Neutrophil Phagocytosis Function and Radical Oxygen Formation and Influencing Factors in Type 2 Diabetes Mellitus Patients

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ABSTRACT

The aim of this study was to investigate the increase in neutrophil count and the decrease in both phagocytosis and neutrophil oxidative burst (formation of radical oxygen) among type 2 diabetes mellitus (DM) patients and the normal range of phagocytosis function and oxidative burst in neutrophil from Non-DM control subjects. The aim of this study is also to investigate the factors that influence neutrophil count, phagocytosis function and neutrophil oxidative burst among type 2 diabetes mellitus (DM) patients. The examination was conducted using a flow cytometry. The study subjects were 142 type 2 DM patients and 65 Non-DM control subjects. Statistical analysis was performed using the Mann Whitney test and linear regression analysis. The results of this study indicated that there is impaired neutrophil function among type 2 DM patients. The results of this study also showed a correlation between Hemoglobin level, age, platelet count, and SGPT vs. neutrophil phagocytosis function, as well as a correlation between HDL cholesterol and (fMLP-stimulated) neutrophil oxidative burst. The study also showed a correlation between sex and stroke and (S. aureus-stimulated) neutrophil radical oxygen formation, a correlation between neutrophil count and platelet count, a correlation of HbA1c and fasting blood glucose level and (fMLP-stimulated) neutrophil radical oxygen formation. In a multivariate analysis, when adjusted to age and sex, there was a correlation between triglyceride and (baseline) neutrophil radical oxygen formation and between HDL cholesterol and (fMLP-stimulated) neutrophil radical oxygen formation.

Key words: *type 2 DM, neutrophil, phagocytosis, oxidative burst*

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INTRODUCTION

Diabetes Mellitus is a chronic disease that patients suffer throughout their life. It is well known that diabetics have a defective immune response, in the form of disturbed neutrophil migration, adhesion, phagocytosis, degranulation, as well as radical oxygen formation. These cause diabetic patients to be more susceptible to infections.[1,2] The goal of this research is to determine the increase in neutrophil count, the reduction of phagocytosis function, and the reduction of neutrophil radical oxygen formation in type 2 diabetes mellitus, and to determine the normal value of phagocytosis function and neutrophil radical oxygen formation in non-diabetic control subjects. The research also aims to determine the factors influencing the number of neutrophils, phagocytosis function, and neutrophil radical oxygen formation in type 2 diabetes mellitus.

MATERIALS AND METHOD

The neutrophil count, phagocytosis function and radical oxygen formation of a non-diabetic control group and a group of type 2 diabetes mellitus patients were compared. This was a cross-sectional research. Samples were taken between February 2002 and September 2002. Informed consent was obtained from all patients after they received information from the researcher. Approval to conduct the research was obtained from the Ethics Comission of the Faculty of Medicine of the University of Indonesia.

There were 65 non-diabetics control adult subjects. The control group consisted of volunteers who did not have diabetes mellitus, randomly chosen from within or outside of the Faculty of Medicine of the University of Indonesia and Cipto Mangunkusumo General Hospital area. Their laboratory tests showed no anemia (males:

hemoglobin > 12 g/dL, females: hemoglobin > 11 g/dL), normal white blood cell count (4500-11000 /μL)[3], normal platelet count (140000-450000 /μL) and no diabetes mellitus (fasting blood glucose level < 126 mg/dL or random blood glucose level < 200 mg/dL).[1]

The number of type 2 diabetes mellitus subjects was 142 adult patients, who suffer from diabetes mellitus with or without complication, chosen randomly from the outpatients clinic of the Metabolics and Endocrinology Subdivision of the Department of Internal Medicine of the Faculty of Medicine of the University of Indonesia / Cipto Mangunkusumo General Hospital. Type 2 DM patients without complication were patients with normal laboratory results without any complications. Type 2 DM patients with complication had one/more abnormal laboratory results with complication. The laboratory tests consisted of screening tests (hemoglobin value, white blood cell count, platelet count, total protein, albumin and globuline value, SGPT), and creatinine, total cholesterol, triglycerides, HDL cholesterol and LDL cholesterol levels. Type 2 DM complications included infection, ulcer, microangiopathies (nephropathy, neuropathy, retinopathy) and macroangiopathies (coronary heart disease, stroke), hypertension, as well as being overweight or underweight. Patients who were pregnant and those taking drugs that alter the immune system such as steroids were excluded from the study.

MATERIALS

From the nondiabetics control subjects, 2mL of coagulated blood was taken after fasting and at random, 2 mL of K_3EDTA blood was taken for hemoglobin level, white blood cell, platelet and neutrophil counts, and 3 mL of lithium heparinized blood was taken for neutrophil phagocytosis function and radical oxygen formation assessments.

The samples taken from each of the subject in the type 2 DM group were 5mL coagulated blood for fasting blood glucose level, total protein, albumin, globulin, SGPT, creatinine, total cholesterol, tryglycerides, HDL and LDL cholesterols tests; 2 mL K_3EDTA blood for hemoglobin level, white blood cell, platelet and neutrophil counts, 3 mL lithium heparinized blood for neutrophil phagocytosis function and radical oxygen formation assessments; and 2 mL coagulated blood taken 2 hours after meal for 2 hours postprandial blood glucose level test. The patients' HBA1c levels were examined 6 weeks afterwards using 2 mL K_3EDTA blood.

NEUTROPHIL PHAGOCYTOSIS FUNCTION ASSESSMENT

Modified Production of FITC Labeled *S. aureus* ATCC 25923[4,5]

S. aureus ATCC 25923 (Difco) was cultured in Tryptic Soy Broth (TSB) (Difco) at 37°C for 18-24 hours, inactivated by heating at 60-70 °C for 60 minutes, rinsed with Phosphate Buffer Saline (PBS) (Sigma), then centrifuged at 1000g for 5 minutes. After 30–50 ug/mL fluorescein isothiocyanate (FITC) (Sigma) was added, the material was incubated at 37 °C for 16 – 24 hours, rinsed twice with PBS for 20 minutes to wipe out unbound fluorescein, resuspended in PBS to concentration of 10^9 bacteria/mL, put into aliquot and sealed with aluminum foil, then put into storage at -20 °C.

Procedure for Neutrophil Phagocytosis Function Assessment[6]

After 100 mL of lithium heparinized blood was put into a tube, 20 mL of FITC labeled *S. aureus* ATCC 25923 was added. The tube was incubated in a water bath at 37 °C for 10 minutes, then placed in ice bath (0 °C) to stop phagocytosis. A volume of 100 mL Trypan blue (Sigma) (concentration of 2.5 mg/mL in PBS) was added. The material was rinsed two times by adding 3 mL PBS, centrifuged at 1000 g for 5 minutes, the supernatant was disposed. The coagulum was rinsed with PBS and resuspended in 1 mL PBS, then measured immediately with flow cytometer.

Flow cytometer: The process was performed using FACS Calibur™, Cell Quest™ software. Gating was performed using a combination of Forward Scatter (FSC) and Side Scatter (SSC) (linier); 10,000 cells were read/observed. The percentage of neutrophils phagocyting *S. aureus* ATCC 25923 was acquired (% gated M2).

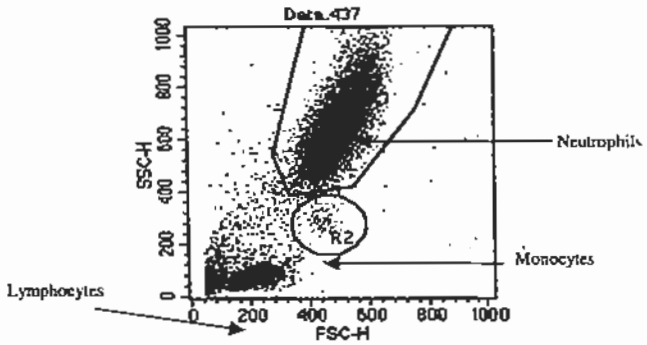

Figure 1. FSC vs SSC Plot. R1 is The Neutrophil Group. The Neutrophils are Further Analyzed as Seen in Figure 2.

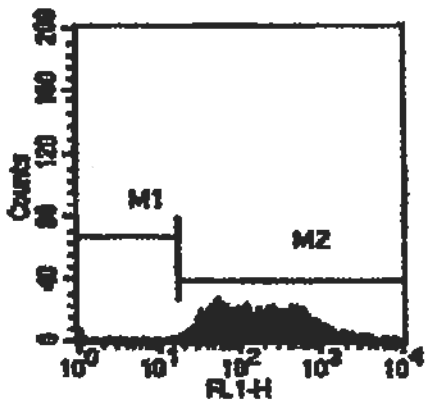

Figure 2. FITC Fluorescence Distribution Following *S. Aureus ATCC* 25923-Stimulation (According to Fluorescence Within The Neutrophils) in Non-DM Control Subjects.

Neutrophil Radical Oxygen Formation Assessment [7]

Three tubes were used for each assessment: Tube I: not stimulated (spontaneous), tube II: stimulated with fMLP, tube III: stimulation with *S. aureus ATCC* 25923. One hundred microliters of lithium heparinized blood was put into each tube. Twenty microliters of PBS was added into the first tube, 20 mL *formyl* –Methionyl –Leucyl - Phenylalanine (5 uM fMLP) (Sigma) into the second tube and 20 mL *S. aureus ATCC* 25923 (10^9 bacteria/ mL) into the third tube. Dihydrorhodamine $_{123}$ substrate (1 mM DHR 123) (Sigma) was added into all tubes .All were incubated in water bath at 37 °C for 10 minutes. Two milliliters of FACS Lysing Solution were added, then incubated for 20 minutes. The tubes were centrifuged at 1000g for 5 minutes, the supernatant was removed. After rinsed with PBS and resuspended in 1 mL PBS, the coagulum was measured immediately with flow cytometer.

After obtaining the group (Figure 1), the neutrophils were analysed as shown in Figure 3, 4, and 5:

Report:

Spontaneous radical oxygen formation (baseline) = result of tube I
Radical oxygen formation (fMLP) = result of tube II – result of tube I
Radical oxygen formation (*S. aureus*) = result of tube III – result of tube I

RESULTS

The assessment of phagocytosis function and (baseline, fMLP-stimulated, and *S. aureus*-stimulated) neutrophil radical oxygen formation were performed 5 consecutive times, resulting variation coefficients (VC) of 1.3 %, 4.05 %, 16.98 % and 1.5 % respectively.

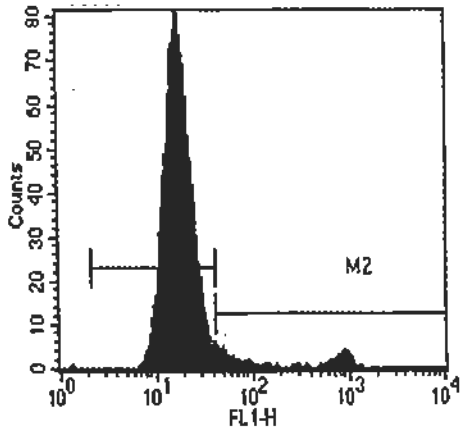

Figure 3. R_{123} Fluorescence Distribution (According to The Amount of Radical Oxygen Within Neutrophils) in Non-DM Control Subjects. Without Stimulation, a Large Portion of The Sample is Located on The Left-Hand Peak.

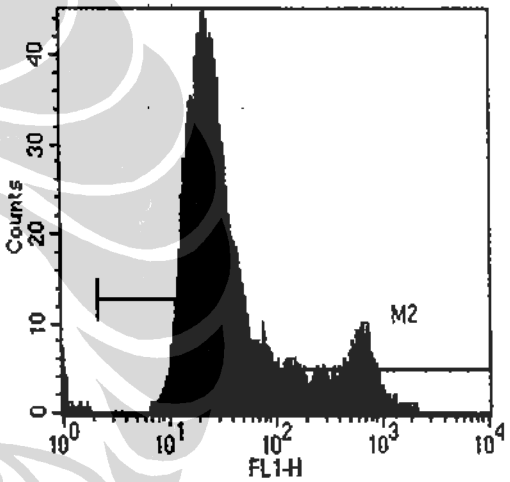

Figure 4. R_{123} Fluorescence Distribution (According to The Amount of Radical Oxygen Within The Neutrophils) in Non-DM Control Subjects. Following Fmlp-Stimulation, a Portion is Located on The Left-Hand Peak and a Portion is Located on The Right-Hand Peak.

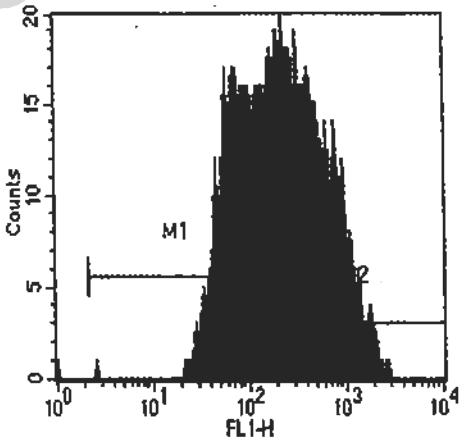

Figure 5. R_{123} Fluorescence Distribution (According to The Amount of Radical Oxygen Within The Neutrophils) in Non-DM Control Subjects. Following *S. Aureus ATCC* 25923-Stimulation, a Large Portion is Located on The Right-Hand Side and a Small Portion is Located on in Left Hand Peak.

The results showed that no significant difference in the independent variables initially assessed in the non-DM and type 2 DM group ($p>0.05$). This indicates that in the early phase of the study, both groups were in balanced condition, thus comparable. The fasting glucose level between the nondiabetics control and the type 2 DM group differed significantly ($p<0.05$) (Table 1).

Table 1. Non-DM Control Group and Type 2 DM Group Characteristics

	Non DM (N = 65)	Type 2 DM (N = 142)	p
Age (years)	52 (30 – 60)	52 (30 – 60)	NS
Duration of DM (years)	-	7 ± 5.4	-
Sex (male/female)	24 / 41	58 / 84	NS
Hemoglobin level (g/dL)	13.4 ± 1.2	13.4 ± 1.6	NS
Leukocyte count (/µL)	7838 ± 1503	8521 ± 2743	NS
Fasting blood glucose (mg/dL)	84 (60 – 106)	141 (54-335)	<0.001
Random blood glucose (mg/dL)	124 (100 – 181)	-	-
Blood glucose 2 hours post meal (mg/dL)	-	247 ± 91	-

Values are presented as mean ± SD, * NS = not significant

In the early phase of the study, statistical analysis showed that neutrophil count, phagocytosis function, fMLP-stimulated and *S. aureus*-stimulated radical oxygen formation differed significantly in both groups ($p<0.01$) (Table 2). This indicates that in the early phase, the nondiabetics group and the type II DM group were not in similar state, thus making it possible for the non DM group to be used as a control group.

Table 2. The Difference Between The Neutrophil Count and Neutrophil Function Between Non-DM Control Subjects and Type 2 DM

	Non DM (N=65)	Type 2 DM (N=142)	p $
	Median (min-max)	Median (min-max)	
Neutrophil count (/µL)	4500 (2800-7500)	4850* (1900-17000)	0.008
Phagocytosis (%)	96.13 (83.40-99.39)	92.33** (62.75-98.70)	0.000
Radical oxygen formation (%)			
Baseline	2.21 (0.62-14.82)	2.31 (0.15-12.51)	0.978
with fMLP-stimulation	1.38* (0.4-12.31)	0.81* (0-11.97)	0.007
with S. aureus- stimulation	93.48** (70.77-97.98)	85.07** (58.94-96.85)	0.000

$ Mann-Whitney Test, *p<0.01 ** p<0.001

Figure 6-9 shows phagocytosis function and neutrophil radical oxygen formation (spontaneous, fMLP stimulated, and *S. aureus* stimulated) and neutrophil count in type 2 DM and non-DM control groups.

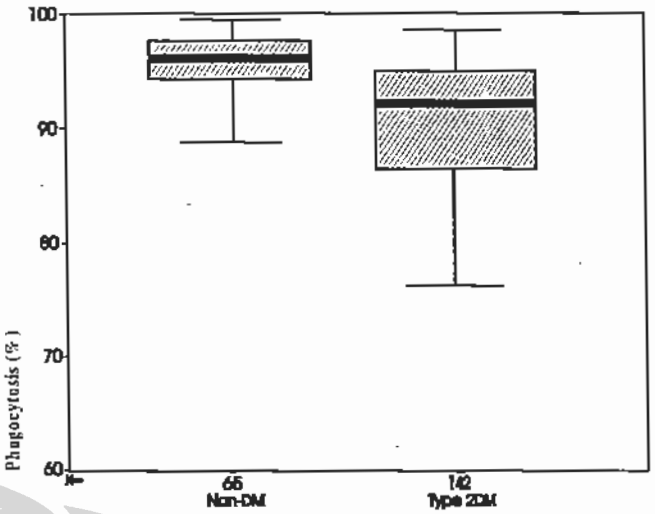

Figure 6. Neutrophil Phagocytosis Function in Non-DM Control Subjects and Type 2 DM Patients (P = 0.000).

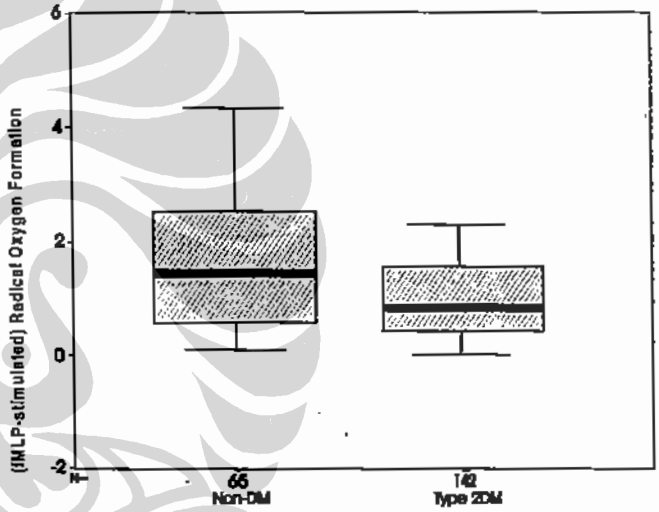

Figure 7. fMLP-stimulated Radical Oxygen Formation in Patients with Type 2 DM is Lowered Compared to that of Non-DM Control Subjects (P = 0.007).

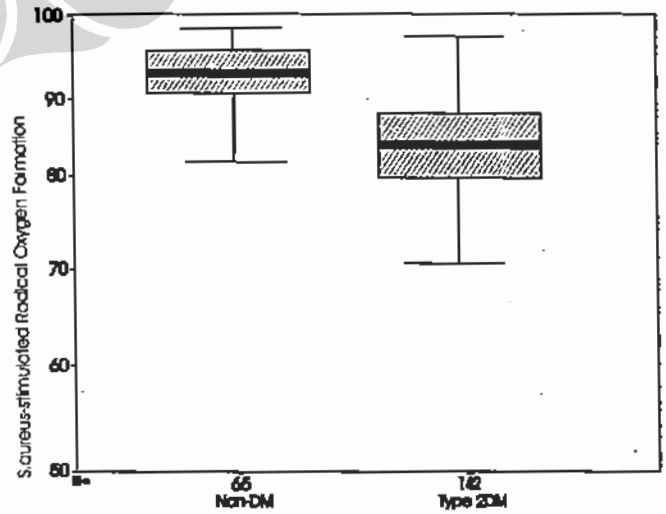

Figure 8. *S. Aureus*-stimulated Radical Oxygen Formation in Patients with Type 2 DM is Lowered Compared to that of Non-DM Control Subjects (P = 0.000).

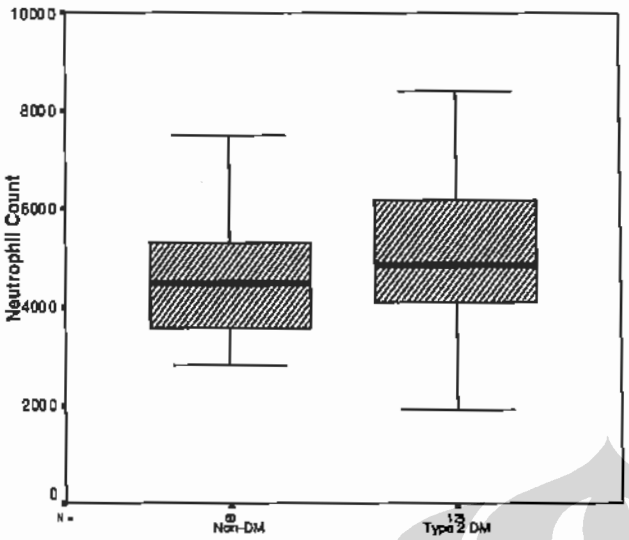

Figure 9. The Neutrophil Count in Non-DM Control Subjects and Type 2 DM Patients (P = 0.008).

In this study, the value range of phagocytosis function and *S. aureus* stimulated neutrophil radical oxygen formation in 65 nondiabetics group were 91.23 % – 98.81 % and 91.47 % - 99.61%, respectively.

FACTORS INFLUENCING NEUTROPHIL COUNT AND NEUTROPHIL FUNCTION IN TYPE 2 DIABETES MELLITUS PATIENTS

The following are the of the type 2 DM patients (Table 3).

Table 3. Characteristics of Type 2 DM Patients

Independent Factor	Type 2 DM (N = 142)
Age (years)	52 (30-60)*
Sex (male/female)	58 / 84
Age DM (years)	44 (24-60)*
Duration of DM (years)	7 ± 5.4
Fasting blood glucose (mg/dL)	141 (54-335)*
Blood glucose 2 hours post meal (mg/dL)	247 ± 91
HbA1c (%)	8.3 ± 1.3
Total cholesterol (mg/dL)	231 ± 64
Triglyceride (mg/dL)	157 (42-1560) *
HDL cholesterol (mg/dL)	46 ± 11
LDL cholesterol (mg/dL)	150 ± 58
BMI (kg/m2)	24 ± 3.3
Hb (g/dL)	13.5 ± 1.6
Leukocyte count (/µL)	8521 ± 2743
Total protein (g/dL)	7.44 ± 0.76
Albumin (g/dL)	3.95 ± 0.43
Globulin (g/dL)	3.49 ± 0.68
SGPT (U/L)	28 (9-117) *
Creatinine (mg/dL)	1.0 (0.3-2) *

Values are presented in the form of mean ± SD, except for Non Gaussian * data, which are presented in the form of median (minimum value-maximum value).

The following table demonstrates a partial correlation between neutrophil count and neutrophil function and various factors in Type 2 DM patients (Table 4).

Table 4. Partial Correlation Between The Neutrophil Count and Neutrophil Function and Various Independent Factors

Independent Factor	Neutrophil Phagocytosis (%)	Neutrophil Radical Oxygen Formation (%)			Neutrophil Count (/µL)
		Baseline	fMLP	S. Aureus	
Age (years)	-0.219*	NS	NS	NS	NS
Sex	NS	NS	NS	-0.196*	NS
Age (years)	NS	NS	NS	NS	NS
Duration of DM (years)	NS	NS	NS	NS	NS
Fasting blood glucose (mg/dL)	NS	NS	NS	NS	NS
HbA1c (%)	NS	NS	NS	NS	NS
Nephropathy	NS	NS	NS	NS	NS
Neuropathy	NS	NS	NS	NS	NS
Retinopathy	NS	NS	NS	NS	NS
Coronary heart disease	NS	NS	NS	NS	NS
Stroke	NS	NS	NS	-0.198*	NS
Hypertension	NS	NS	NS	NS	NS
BMI (kg/m2)	NS	NS	NS	NS	NS
Ulcer	NS	NS	NS	NS	NS
Hb (g/dL)	0.202*	NS	NS	NS	NS
Leukocyte count (/µL)	NS	NS	NS	NS	NA
Platelet count (/µL)	-0.198*	NS	NS	NS	0.271*
HDL cholesterol (mg/dL)	NS	NS	-0.168*	NS	NS
Triglyceride (mg/dL)	NS	NS&	NS	NS	NS
Albumin (g/dL)	NS	NS	NS	NS	NS
SGPT (U/L)	-0.184*	NS	NS	NS	NS
Creatinine level (mg/dL)	NS	NS	NS	NS	NS

** p<0.01 (2-tailed),*p<0.05 I (2-tailed),NS: not significant, excluded

The regression line formula is as follows:
Phagocytosis (%) = 94.55 + 0.94 Hb – 0.18 Age – 0.02 Platelet – 0.09 SGPT;
Radical oxygen formation (fMLP) = 2.45 – 0.03 HDL cholesterol;
Radical oxygen formation (*S. aureus*) (%) = 85.45 – 6.02 (with persisting stroke) – 2.64 (if male);
Neutrophil /µL = 3142.93 + 7.91 platelet

Figure 10 shows a correlation between hemoglobin and neutrophil phagocytosis function in type 2 DM and Figure 11 shows a correlation between age and neutrophil phagocytosis function in type 2 DM.

The results of multivariate analysis of factors influencing fMLP-stimulated, *S. aureus*-stimulated neutrophil radical oxygen formation; and neutrophil count in type 2 DM are as follows (Table 4):

There was a correlation between HDL cholesterol and fMLP stimulated neutrophil radical oxygen formation in type 2 DM (Figure 12). A correlation was also found between stroke (Figure 13) and *S. aureus*-stimulated neutrophil radical oxygen formation in type 2 DM , and also between sex (Figure 14) and *S. aureus* stimulated neutrophil radical oxygen formation in type 2 DM. Figure 15 shows the correlation between platelet count and neutrophil count in type 2 DM.

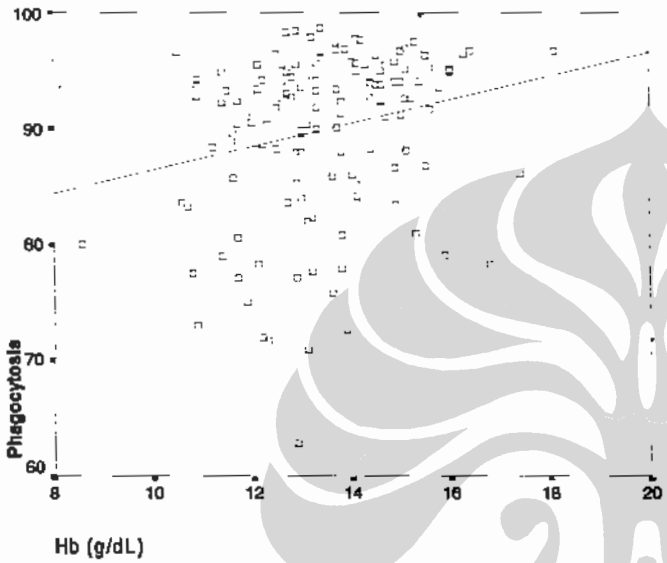

Figure 10. The Correlation Between Neutrophil Phagocytosis Function and Hemoglobin Level in Type 2 DM.

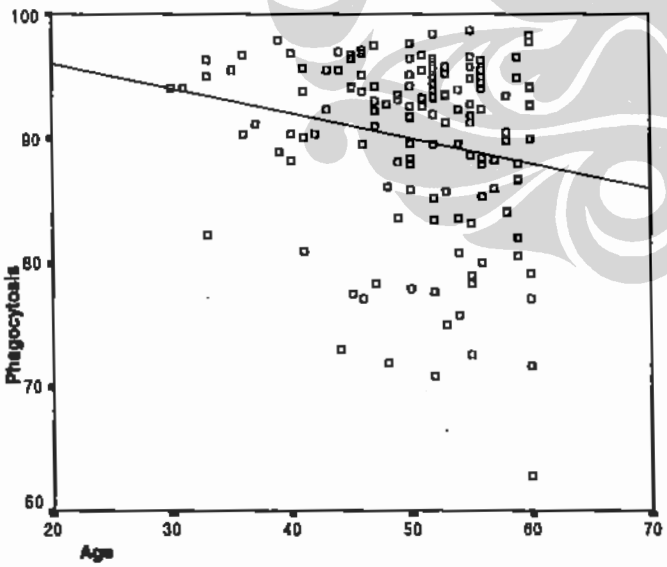

Figure 11. The Correlation Between Neutrophil Phagocytosis Function and Age in Type 2 DM.

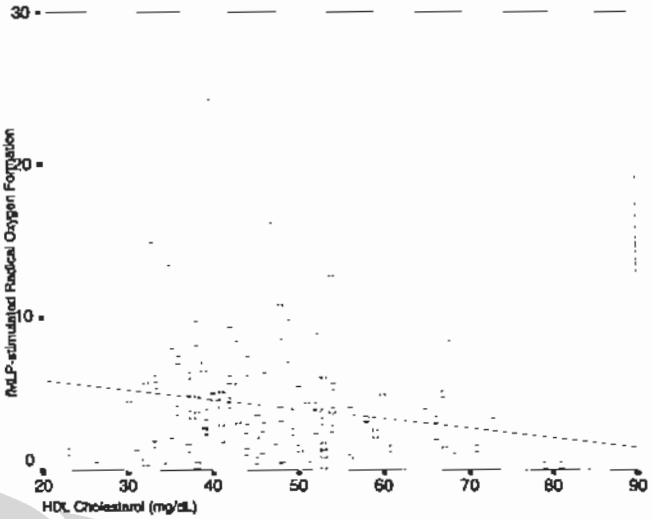

Figure 12. The Correlation Between fMLP-stimulated Radical Oxygen Formation and HDL Cholesterol in Type 2 DM

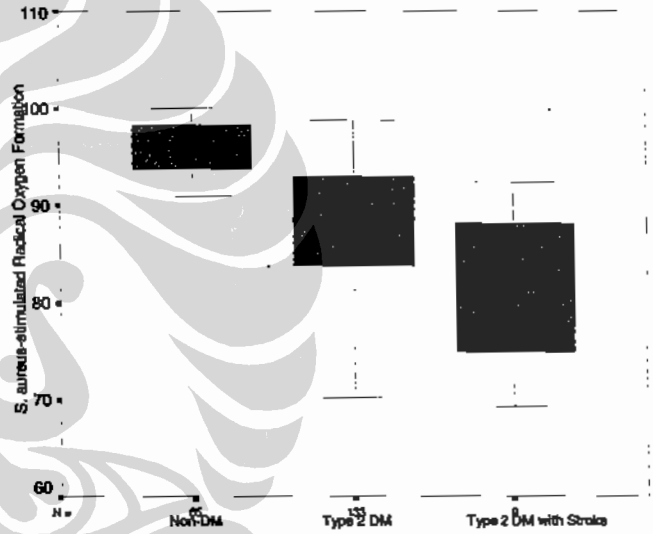

Figure 13. S. aureus-stimulated Radical Oxygen Formation in Non-DM Control Subjects, Type 2 DM, and Type 2 DM with Stroke

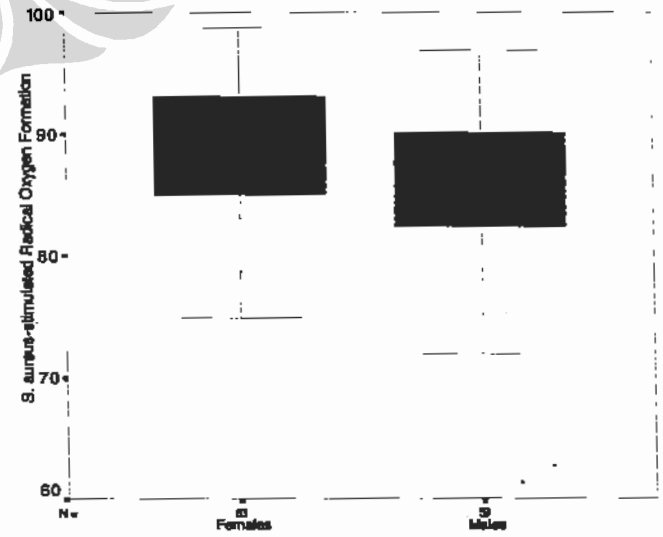

Figure 14. *S. aureus*-stimulated Radical Oxygen Formation in Females and Males with Type 2 DM.

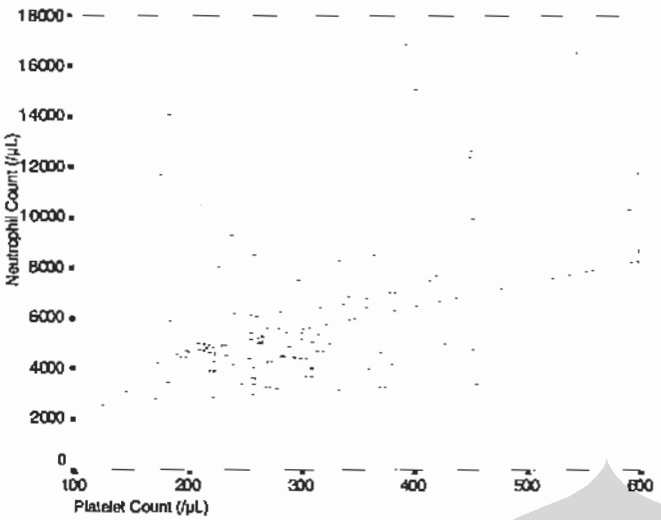

Figure 15. The Correlation Between Neutrophil Count and Platelet Count in Type 2 DM.

Table 5 demonstrates a correlation between a controlled state and neutrophil function. There are correlations between HbA1c and fasting blood glucose and fMLP-stimulated neutrophil radical oxygen formation.

Table 5. The Correlation Between a Controlled State (of Hemoglobin Level A1c and Fasting Blood Glucose) and Neutrophil Function

	HbA1c (%)	Fasting blood glucose (mg/dL)
Phagocytosis (%)	0.167	0.022
Radical oxygen formation (%)		
Baseline	0.232	0.095
with fMLP-stimulation	0.313**	0.285*
with S. aureus-stimulation	0.005	-0.007
Neutrophil count (µL)	-0.017	0.166

Values in this table are presented in the form of rho Spearman correlation coefficient, * p<0.05, ** p<0.01
Data of the placebo group, final data (N = 71).

Table 6 demonstrates partial correlation and control for age and sex. The results of this study demonstrate that baseline radical oxygen formation increases along with triglyceride level, thus increasing oxidative burden, while fMLP-stimulated radical oxygen formation has a negative correlation with HDL cholesterol.

Table 6. Partial Correlation and Control for Age and Sex

Phagocytosis	Radical Oxygen Formation			
	baseline	fMLP	S. aureus	
Total cholesterol	0.0325	0.0228	-0.0198	0.0394
	p= 0.703	p= 0.790	p= 0.816	p= 0.644
Triglyceride	0.0118	0.1758	0.0612	-0.0320
	p= 0.889	p= 0.038	p= 0.472	p= 0.707
HDL cholesterol	-0.0606	-0.1349	-0.1789	-0.0960
	p= 0.477	p= 0.112	p= 0.034	p= 0.259

DISCUSSION

In the early phase of the study, we performed a within run test of neutrophil phagocytosis and radical oxygen formation function. A subject was chosen from the non-diabetics control group and examined five consecutive times using flow cytometry. Orpegen Pharma, Lun, and this study obtained different variation coefficient results.[6,7,9] This may have occured due to the difference in the number of samples used. Orpegen Pharma performed the tests three times for each sample, Lun twice, while in this study, we tested each sample five times. This may also be the cause of the difference. The VC from this research was smaller than the one from the the study by Lun.

We found a significant decrease in neutrophil phagocytosis function in type 2 DM. The number of neutrophils phagocytosing *S. aureus* in type 2 DM was significantly lower than in the control group. A similar result was also reported by Marhoffer et al, who conducted the study using radiometrically measured ^{3}H-thymidin- labeled *S. aureus*. The study showed that neutrophil phagocytosis function in diabetics was significantly lower than nondiabetics (50.7 ± 4.1 % vs. 76.6 ± 4.6 %, p<0.001).[10] The reduction of neutrophil phagocytosis activity is caused by intrinsic neutrophil defect. An increase in cyalidase enzyme secretion will result in defect of cell membrane, thus altering neutrophil phagocytosis activity. Other researchers stated that high levels of blood glucose would destroy lectin receptors, causing neutrophils to lose their capacity to recognize target cells, therefore resulting in phagocytosis dysfunction.[11]

Neutrophil radical oxygen formation was measured under spontaneous condition, fMLP stimulation (a tripeptide acting as chemotactic factor) and *S. aureus* stimulation.[12] There was no stimulation condition depicted neutrophil activation occurring in vivo, causing spontaneous radical oxygen formation. Those under fMLP stimulation, i.e. weak stimulation, depicted neutrophil activation in vivo (priming), which makes it easier activated to produce radical oxygen. Stimulation with S. aureus, i.e. strong stimulation, depicted neutrophil capacity to produce radical oxygen.

In this study, we found that in the basal state, there was no significant difference in baseline neutrophil radical oxygen formation between those with type 2 DM and the non-DM control group (2.31 % and 2.21 %, p=0.978). Similar studies reported different results. Ihm et al measured spontaneous neutrophil radical oxygen formation using flow cytometry on 79 DM patients and 48 non-

diabetics control subjects. The result showed a significant increase of spontaneous neutrophil radical oxygen formation in diabetics compared with the non-diabetics control subjects (111 ± 29 mmol/L and 94 ± 21 mmol/L, p<0.05).[13] A study by Wykretowicz et al on 20 type 2 DM patients and 15 non- DM control subjects showed that spontaneous neutrophil superoxide production in type 2 DM group was significantly higher than in the non-DM group (3.48 ± 0.09 nmol $O2^-$/ 2.5 x 10^6 PMN/10 minute and 3.01 ± 0.12 nmol $O2^-$/ 2.5 x 10^6 PMN/10 minute, p<0.05).[14] Wierusz et al conducted a study on 20 diabetic patients. The results showed that spontaneous neutrophil superoxide production in type 2 DM group was significantly higher than in the non DM group (11.7 ± 1.25 nmol $O2^-$/ 10^6 PMN/10 minute and 4.15 ± 1.24 nmol $O2^-$/ 10^6 PMN/10 minute, p<0.01).[15] All of the results mentioned generally showed that spontaneous neutrophil radical oxygen formation in type 2 DM group was higher than in the non DM control. The reason for this condition may be hyperglycemia that diabetics experience in their basal state, which may increase reactive oxygen species (ROS) production. The mechanism of the increase of ROS production also relates to the increase of basal state neutrophil radical oxygen formation. [16] This study demonstrated no significant difference of baseline radical oxygen formation between non DM control and type 2 DM, while other researches generally showed a significant difference.The reason for this might be because we used a different technique and apparatus than other researchera. This might also have happened due to the great variation of daily baseline radical oxygen formation between the control and the type 2 DM group.

Our results showed that fMLP stimulated neutrophil radical oxygen formation in type 2 DM was significantly lower than the non DM control (0.81 % and 1.38 %, p= 0.007). This is similar with results from other researchers. Bernheim et al conducted a study on in vitro AGE effect by measuring fMLP stimulated neutrophil superoxide production. Different AGE-BSA levels were added to neutrophils. The higher AGE-BSA level given, the lower fMLP stimulated neutrophil superoxide production. At an AGE-BSA level of 2.5 mg/mL, the production was 0.54 ± 0.05 nmol $O2^-10^{-6}$ cell/minute (p<0.028), at an AGE-BSA level of 5 mg/mL, the production was 0.48 ± 0.09, with a control of 1.08 ± 0.05 nmol $O2^-10^{-6}$ cell/minute (p<0.046). In diabetics, AGE lowers fMLP stimulated neutrophil superoxide production. Superoxide production plays a role in killing bacteria. This is the cause for the decrease of fMLP stimulated neutrophil radical oxygen formation. [17]

Our results showed that *S. aureus*-stimulated neutrophil radical oxygen formation in type 2 DM was significantly lower than in the non-DM control group (85.07 % and 93.48 %, p=0.000). The following are mentioned results of other researches of neutrophil radical oxygen formation using various other stimulations and methods. In our past research, we have measured *S. aureus* stimulated neutrophil radical oxygen formation. The apparatus we used was flow cytometer. The 18 type 2 DM subjects were treated in Cipto Mangunkusumo General Hospital patients, both outpatients and inpatients from inpatient wards A and B. The control group consisted of 10 Faculty of Medicine University of Indonesia students and laboratory workers who did not have any signs and symptoms of the disease at the time of the examination. The study demonstrated that *S. aureus*-stimulated neutrophil radical oxygen formation in type 2 DM patients was significantly lower than in the non DM control (46.7 ± 18.8 % and 85.6 ± 10.4 %, p<0.001).[18] Ihm et al measured neutrophil radical oxygen formation with stimulation of Phorbol Myristate Acetate (PMA). The number of subjects was 79 DM patients and 48 non DM control subjects. A flow cytometer was used to measure neutrophil radical oxygen formation. The results indicated that PMA stimulated neutrophil radical oxygen formation decreased significantly in DM patients compared with the control group (137 ± 45 mmol/L and 192 ± 77 mmol/L, p<0.05).[13] Bernheim et al investigated in vitro AGE effect by measuring superoxide production in PMA stimulated neutrophils. Different levels of AGE-BSA were added to neutrophil. The higher the level of AGE-BSA given, the PMA stimulated neutrophil produced significantly lower superoxide. At the level of 2.5 mg/mL AGE-BSA, the production was 2.89 ± 0.54 nmol $O2^-10^{-6}$ cell/minute (p<0.028), at the level of 5 mg/mL AGE-BSA, the production was 2.26 ± 0.54 nmol $O2^-10^{-6}$ cell/minute (p<0.01), with control 4.46 ± 0.77 nmol $O2^-10^{-6}$ cell/minute (p<0.046).[17] In this study, we used a different technique and apparatus from other researchers, thus yielding different results. However, the results showed a similarity with others, i.e. there was a reduction in radical oxygen formation in neutrophils given strong stimulation in type 2 DM group. The reduction of radical oxygen formation may be caused by the hyperglycemia that occurs in diabetics. [10,11] During, hyperglycemia HMPS and polyol tract activity increases, causing increased usage of NADPH to convert glucose into sorbitol. This reduces cell NADPH levels, causing

a reduction in NADPH dependent production of radical oxygen. [10,20]

In this study, the results showed that the reduction of neutrophil radical oxygen formation *(S. aureus)* in type 2 DM was greater compared to the reduction of neutrophil phagocytosis function (85.07 % and 92.33 %). A similar result was reported by Marhoffer et al. [10] Pickup stated that phagocytosis function reduction in type 2 DM does not easily occur, because the process of phagocytosis is strong, in that the process should not easily be disturbed. During earlier stages, neutrophil radical oxygen disturbance will occur in type 2 DM. This will result in a normal or slightly decreased neutrophil phagocytosis function compared with the decrease in neutrophil radical oxygen formation.[19]

Our study demonstrated a significantly higher neutrophil count in type 2 DM than in the control group (4850 /mL and 4500 /mL, p=0.008). Similar studies have reported similar results. Swirski et al conducted neutrophil count examination in 18 type 2 DM patients and 16 non DM control subjects. The results showed that neutrophil count was significantly higher in type 2 DM compared to control subjects (5000 ± 400 /mL and 3200 ± 300 /mL, p<0.001).[2] Pecsvarady et al examined the neutrophil count in 33 type 2 DM patients and 22 non-DM control subjects. The research indicated a higher neutrophil count in type 2 DM compared to in control subjects, although the result was not statistically significant (3200 ± 200 /mL dan 3100 ± 300 /mL).[21] Diabetes mellitus is a chronic disease that causes susceptibility to infection or inflammation. During inflammation, neutrophils in the marginal circulating pool migrate to the inflammation area, causing a decrease in the absolute number of blood circulating neutrophil. The release of IL-1 and TNFa will trigger proliferation and differentiation of bone marrow cells that will release more neutrophils to the blood circulation,[22] thus causing an increase in the absolute number of neutrophils in the circulation. [12,23]

Not many factors influencing neutrophil count and function have been discussed in references. This study reveals several factors that may influence neutrophil count and function in type 2 DM patients.

Wenish stated that in general, the aging process will make it people more susceptible to infection. Neutrophil phagocytosis function is one of the body's defense mechanisms against bacteria. This function is also affected by a person's age because older people have higher intracellular calcium levels than younger people.[24]

In this study, we found that fMLP-stimulated neutrophil radical oxygen formation decreased as HDL cholesterol level increased. According to Vosbeck et al, lipopolysaccharide (LPS) stimulates neutrophil priming. LPS binds with HDL cholesterol making a complex PS-HDL. The LPS-HDL complex reduces neutrophil fMLP receptors. As neutrophils are stimulated with fMLP, their ability to form radical oxygens is also decreased. This shows that HDL cholesterol impedes fMLP-stimulated neutrophil radical oxygen formation.[25]

CONCLUSION

Our results demonstrate an increase in neutrophil count and a decrease in neutrophil phagocytosis function and neutrophil radical oxygen formation in type 2 DM compared with nondiabetics control subjects (p<0.05). Of all the independent factors, there was a correlation between neutrophil phagocytosis activity and hemoglobin level, age, platelet number, and SGPT in type II DM patients. There were correlations between fMLP-stimulated radical oxygen formation and HDL cholesterol, between *S. aureus*-stimulated radical oxygen formation and sex and stroke, as well as between neutrophil count and platelet count. The results showed a correlation between fMLP-stimulated radical oxygen formation and HbA1C and fasting blood glucose level. With age and sex as control, we found a correlation between triglyceride level and baseline radical oxygen formation. There was also correlation between HDL cholesterol and fMLP stimulated radical oxygen formation.

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