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ORIGINAL PAPER

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Evaluation of neutrophil phagocytic, complement functions, and cytokines expression among diabetic patients in Abuja, Nigeria

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ABSTRACT

Introduction. Inflammatory response in Diabetes Mellitus (DM) begins with chronic sub-clinical inflammations as a result of insulin resistance and activation of both innate and adaptive immune system as the disease progresses to complicated diabetes. Hence, the present study investigated the neutrophil phagocytic, complement function (CH50), and some cytokine profiles among diabetic and non-diabetic patients attending the National Hospital in Abuja, Nigeria.

Aim. To evaluate the neutrophil phagocytic, complement function (CH50), and some cytokine profiles among post-operative septic diabetic and post-operative septic non-diabetic patients at the National Hospital in Abuja, Nigeria.

Material and methods. Subjects were recruited by convenient sampling technique through interviewer-administered questionnaires. Subsequently, blood samples were collected. Fasting blood sugar (FBS) (mmol/L) was determined using glucose oxidase method. Neutrophil function test (Fmol/phag) was assayed using nitroblue tetrazolium reduction test (NBT). Hemolytic complement function (CH 50) test was conducted using serum harvested from sheep sensitized with human group (O^{Rh} D +ve) red blood cells. While serum Interleukin-4, -6, -10 and TNF- α were determined using Enzyme Linked Immunosorbent Assay (ELISA).

Results. Mean ± Standard deviation (SD) of FBS concentration of 10.5 ± 1.3 (mmol/|L) among diabetic and 4.7 ± 0.9 (mmol/L) among non-diabetics was recorded. There is a decrease in neutrophil phagocytic function with a mean ± SD of 5.4 ± 2.1 (Fmol/phag) in diabetics compared to 9.2 ± 2.1 (Fmol/phag) in non-diabetics. Similarly, complement (CH 50) function and C-reactive protein were significantly lower in diabetics when compared to non-diabetics ($p<0.001$). There was a significant difference in

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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IL-6 concentration between diabetics and non-diabetics groups, but no significant difference was observed in TNF-α, IL-4 and IL-10 concentrations between study groups (p>0.05). TNF-α and IL-6 was significantly higher in diabetics with cardiovascular disorders compared to non-diabetics subjects with cardiovascular disorders (p<0.001).

Conclusion. Findings from this study revealed the association of complement, neutrophil phagocytic function, CRP and IL-6 among septic diabetic patients,. In addition TNF-α and IL-6 expression was higher in DM patients with cardiovascular disorders.

Keywords. diabetes mellitus, effector molecules, pro-inflammatory markers

Introduction

Diabetes mellitus (DM) causes immune-suppression with an increased risk of either partial or permanent complications of certain tissues or organs such as the eyes, kidneys and heart.[1] Inflammatory response in DM begins with chronic sub-clinical inflammations as a result of insulin resistance and activation of both innate and adaptive immune system as the disease progresses to complicated diabetes over a period of 10–20 years.[2]

Cytokines regulate inflammatory and immune response through their activity on cells and they provide important signals in the pathogenesis of a range of diseases, including type 2 diabetes mellitus.[3] It has been reported by many studies that DM compromises the immune system by impairing leukocyte migration, phagocytosis and poor signal transduction that leads to increased risk of oxidative stress and increased cellular loss of function of which pro and anti-inflammatory cytokines play key role in the development and progression of diabetic complications over time.[4]

Cytokine secretions occur either in autocrine or paracrine manner in cells and trigger several cellular responses depending on diverse factors, such as cell type, timing, acting synergistically in many contexts to markedly amplify their effects.[4] They also have the capacity to induce expression of cytokine receptors on other cytokines.[5] In addition to being cells of acute inflammation and primary phagocytes of innate immune systems, neutrophils secrete cytokines and chemokines that further mobilize monocytes and macrophages to the site of inflammation.[5]

Metabolic and hemodynamic alteration associated with inflammatory response were hypothesized to be the major causes of cardiovascular and renal injuries.[6] Complications such as diabetic cardiomyopathy occur in 80% of all diagnosed type 2 diabetic patients, while 15% develop diabetic nephropathy and other complications account for 5%.[7]

Preventing diabetic complication is one of the major challenges in diabetes management, though previous diagnostic and treatment strategies in Nigeria and most developing countries do not include targeting immunological markers. The recent use of inflammatory cytokines is pivotal and very useful in monitoring diabetic patients during sub-clinical transformation to a life threatening diabetic complication.[8] Inflammatory cytokines, mainly IL-4 and TNF- α are involved in the development and progression of type 2 diabetes.[9]

IL-6 was initially thought to be a proinflammatory cytokine mainly with effects within the immune system, but this understanding of IL-6 was soon found to be too simplistic.[10] In the adaptive and innate immune systems, IL-6 is involved in both amplification of and protection against inflammation.[10] Thus, inappropriate regulation of IL-6 may play a direct protective or deleterious role in both antigen-specific immune-mediated diseases and in diseases where IL-6 or other inflammatory factors cause a low-grade inflammation (as seen in obesity and type 2 diabetes), which is likely to be involved in the pathogeneses of these diseases.[10] IL-6 has been suggested to be involved in the development of obesity-related and T2DM-related insulin resistance.[11]

TNF-α are pro-inflammatory cytokines whose expression increases chemotactic factors and adhesion molecules on vascular endothelium to effect the process of extravasation of leucocytes (especially neutrophils) in response to specific inflammatory stimulus such as bacterial infection.[12] IL-6, in more recent studies in type 2 diabetic patients in India, was demonstrated to be a strong predictor of progression to diabetic complications which has been related to alterations in endothelial permeability, induction of messengial cell proliferation and increased fibronectin expression.[12] Among various pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) has attracted the most attention, since it amplifies the inflammatory network of cytokines leading to a worsening progression of diabetes.[13]

In particular, it has been suggested that TNF-α is associated with insulin resistance and type 2 diabetes, given that TNF-α down-regulates the tyrosine kinase activity of the insulin receptor. Interleukin-10 (IL-10) is a centrally operating anti-inflammatory cytokine that plays a crucial role in the regulation of the innate immune system. It has strong deactivating properties on the inflammatory host response mediated by macrophages and lymphocytes, and potently inhibits the production of pro-inflammatory cytokines such as IL-6 and TNF-α.[14] IL-10 is produced by T-cells, B-cells, monocytes, and macrophages, under tight genetic control. The key roles of IL-10 as an inhibitory cytokine of autoimmunity and inflammation raise questions concerning the impacts of this cytokine on the pathogenesis of other diseases including type 2 DM and its nephropathic complications.[15]

Understanding the roles of inflammatory cytokines in the development and progression of diabetic cardiomyopathy and nephropathy is of critical importance. Early quantification of these markers may constitute a pivotal target in DM management and might open the possibility of new potential therapeutic targets.

There is paucity of studies on the roles of pro-inflammatory markers in predicting the risks for diabetes complications in subpopulations of interest, especially in Nigeria. Hence the need to carry out this study to evaluate the neutrophil phagocytic, complement function (CH50) and some cytokine profiles among post-operative septic diabetic and post-operative septic non-diabetic patients at the National Hospital in Abuja, Nigeria.

Material and methods

Study area

The study was carried out in the National Hospital Abuja, a referral tertiary health care facility with a 500 bed capacity.

Ethical approval

The ethical approval for this research was obtained from the Ethics and Research Committee of National Hospital Abuja (NHA) and the study was conducted in accordance with the declaration of Helsinki.

Study sample size

The sample size for the study was determined using the standard formula for calculation of minimum sample size by Rodrigues et al.[15] As calculated, the sample size was n = 38 case and n= 38 controls. In order to increase the precision, an attrition rate of 10% was added to each group. Hence, the sample size was increased to 45 case subjects and 45 controls. All eligible type 2 diabetic patients were within the age range of 25-60 years in both case and control groups.

For the purpose of this study, subjects were classified (inclusion criteria) as follows:

Groups (n = 45)	Case group (n = 45)	Control Group (n = 45)
Group 1: (n = 15)	Diabetic patients ≤ 5 years on treatment	Apparently healthy subjects
Group 2: (n = 15)	Diabetic patients with cardiovascular disease	Non-diabetic patients with cardiovascular disease
Group 3: (n = 15)	Diabetic patients with nephropathy	Non-diabetic patients with nephropathy

Exclusion criteria

a. Patients > 5 years on diabetic treatment without diabetic complication were excluded.
b. Patients with diabetic complication that were not clinically confirmed were excluded.
c. Diabetic patients with terminal complications (stroke, cardiomyopathy, myocardial infarction and renal dialysis) were excluded in this study.
d. Diabetic patients on anti-TB drugs, anti-retroviral drugs and cancer chemotherapy.
e. Patients with type 1 diabetes and gestational diabetes were excluded from the work.

Blood sample collection

8 mL of blood was collected from each patient, and 4 mL was collected into a sterile plain vacutainer tube. The blood was allowed to clot and was later centrifuged at 12000×g for 5 minutes and the clear sera was harvested and rapidly stored at -20°C until used for the determination of serum cytokines. 2 mL of blood was collected into EDTA vacutainer tube (BD® New Jessy, USA) and samples were immediately used for NBT assay. The remaining 2 mL of blood was collected into fluoride oxalate tube and later centrifuged at 12000×g for 5 minutes and the plasma was used for glucose determination and complement studies within 1 hour.

Table 1. Age and Sex Characteristics of Diabetic and non-diabetics subjects

Characteristic	Case (n = 45)			Controls (n = 45)			Total (n=90)
	Group 1 β (%)	Group 2 β (%)	Group 3 β (%)	Control 1 β (%)	Control 2 β (%)	Control 3 β (%)	
Age (years)							p<0.001
27 – 36	1 (2.2)	2(2.2)	0 (0.00)	0 (0.00)	0 (0.00)	9 (20.0)	12 (13.3)
37 – 46	4 (8.8)	3 (6.6)	3 (6.6)	4 (8.8)	2 (4.4)	2 (2.2)	18 (20.0)
47 – 56	8 (17.7)	6 (13.3)	6 (13.3)	7 (15.5)	4 (8.8)	4 (8.8)	35 (38.8)
≥ 57	2 (4.4)	4 (8.8)	6 (13.3)	4 (8.8)	9(20.0)	0 (0.00)	25 (27.7)
Mean ± SD	42 ± 1.3	29 ± 2.2	44 ± 2.4	42 ± 1.9	45 ±2.3	43 ± 1.5	p<0.001
Sex							P=8834
Male	9 (20.0)	8 (17.7)	8(17.7)	7 (15.5)	10(22.2)	9 (20.0)	51 (56.6)
Female	6 (13.3)	7 (15.5)	7(15.5)	8 (17.7)	5 (11,1)	6 (13.3)	39 (43.3)

n = number of case and control, group 1 = diabetic patients on treatment, group 2 = diabetic patients with cardiovascular disease, group 3 = diabetic patients with nephropathy, β = number of occurrence, (%) = percentage in parenthesis

Analytical Laboratory procedures

Patients were prepared for Fasting blood glucose (mmol/L). Plasma glucose was determined using glucose oxidase kit procured from Randox Laboratories, England. Neutrophils phagocytic function test was performed using the modified method of Onyenekwe et al.[16]. Serum samples were used to estimate the level of IL-4, IL-6, and IL-10 using ELISA Kits from Enzo-life-sciences (UK) LTD according to manufacturer's instructions. Serum TNF-alpha was quantified using sandwich ELISA by kits supplied by WKEA Med Supplies Corp, China. Human C-reactive protein (CRP) was estimated based on ELISA kit from Cayman Chemical (UK) LTD.

Data analysis

The data obtained were computed using Microsoft office Excel 2013 and SPSS version 20. The results were expressed as percentage and mean ± SD. All data were checked for distribution of variables and feasibility of using a parametric test. If the data is normally distributed and there is no violation of assumptions (normal or Gaussian distribution) independent t-test or one way ANOVA (parametric) was used to find the association between the variables. A p value less than 0.05 ($p < 0.05$) was considered statistically significant.

Results

The mean ± SD age (years) of 42 ± 1.3, 44 ± 0.5 and 45 ± 2.3 in diabetic patients with ≤ 5 years of management, diabetic patients with cardiovascular diseases and patients with diabetic nephropathy, respectively, was recorded. In addition, 29 ± 2.2, 42 ± 1.9 and 43 ± 1.5 among apparently healthy controls, non-diabetic patients with cardiovascular disease, and non-diabetic patients with nephropathy, respectively, was recorded (Table 1). The age range between 47–56 years had the highest number of participants, 35 (38.8 %), while age range 27–36 years had the least participants, 12 (13.3%). From this study, 51 (56.6 %) of the participant were male and 39 (43.3%) were female (Table 1).

Findings from this study showed that neutrophil function consistently decreases among diabetic patients with ≤ 5 years treatment, diabetic patients with cardiovascular disease, and diabetic nephropathy, respectively. However, higher values were observed among the control subjects with 9.2 ± 2.1, 7.3 ± 1.9 and 8.5 ± 3.5 (fmol/phag) with no significant difference of neutrophil function among the groups ($p>0.05$) (Table 2). The findings also indicated that FBG of 10.5 ± 1.3, 10.2 ± 1.6 (mmol/L) and 8.6 ± 1.7 (mmol/L) in diabetic patients with ≤ 5 years on treatment, patients with cardiovascular disease, and patients with diabetic nephropathy, respectively.

Consistent normal FBG concentrations were observed among the control group. There was significant differences in FBG among the study groups ($p<0.05$) (Table 2). The serum CRP concentrations of 8.5 ± 1.8 (ng/L) and 3.5±2.8 (ng/L) was recorded in diabetic and non-diabetic subjects with cardiovascular disease while serum CRP concentrations of 5.2 ± 0.3 (ng/L) and 3.7 ± 0.3 (ng/L) was observed in diabetic and non-diabet-

Table 2. Neutrophil Phagocytic Activity, Fasting Blood Glucose, Serum C-Reactive Proteins and BMI (Mean ± SD) among Diabetic Patients and Controls

Analyte	Case (n=45)	Controls (n=45)	p value
NBT (fmol/phag)			
Group 1	5.4 ± 2.1	9.2 ± 2.1	0.0138
Group 2	6.7 ± 2.0	7.3 ± 1.9	0.2860
Group 3	6.8 ± 3.9	8.5 ± 3.5	0.2308
FBG (mmol/l)			
Group 1	10.5 ± 1.3	4.7 ± 0.9	<0.0001
Group 2	10.2 ± 1.6	4.8 ± 1.2	<0.0001
Group 3	8.6 ± 1.7	4.8 ± 0.9	<0.0001
CRP (ng/l)			
Group 1	4.2 ± 0.3	3.5 ± 1.3	<0.0002
Group 2	8.5 ± 1.8	3.5 ± 2.8	0.0678
Group 3	5.2 ± 0.3	3.7 ± 0.3	<0.0001
BMI (kg/m^2)			
Group 1	32.7 ± 1.3	26.3 ± 2.4	<0.0001
Group 2	39.2 ± 3.5	32.3 ± 1.0	0.05
Group 3	34.7 ± 2.5	29.2 ± 1.5	0.0723

n – number of case and control, group 1 – diabetic patients on treatment, group 2 – diabetic patients with cardiovascular disease, group 3 – diabetic patients with nephropathy, NBT – Nitro blue tetrazolium reduction test, FBG – fasting blood glucose, CRP – C-reactive proteins and BMI – body mass index

ic patients with nephropathy. There was a significant difference between CRP level between the study group ($p<0.05$) and significant BMI among the group of diabetic patients with ≤ 5 years of treatment, and diabetic patients with cardiovascular disease group ($p < 0.05$). There was no significant BMI difference among diabetic and non-diabetic patients with nephropathy respectively 34.7 ± 2.5 (kg/m^2) and 28.2 ± 1.5 (kg/m^2) ($p>0.05$) (Table 2).

Serum IL-6 concentration was significant higher (30.2 ± 2.1ng/L) among diabetic patients with cardiovascular disease and non-diabetic patients with cardiovascular disease (21.4 ± 1.3 ng/L) ($p<0.05$) (Table 3). However, no significant difference in serum IL-6 concentration was observed among diabetic and non-diabetic patients with nephropathy ($p = 0.1040$) (Table 3). The serum concentration of TNF-α (20.2±2.4 ng/L) among diabetic patients with ≤ 5 years on treatment was significantly higher than apparently healthy controls (18.1 ± 1.4ng/L) ($p=0.012$) (Table 2). Serum TNF-α concentration of 35.5 ± 2.5ng/L was observed among diabetic patients with cardiovascular disease and 29.4 ± 2.5ng/L among non-diabetic patients with cardiovascular disease. There was significant difference in TNF- α between the two groups ($p<0.05$). No significant difference in TNF-alpha was observed among diabetic patients and non-diabetic patients with nephropathy ($p=0.421$) (Table 3).

The mean serum IL-4 concentrations in diabetic patients ≤ 5 years on treatment (5.8 ± 3.1 ng/L), diabetes with cardiovascular disease (5.9 ± 0.6 ng/L) was not significantly different from diabetic patients with nephropathy (5.1 ± 4.7 ng/L) and the control subjects ($p=0.2443$). Similarly, serum IL-10 concentration of 8.4 ± 3.1ng/L among diabetic patients with ≤ 5 years on treatment was not significantly different from 8.8 ± 0.9 ng/L and 8.1 ± 0.6ng/L among diabetic patients with cardiovascular disease and nephropathy, respectively ($p=0.9849$) (Table 4). Similarly, no significant difference between serum IL-10 concentration among diabetic patients (8.0 ± 7.4ng/L) and non-diabetic patients with nephropathy (14.5 ± 7.4 ng/L) ($p=0.2146$) (Table 4).

Discussion

Nigeria is one of the countries most affected by type 2 diabetes mellitus. Diabetic patients suffer from multiple immunological disorders with increased risk of complications. This study revealed that neutrophil phagocytic activity significantly decreased consistently in DM patients compared to non-Diabetic controls. A Previous study established that insulin insensitivity affects glucose uptake into cells and peripheral tissues, and thus affects the neutrophil functions of the innate immune system.[17] These findings also agree with Oni et al. and Chenxiao et al., who reported that high

Table 3. Serum Pro-inflammatory Cytokines IL-6 and TNF-α Concentration (Mean ± SD) among Diabetic Patients and Controls

Cytokines	Case (n = 45)	Controls (n = 45)	p value
IL-6 (ng/l)			
Group 1	21.9 ± 1.4	18.1 ± 1.5	0.001
Group 2	30.2 ± 2.1	21.4 ± 1.3	0.001
Group 3	22.8 ± 2.4	20.5 ± 2.1	0.104
TNF-α (ng/l)			
Group 1	20.2 ± 2.4	18.1 ± 1.4	0.061
Group 2	35.5 ± 2.5	29.4 ± 2.5	0.001
Group 3	23.7 ± 2.3	22.7 ± 1.9	0.421

n - number of case and control, group 1 - diabetic patients on treatment, group 2 - diabetic patients with cardiovascular disease, group 3 - diabetic patients with nephropathy, the figures in parenthesis (%) - percentage

Table 4. Serum Anti-inflammatory Cytokines IL-4 and IL-10 Concentrations among Diabetic Patients and Controls

Cytokines	Case (n = 45) Mean ± SD	Controls (n = 45) Mean ± SD	p value
IL-4 (ng/L)			
Group 1	5.8 ± 3.1	6.1 ± 0.3	0.1648
Group 2	5.9 ± 0.6	5.6 ± 0.5	0.4193
Group 3	5.1 ± 4.7	8.9 ± 4.7	0.2443
IL-10 (ng/L)			
Group 1	8.4 ± 0.9	8.5 ± 0.7	0.9849
Group 2	8.8 ± 0.6	8.1 ± 0.6	0.1598
Group 3	8.0 ± 7.4	14. 5 ± 7.4	0.2146

n - number of case and control, group 1 - diabetic patients on treatment, group 2 - diabetic patients with cardiovascular disease, group 3 - diabetic patients with nephropathy, the figures in parenthesis (%) - percentage

blood glucose concentrations impaired superoxide production from isolated blood neutrophils in diabetic patients.[18,19] These authors further reported that high glucose concentration rapidly affects membrane receptors responsible for the activation of NADPH oxidase in neutrophils. Anelise et al. and Harshad et al. also reported similar observations in an animal study.[20,21] The result from this study confirmed high glucose concentration among diabetic patients. However, insulin inaction in type 2 diabetes leads to insulin toxicity and production of reactive oxygen species which changes the hemodynamic. This causes damage to much tissue especially the vascular endothelium and glomerular basement membrane.[22] This is in consonance with the findings of Dwijo et al., Cathy et al., Juan et al., Joachim et al. and Marisa et al.[4,6,23-25]

Findings from this present study revealed that the serum level of C-RP is high in both diabetic and non-diabetic patients with cardiovascular diseases, while it is relatively low in diabetic and non-diabetic patients with nephritic syndromes (p=0.001). It has been confirmed that C-RP in diabetes plays a central role in pathogenesis of atherosclerosis and athero-thrombotic conditions associated with huge prevalence of cardiovascular disease. It does also predict the risk of cardiovascular event in both diabetic and non-diabetic patients.[26] This result is in consonance with findings of Cathy et al. and Zaid et al.[6,26] However, this finding disagrees with report from Youn-Hee et al. who reported that C-reactive protein production is independent of diabetic complication or occurrence of cardiovascular diseases or nephritic syndrome.[27] Joachim et al. reported that high serum level of CRP is an indicator for diabetic progression toward complication especially in DM associated cardiovascular disease.[24]

This study showed a consistent increase in serum level of IL-6 in DM and diabetic patients with cardiovascular disease. Many studies established that increase serum concentration of pro-inflammatory cytokines IL-6 is associated with insulin resistance diabetes and is related to progression of vascular thrombotic accident, endothelial damage, cardiovascular disease and renal injury.[28-30] This finding is in consonance with reports from Weijiang et al. and Ingrid et al. that there is high concentration of IL-6 in diabetic, cardiovascular disease and patients with nephritic syndrome.[31,32]

This study showed that serum concentration of TNF-α is lower among DM than non-diabetic subjects. However, high TNF-α levels were recorded in DM patients with cardiovascular disease as compared with other groups and controls. This could be because TNF-α is associated with progression of vascular thrombotic and endothelial damage as seen in many cardiovascular diseases.[31] Thus, an anti-TNF-α drug can provide a promising therapy for insulin resistance in pre-diabetic stage. This is in agreement with findings from Weijiang et al. and Ingrid et al.[31,32]

Findings from this study showed that the serum concentration of IL-4 was significantly higher in DM and non-diabetic patients with cardiovascular diseases, but no significant difference was observed in diabetic and non-diabetic patients with nephritic syndromes. This could be because IL-4 has been demonstrated to be involved in immune-modulation of Th1 and Th2 in DM.[32]

There was no significant difference in the IL-10 expression all group of subjects studied. However, it was relatively high in non-diabetics with nephropathy. Even though IL-10 has anti-inflammation role, findings from a study did not associate IL-10 with nephropathic complications.[14] Notwithstanding, a cohort prospective study might give better insight into the end effects of IL10 levels in these categories of subjects.

Conclusion

Findings from this study revealed the association of complement, neutrophil phagocytic function, CRP and IL-6 among septic diabetic patients. In addition TNF-α and IL-6 expression was higher in DM patients with cardiovascular disorders.

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