 $\pm$ 0.87*
IL-10 (pg/mL)	53.85 $\pm$ 10.56	17.24 $\pm$ 4.14*
HbA1c (µmol/L)	4.96 $\pm$ 0.22	10.64 $\pm$ 3.17*

Note: \*  $p < 0.05$  (independent samples t-test).



Path analysis using multiple regression showed that IL-10, SOD, and MDA levels simultaneously affected HbA1c levels in T1DM patients by as much as 99.4%. Briefly, IL-10 directly increased SOD levels ( $R^2 = 0.853$ ) and directly decreased MDA levels ( $R^2 = -0.866$ ). IL-10 also indirectly decreased HbA1c levels via MDA ( $R^2 = -0.980$ ).

In this study, a total of 25 subjects were included in each of the T1DM and control groups. There were no significant differences in subject characteristics between the two groups, indicating that the T1DM and control groups were appropriately matched. IL-10 levels were significantly lower in the T1DM group as compared with the control group. This result was discordant with a previous study, which reported that IL-10 was produced at higher levels in high-risk diabetes mellitus (DM) patients, suggesting an autocrine role of this cytokine in islet cell destruction [5]. IL-10 regulates inflammatory processes through suppression of proinflammatory cytokines, chemokines, adhesion molecules, APC (antigen presenting cells), and costimulatory molecules in monocytes/macrophages, neutrophils, and T cells [12]. IL-10 has been reported to act in the phosphatidylinositol 3-kinase (PI3K) and Akt/PKB (Protein Kinase B) pathways, whereas PI3K acts in gluconeogenesis suppression and glycogen synthesis stimulation [13]. The exogenous IL-10 administration could inhibit the progression of T1DM in NOD rats [14]. Another study showed that IL-10, which is produced by pancreatic Th2 cells was not effective in protecting NOD rats from the onset of T1DM [15].

In this study, SOD levels were significantly higher in the control group as compared with the T1DM group. This result was in accordance with a previous study suggesting that SOD activity in DM was inhibited [16]. Furthermore, another study showed that serum superoxide levels were higher in 47 T1DM patients as compared with the control group, suggesting that oxidative stress was occurring in T1DM patients [17]. Another previous study reported that SOD, CAT, and Cp activity was decreased in type 1 and 2 DM as compared with the control. A lower level of SOD predicts vascular dysfunction in DM patients [18]. A previous study reported that SOD levels and activity were higher in pediatric T1DM patients and associated with flow-mediated dilatation. High SOD levels in the circulation could protect children and adolescents with T1DM from endothelial dysfunction [19]. The highest activity of SOD was found in pediatric DM patients at the clinical onset of disease [20].

Superoxide anion reacts with nitric oxide to form reactive peroxynitrite [21]. Abundant production of superoxide anion-induced by hyperglycemia could stimulate protein kinase C, the hexosamine and polyol pathways, and the formation of advanced glycation end products (AGE). AGE was important in pathogenesis of DM complications [22, 23].

In this study, MDA levels were significantly higher in the T1DM group as compared with the control group. MDA as a product of lipid peroxidation was increased as a result of oxidative stress, and its level was elevated in DM patients [16, 20, 24]. Furthermore, oxidative stress contributed to the formation of oxidized LDL and worsened vascular complications in DM patients [25]. Lipid peroxidation affects membrane function and elasticity, which in turn converts enzymatic and receptor activity and decreases membrane fluidity [26, 27].

In this study, glycemic control of T1DM was reflected in HbA1c levels. As many as 20 of 25 subjects (T1DM group) had poor glycemic control ( $HbA1c > 7.5\%$ ). HbA1c was reflected in blood glucose concentration within 6 – 12 weeks [28, 29]. This result was in accordance with a previous study [16, 17, 30]. Higher HbA1c levels could be caused by a limited supply of insulin and poor blood glucose monitoring [31].

A correlation study of IL-10, SOD, and MDA was carried out. In this study, IL-10 directly increased the SOD level. IL-10 played an important role in endothelial protection after acute inflammation induced by higher superoxide levels. Restoration of vasorelaxation after Polyethylene glycol-superoxide dismutase (PEG-SOD) or allopurinol administration confirmed that endothelial protection was likely mediated by decreased superoxide via xanthine oxidase [32]. A similar study showed that endothelial relaxation in inflammation, atherosclerosis and the diabetic condition was recovered by SOD administration [33]. Furthermore, IL-10 could inhibit proinflammatory cytokine production, which in turn inhibited ROS production [34, 35].

IL-10 directly decreased MDA levels in T1DM patients. A previous study reported that micronutrient administration could increase peripheral/lymphatic IL-4 and IL-10, decrease blood glucose levels, increase pancreatic  $\beta$ -cell function, increase pancreatic insulin levels, increase GSH-Px activity, and decrease pancreatic MDA levels. This result indicated that micronutrients could decrease islet cell lesions through elevated production of IL-4 and IL-10 and decrease oxidative stress in diabetic mice, reflected in higher antioxidant levels and lower MDA levels [36].



Results also showed that IL-10 decreased HbA1c levels in T1DM patients. A previous study reported that IFN- $\gamma$  and IL-10 production in diabetic peripheral blood mononuclear cells were associated with metabolic control. This result indicated that good metabolic control in diabetic patients would improve activation and maintenance of the immune response and decrease vulnerability to infection [37].

Superoxide dismutase levels were negatively correlated with HbA1c levels in T1DM patients in this study. This result was in accordance with a previous study, which reported that SOD, both in serum and saliva, was higher in diabetic patients and negatively correlated with glycemic control [38]. A similar study showed that total antioxidant capacity in diabetic patients was significantly lower as compared with the control. Furthermore, in diabetic patients, there was a significant correlation between total antioxidant capacity and HbA1c levels, fasting blood glucose levels, and duration of DM. However, there was no correlation between SOD and GPx with those parameters as previously stated. The conclusion of this study was that total antioxidant capacity could be used as an indicator of glycemic control and progression of complications [39]. Another study considered that the main factor in decreased salivary SOD activity was elevated glycation of this enzyme or a diminished effect of free radicals by a glycated protein on SOD activity [40].

Malondialdehyde and HbA1c levels were significantly correlated in this study. A previous study showed similar results [16]. Another study reported significant elevation of total cholesterol, LDL, apolipoprotein A, apolipoprotein B, and MDA levels in T1DM patients. Furthermore, serum MDA levels and the MDA/LDL index were elevated and significantly correlated with metabolic control in T1DM [41]. A previous study showed that elevated salivary and serum antioxidant levels depended on HbA1c levels and the severity of diabetes. Moreover, MDA levels were also correlated with fasting plasma glucose [38]. In accordance with this study, a previous study also showed decreased activity of several antioxidant enzymes such as SOD, GPx, and CAT in T1DM patients, but MDA was increased significantly in the T1DM group relative to the control. Furthermore, MDA was positively correlated with HbA1c, but SOD was negatively correlated with HbA1c [42]. Another study found no correlation between MDA and glycemic control and found that elevation of MDA levels in nondiabetic patients was associated with age, periodontal status, and smoking [43, 44].

## CONCLUSION

This study can be concluded that IL-10 and SOD levels were significantly decreased, but MDA and HbA1c levels were significantly elevated in the T1DM group. IL-10 levels were positively correlated with SOD levels and negatively correlated with MDA and HbA1c. SOD levels were negatively correlated with HbA1c levels. MDA was positively correlated with HbA1c levels.

## ACKNOWLEDGMENT

The authors thank to Saiful Anwar Hospital to facilitated this research.

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