



Research Article

## Plasma cholesterol modulate functions of neutrophils in streptozotocin-induced type 1 diabetic rats

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### **Abstract**

**Objectives:** Our previous study demonstrated neutrophil dysfunction in streptozotocin (STZ)-induced diabetic rats. This study was aimed to investigate the biochemical indices such as plasma levels of cholesterol, triglycerides, creatinine, urea, alanine transaminase and aspartate transaminase in diabetic and control rats and thus, investigate their relationship with neutrophil functions. **Methods:** Diabetes was induced in Long Evans rats by an intraperitoneal injection of citrate buffer dissolved streptozotocin (STZ). Age matched control rats were injected with citrate buffer only. Neutrophils were isolated from blood using standard dextran sedimentation followed by Ficoll-Hypaque centrifugation; morphological changes in neutrophils, their ability to reduce nitroblue tetrazolium (NBT) dye and phagocytic activity from both the groups of rats were evaluated formerly. Biochemical indices were measured by standard colorimetric methods. **Results:** The average levels of glucose, triglycerides, cholesterol, creatinine, urea in the plasma of diabetic and control rats were  $302.6 \pm 87.5$  vs  $100.7 \pm 11.5$  mg/dL,  $174.9 \pm 18.6$  vs  $82.2 \pm 10.2$  mg/dL;  $250.8 \pm 22.3$  vs  $165.2 \pm 24.1$  mg/dL;  $0.94 \pm 0.19$  vs  $0.81 \pm 0.05$  mg/dL;  $77.1 \pm 9.7$  vs  $26.8 \pm 5.8$  mg/dL, respectively. The mean values of aspartate transaminase (AST) and alanine transaminase (ALT) in diabetic and control rats were  $141.4 \pm 28.0$  vs  $61.6 \pm 18.6$  IU/L and  $61.4 \pm 13.6$  vs  $48.5 \pm 6.0$  IU/L, respectively. Biochemical parameters measured in diabetic rats varied significantly ( $p < 0.001$ ) compared to those of control rats. Plasma indices such as triglycerides, cholesterol, creatinine, urea, AST and ALT had no relation with the functions of neutrophils. However, multidimensional scaling found a close relation between plasma cholesterol and phagocytic activity of neutrophils from diabetic rats. Ability to reduce NBT dye was closely related to the morphology of the activated neutrophils. On the other hand, levels of plasma glucose were distantly related to the functions of neutrophils. **Conclusion:** Thus, important liver and kidney functions indices, lipid profile parameters were significantly altered in diabetic rats and plasma cholesterol modulated the phagocytic activity of neutrophils.

**Keywords:** Diabetic rats, phagocytic activity, plasma cholesterol, STZ-diabetes, neutrophils.

## INTRODUCTION

Neutrophils, part of the innate immune response, are the first line of defense in hosts. Numerous studies have demonstrated altered neutrophil functions in diabetes and it is suggested that the impaired neutrophil functions [e.g., chemotaxis, phagocytosis, nitroblue tetrazolium (NBT) dye reduction ability etc.] cause the susceptibility to infections in diabetics (Coopan, 1985; Reeves and Wilson, 1992). The chemotactic activity of neutrophils from diabetic patients is significantly lower compared to those of healthy controls (Mowat and Baum, 1971). Studies of the phagocytic and microbicidal activities of neutrophils from diabetic patients reveal, with few contrasting results, an impairment of these functions. Decreased bactericidal activity (Tan *et al.*, 1975), impairment of phagocytosis and decreased release of lysosomal enzymes (Bagdade *et al.*, 1972), and reduced production of reactive oxygen species (Nielson and Hindson, 1989) by neutrophils of diabetic patients have been described. Furthermore, reduction in leukocyte phagocytosis and bactericidal activity showed a significant correlation with increases in blood glucose levels (Jakelic *et al.*, 1995).

Hyperglycemia, one of the characteristic manifestations of diabetes mellitus, has been found to be associated with neutrophil dysfunctions (Nabi *et al.*, 2005). Formation of advanced glycation end products (AGEs) through an interaction between glucose and lipids and/or proteins is one of the causes of long-term complications in diabetes (Brownlee, 2001). Glycosylated proteins isolated from the serum of diabetic rats affect membrane permeability and migration, reduce the rolling and adhesion abilities of leukocytes in alloxan-induced diabetic rats (Sannomiya *et al.*, 1997; Masuda *et al.*, 1990). Further, AGEs in human is linked to a rise in intracellular  $Ca^{2+}$  and to actin polymerization (Collison *et al.*, 2002). Actin polymerization in neutrophil plays important role in chemotactic action of neutrophils that is crucial to exhibit its normal functions. A positive association between polyol pathway activation and leukocyte dysfunction in experimental diabetes mellitus has been reported and hypothesized that the accelerated formation of sorbitol in diabetic animals may increase the intracellular osmolarity or decrease the availability of the enzyme co-factor

NADPH, leading to a disturbance of endothelial cell functions that might alter leukocyte-endothelial cell interactions (Cruz *et al.*, 2000).

Epidemiological studies have established a strong correlation between elevated total cholesterol levels in serum and morbidity and mortality from myocardial infarction (Thomas *et al.*, 1993). Elevated numbers of circulating neutrophils have been shown to be predictive of cardiovascular events independent of serum cholesterol levels (Guasti *et al.*, 2011) In a more recent study, a direct mechanistic link between hypercholesterolemia and proliferation of myeloid progenitor cells and, hence, neutrophilia and monocytosis has been identified (Murphy *et al.*, 2011 and Weber *et al.*, 2011). High dose of Statin, a cholesterol lowering agent, has been demonstrated to lower the migration ability of neutrophils significantly in healthy volunteers (Kinsella *et al.*, 2011). Further, free cholesterol has been found to be associated with the altered lipid raft structure of cell membrane and function regulating neutrophil  $Ca^{2+}$  entry and respiratory burst (Kolenkode *et al.*, 2007). Our previous study demonstrated neutrophil dysfunction in streptozotocin (STZ)-induced diabetic rats (Nabi *et al.*, 2005). This study was aimed to investigate the biochemical indices such as plasma levels of cholesterol, triglycerides, creatinine, urea, alanine transaminase and aspartate transaminase in diabetic and control rats and thus, investigate their relationship with the previously studied neutrophil functions.

## MATERIALS AND METHODS

### Preparation for streptozotocin-induced type 1 diabetes mellitus in the rats and collection of blood and plasma

A total of 30 Long Evan rats (Male: 15; Female: 15) each 2 weeks of age were kept in the plastic cages with even floors covered with wood shavings in the animal house of the department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh. The initial average body weight of the male rats was  $145.3 \pm 5.6$  gm and of the female rats was  $140.85 \pm 4.3$  gm. These animals were kept under constant temperature with a 14 hour light and

10 hour dark cycle. About 5-6 gm of balanced pelleted rat food was supplied thrice a day. These animals had also free access to drinking water. These conditions were maintained for the next 4 weeks.

Protocol for the preparation of streptozotocin-induced type 1 diabetic rats has been described in our previous study (Nabi *et al.*, 2005) using intraperitoneal injection of streptozotocin (STZ) dissolved in citrate buffer (65 mg/Kg body weight) at the age of 4 weeks ( $185.5 \pm 10.2$  gm and  $180.7 \pm 11.2$  gm body weights for 10 male and 10 female rats, respectively). Age matched control rats (5 male and 5 female) were injected with citrate buffer only. After anesthetizing in a chamber containing diethyl ether, blood samples were collected by sacrificing each diabetic and control rats into a heparin-containing falcon tube. Immediately after collection, 2.0 ml of blood was transferred into fresh tube and centrifuged at 3000 rpm for 10 minutes. The plasma was collected and stored at  $-20^{\circ}\text{C}$  until further analysis.

#### **Isolation of neutrophils, polarization assay and NBT dye reduction tests**

Neutrophils were isolated from the freshly collected blood samples of control and diabetic rats by dextran (Sigma) sedimentation followed by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) as described elsewhere (Islam and Nabi, 2003; Nabi *et al.*, 2005). The separated neutrophils were resuspended in a minimal volume (1.0 – 1.5 ml) of Hank's Balanced Salt Solution mixed with 3-(N-morpholino)-propanesulfonic acid (HBSS-MOPS). The percentage of polarized cells was assessed by viewing the cell preparation under Light microscope using a 40X objective (Nabi and Islam, 2001; Islam and Nabi, 2003; Nabi *et al.*, 2005). At least 300 cells were counted from each preparation. NBT dye reduction test was performed according to the protocol described earlier (Nabi and Islam, 2001; Nabi *et al.*, 2005). Briefly, neutrophils ( $2 \times 10^6$  cells/mL) treated with yeast activated serum, incubated for 30 minutes at  $37^{\circ}\text{C}$  and then, aliquots of NBT (Sigma) solution was added into the cells and incubated for 1 hour at  $37^{\circ}\text{C}$ . The unused NBT was removed through washing and the reduced dye was extracted in dioxan (Sigma) and quantitated at 520 nm.

#### **Biochemical analyses of the plasma samples**

Total plasma cholesterol, triglycerides, plasma creatinine, urea and activities of plasma AST and ALT were measured by standard colorimetric method in an Autoanalyzer (UK) using kits purchased from RANDOX, UK. Briefly, plasma cholesterol was measured by oxidation of cholesterol using cholesterol oxidase, triglycerides by enzymatic hydrolysis using lipases, creatinine by Jaffe method using alkaline picrate, urea using urease method, ALT and AST by kinetic methods using lactate dehydrogenase and NADH. By means of respective units, results of each parameter were expressed.

#### **Phagocytosis assay**

Baker's yeasts (*Saccaromyces cerevisiae*; Gist brocades, Holland) were preincubated with fresh control serum for opsonization to perform phagocytosis assay. Neutrophils ( $1 \times 10^6$  cells/mL, from control and diabetic rats) were taken onto clean glass slides and incubated for 5 minutes at  $37^{\circ}\text{C}$ . A few drops of prepared yeasts at  $1 \times 10^8$ /mL were then added to the neutrophils and incubated for a further 5 minutes at  $37^{\circ}\text{C}$ . The scoring was done according to our previous protocol (Islam and Nabi, 2003; Nabi *et al.*, 2005). The percentage of phagocytic cells and the number of yeast cells attached per 100 randomly chosen neutrophils were counted by examining at least 300 neutrophils from each preparation (controls and diabetics) under the oil immersion lens.

#### **Statistical analyses**

The results were expressed as mean  $\pm$  SD. To compare the differences between neutrophils from the control and diabetic rats, independent Student's *t*-test was performed. Correlation was determined by using non-parametric Spearman's rho test. A *p* value of less than 0.05 was considered significant. Multi dimensional scaling or Euclidean distance model was performed using SPSS package.

**RESULTS**

**Biochemical analyses**

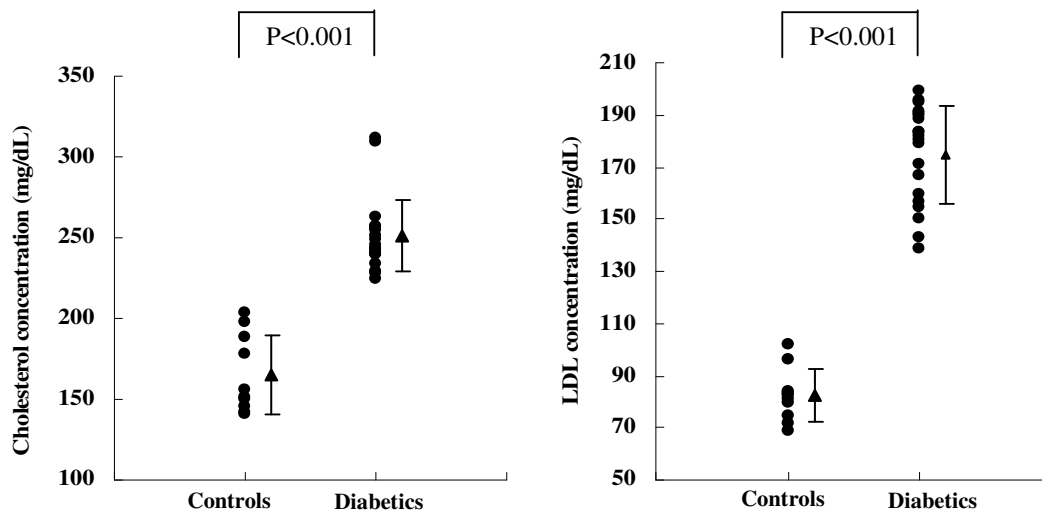
The development of diabetes was confirmed by the presence of hyperglycemia (blood glucose level > 230 mg/dL) as described previously (Nabi *et al*, 2005). Plasma glucose levels were determined by the glucose oxidase method using blood samples obtained from the animal tail. The rats were used for the experiments 1 week after receiving STZ injection. The average levels of glucose in the plasma of diabetic and control rats were 302.6 ± 87.5 and 100.7 ± 11.5 mg/dL, respectively. Other biochemical indices examined from the plasma of both the groups of rats have been presented in Table 1 and shown in Figure 1. In diabetic and control rats, the

average levels of plasma triglycerides were 174.9 ± 18.6 vs 82.2 ± 10.2 mg/dL; total cholesterol were 250.8 ± 22.3 vs 165.2 ± 24.1 mg/dL; plasma creatinine were 0.94 ± 0.19 vs 0.81 ± 0.05 mg/dL; plasma urea were 77.1 ± 9.7 vs 26.8 v 5.8 mg/dL, respectively. The mean values of aspartate transaminase and alanine transaminase in diabetic and control rats were 141.4 ± 28.0 vs 61.6 ± 18.6 IU/L and 61.4 ± 13.6 vs 48.5 ± 6.0 IU/L, respectively.

Statistical analysis using student’s *t* test showed an increase levels of all the biochemical parameters that measured in diabetic rats varied significantly (*p* < 0.001) compared to those of control rats (Table 1 and Figure 1).

**Table 1.** Levels of plasma glucose, creatinine, urea, alanine transaminase (ALT), aspartate transaminase (AST) in control and STZ-induced diabetic Long Evan rats.

Long Evan rats	Glucose (mg/dL)	Creatinine (mg/dL)	Urea (mg/dL)	ALT (IU/L)	AST (IU/L)
Control	100.7 ± 11.5	0.81 ± 0.05	26.8 v 5.8	48.5 ± 6.0	61.6 ± 18.6
Diabetic	302.6 ± 87.5	0.94 ± 0.19	77.1 ± 9.7	61.4 ± 13.6	141.4 ± 28.0
<i>p</i> values	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001



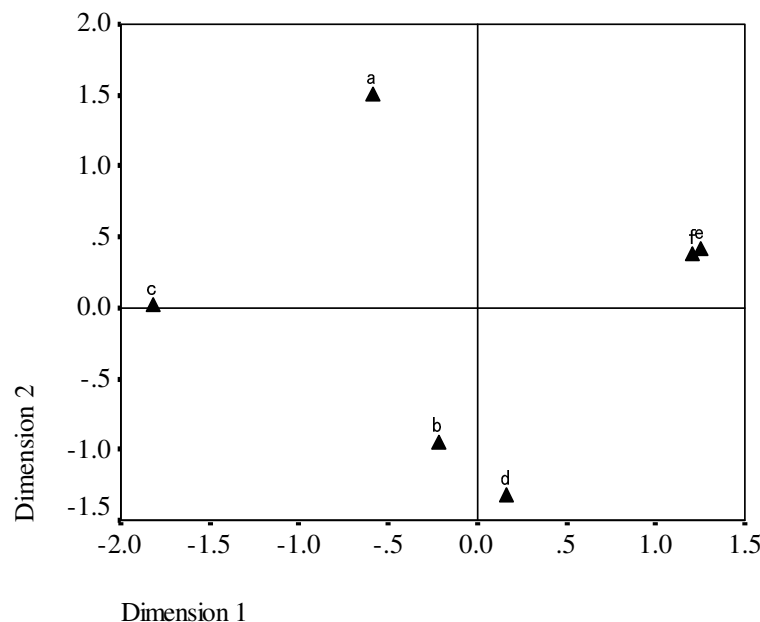
**Figure 1.** Levels of cholesterol (A) and triglycerides (B) in the plasma of diabetic and control rats. The mean levels of plasma triglycerides were 174.9 ± 18.6 and 82.2 ± 10.2 mg/dL in diabetic and control rats, respectively that varied significantly (*p* < 0.001).

### Association of biochemical indices with polarization of neutrophils and NBT dye reduction

Our previous reports revealed that at the base line level, neutrophils from diabetic rats were significantly more polarized ( $p < 0.001$ ) compared to those from control rats ( $30 \pm 4$  vs  $13 \pm 3$ ) (Nabi *et al*, 2005). We also showed that neutrophils from diabetic rats could reduce significantly more NBT dye ( $0.12 \pm 0.03$  vs  $0.04 \pm 0.01$ , respectively;  $p < 0.001$ ) than those from control rats at the baseline level (Nabi *et al*, 2005). These functions exhibited by diabetic and control neutrophils did show any relationship with the biochemical indices examined (Table 2 and 3). However, Euclidean distance model represented in Figure 2 indicated that the ability to reduce nitroblue terazolium dye by the neutrophils (e) of the study rats was closely related to the percentages of the polarized neutrophils (f).

### Association of biochemical indices with the phagocytic activity of neutrophils

According to our previous study, it was perceived that neutrophils from the diabetic rats were statistically less phagocytic than those from the control rats ( $61 \pm 7\%$  vs  $87 \pm 4\%$ , respectively;  $p < 0.001$ ) by phagocytosing significantly ( $p < 0.001$ ) less number of opsonized yeast particles ( $282 \pm 16$ ) compared to those of the control cells ( $381 \pm 17$ ) (Nabi *et al*, 2005). Though our previous findings demonstrated that percentages of phagocytic neutrophils as well as efficiency of the neutrophils i.e., number of yeast particles phagocytosed by the cells were inversely related to the levels of plasma glucose in diabetic rats (Nabi *et al*, 2005), we did not find any relationship between the immune function mediated by the neutrophils and plasma cholesterol/triglycerides using bivariate Spearman's rho analysis in case of neither diabetic nor control rats (Table 2 and 3). Interestingly, multidimensional scaling plot demonstrated a close relation between the levels of plasma total cholesterol and the percentages of phagocytic neutrophils as shown in Figure 2.



**Figure 2.** Euclidean distance model or multiple distance scaling indicated that the ability to reduce nitroblue terazolium dye by the neutrophils (e) of the study rats was closely related to the percentages of the polarized neutrophils (f). On the other hand, phagocytic activity of the neutrophils (b) was closely related to that of the plasma endogenous cholesterol (d). This model further showed that plasma glucose (a) and total number of yeasts phagocytosed by neutrophils (c) are distantly related.

**Table 2.** Correlation of the plasma glucose, triglycerides, total cholesterol, creatinine and urea with functional indices of neutrophils such as percent polarized, percentages of phagocytic neutrophils, total number of opsonized yeasts phagocytosed and ability to reduce NBT dye by neutrophils from diabetic rats.

	Glucose (mg/dL)	Creatinine (mg/dL)	Urea (mg/dL)	%Phagocytic c cells	Total number of yeast phagocytose d	NBT reductio n	%polarize d cells	Plasma triglycerid e (mg/dL)	Plasma cholesterol l (mg/dL)
Glucose (mg/dL)	1.000								
Creatinine (mg/dL)	0.133	1.000							
Urea (mg/dL)	0.170	-0.081	1.000						
%Phagocytic cells	-0.497* a	0.013	-0.406	1.000					
Total number of opsonized yeast phagocytosed	-0.500* a	0.264	-0.230	0.245	1.000				
NBT reduction	0.044	-0.132	0.111	0.132	-0.179	1.000			
%polarized cells	-0.193	0.007	-0.034	0.343	0.113	-0.230	1.000		
Plasma triglyceride (mg/dL)	-0.175	-0.012	0.449*	0.163	-0.026	0.003	0.172	1.000	
Plasma cholesterol (mg/dL)	0.185	-0.429	-0.213	0.067	-0.193	0.225	0.030	-0.017	1.000

\*Correlation is significant at the 0.05 level (2-tailed).

<sup>a</sup>Nabi *et al.*, 2005.

## DISCUSSION

The levels of biochemical indices such as important metabolites of lipid profile panel e.g., plasma cholesterol, triglycerides; of kidney function e.g., creatinine and urea; of liver function activities of serum enzymes such as aspartate transaminase and alanine transaminase were studied in streptozotocine-induced type 1 diabetic rats and thus, association of these indices with the possible alteration of the functions of neutrophils were investigated in this study. Plasma ALT and AST levels were measured to evaluate the hepatic functions. This study reveals statistically significant ( $p < 0.001$ ) increased levels of AST and ALT in the plasma of diabetic rats compared to that of control rats (Table 1). The increase in aminotransferases levels may be due to

the cellular damage in the liver caused by STZ-induced diabetes. Thus, our result supports the data of other researchers who found elevated levels of ALT and AST in diabetic rats (Zafar *et al.*, 2009; Baxter and Schofield, 1980). Voss *et al.* (1988) also reported similar finding by proposing time dependent rise in AST, ALT, and alkaline phosphates (ALP) levels in STZ-induced diabetic rats. Moreover, we also found that levels of creatinine and urea were significantly elevated in the plasma of diabetic rats (Table 1) which matched with the data reported by Voss *et al.* (1980). Thus, this data indicated that normal metabolic processes are altered in STZ-induced diabetic rats by affecting both liver and kidneys.

*In vitro* animal and clinical research indicated extensive relationship between serum lipids and the immune system

(Sullivan, 1994; Kelley and Bendich, 1996). From the basic metabolic standpoint of view, we know that lipoprotein particles deliver cholesterol, various phospholipids and fat soluble antioxidants to cells which are essential for maintaining the integrity of cell membrane and optimal immune function through the production of eicosanoid and anti-oxidants (Heiniger *et al*, 1978; Pace and Eshfahani, 1987; Bendich, 1990).

Antigen presenting activity of monocytes and chemotactic activity of some cell lines can be increased by cholesterol (Hughes *et al*, 1992; Kreuzer *et al*, 1991). Another study reported that diet-induced hypercholesterolemia in animals reduce macrophage response and phagocytic function (Kos *et al*, 1979).

**Table 3.** Correlation of the plasma glucose, triglycerides, total cholesterol, creatinine and urea with functional indices of neutrophils such as percent polarized, percentages of phagocytic neutrophils, total number of opsonized yeasts phagocytosed and ability to reduce NBT dye by neutrophils from control rats.

	Glucose (mg/dL)	Plasma triglyceride (mg/dL)	Plasma cholesterol (mg/dL)	Creatinine (mg/dL)	Urea (mg/dL)	%Phagocytic cells	Total number of yeast phagocytosed	NBT reduction	%polarized cells
Glucose (mg/dL)	1.000								
Plasma triglyceride (mg/dL)	0.078	1.000							
Plasma cholesterol (mg/dL)	0.267	0.237	1.000						
Creatinine (mg/dL)	-0.237	-0.585	0.061	1.000					
Urea (mg/dL)	-0.284	0.030	0.607	0.469	1.000				
%Phagocytic cells	-0.103	-0.201	-0.568	0.116	-0.390	1.000			
Total number of opsonized yeast phagocytosed	0.187	-0.369	-0.158	0.048	-0.296	0.024	1.000		
NBT reduction	0.466	-0.090	0.413	0.012	0.163	0.310	-0.248	1.000	
%polarized cells	0.613	0.328	0.103	-0.733*	-0.492	-0.397	0.200	0.042	1.000

\*Correlation is significant at the 0.05 level (2-tailed).

Cholesterol is present in human plasma at concentrations of milligrams per milliliter. The vast majority is complexed in lipoproteins. However, although clinical free cholesterol is albumin-bound and albumin can delay cholesterol's effects on signaling *in vitro*, cells are continuously exposed to cholesterol *in vivo* in equilibrium where cholesterol desorbs from albumin to cell membranes. Moreover, albumin concentrations vary markedly from plasma to the interstitium in health, and may vary further as a function of disease states. It is reported that high serum or tissue-free cholesterol

availability could activate immune cells that contribute to the progression of atherosclerotic lesions (Kolenkode *et al*, 2007). With respect to lipid effects on neutrophil phagocytosis, the phagocytic capacity in most human and animal studies appears to be decreased *in vitro* by lower chain triglycerides or LCT (Wiernik *et al*, 1983; Usmani *et al*, 1988; Rasmussen *et al*, 1988). Also, depressed phagocytosis, oxygen radical production and Fc-receptor expression have been observed after exposure of neutrophils to LCT (Cleary and Pickering, 1983).

The earliest morphological response of leucocytes to a chemoattractant is a change from a spherical to a polarized shape with formation of an extended, ruffled, anterior veil or lamellipodium. This polarization is accompanied by functional polarization, since the lamellipodium remains organelle-free, is rich in filamentous actin, and several cell surface proteins have been reported to become concentrated at the front of the cell. In various leucocytes these proteins include Fc receptors (Walter *et al.*, 1980; Wilkinson *et al.*, 1980), Thy-1 (Shields and Haston, 1985), CD15 and CD45 (Haston and Maggs, 1990), and urokinase-type plasminogen activator receptor (Estreicher *et al.* 1990). In the present study, it has been demonstrated through multi-dimensional scaling that phagocytic activity of the neutrophils are closely related to that of the levels of plasma cholesterol (Figure 2). Thus, it is possible that the equilibrium between the albumin-bound cholesterol and membrane cholesterol has been disturbed which might have altered baseline morphology of neutrophils followed by change in the normal distribution of receptors responsible for phagocytosis as reported by Cleary and Pickering (1983). This has also been reflected by the significantly lower percentages of phagocytosis mediated by diabetic neutrophils. Previous findings revealed enhanced phagocytic activity by the activated neutrophils (Kozel *et al.*, 1987). Thus, it was expected that activated diabetic neutrophils at the baseline level should demonstrate higher phagocytic activity. However, decreased phagocytic activity of the diabetic neutrophils as well as their decreased efficiency and its close association with plasma cholesterol might be attributed to the possible i) shedding of the receptors and/or ii) sequestration of the receptors and/or iii) alteration of membrane-bound receptor mediated cell signaling required to maintain optimal phagocytic activity. Thus, endogenous plasma cholesterol elevated due to type 1 diabetes in STZ-induced diabetic rats may alter the functions of neutrophils *in vitro*. The short coming of the current study is that we could not evaluate the effects of exogenous cholesterol on the neutrophil functions. However, future research work should be performed to observe the dose-dependent and time-dependent effects of cholesterol on neutrophil functions, status of different

functional receptors on the plasma membrane as well as on the cell signaling mechanism.

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