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ORIGINAL RESEARCH

Candida albicans effects the tissue factor activity of blood cells in Type II Diabetes Mellitus

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Abstract

Disorders of hemostatic mechanism play a role in the development of diabetic complications and cardiovascular events. *C. albicans* infection is frequently found in diabetic individuals compared to nondiabetic individuals. In this study, the *in vitro* effect of *Candida albicans* on the tissue factor activity of the PMNS, MNS and platelet cells, that were isolated from the blood samples of healthy and diabetic individuals, was determined. The isolated blood cells were counted after the viability test has been checked. *C.albicans* suspension was added to one part of the cells. An equal volume phosphate buffer was added to other part of the cells instead of *C.albicans* suspension. Tissue factor activity of all blood cells was determined by the method of Quick. Tissue factor activity of these cells was found to be significantly changed in the presence of *C.albicans*. The presence of diabetes was also significantly changed tissue factor activity of the PMNs, MNs and platelet cells when compared to the healthy individuals. The findings of this study will contribute to the treatment of diabetic patients with or without candida infection in terms of tissue factor activity.

Keywords: Tissue factor activity, diabetes, *Candida albicans,* leukocyte, platelet

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INTRODUCTION

Diabetes mellitus (DM) is one of the most prevalent metabolic syndromes that is characterized by hyperglycemia resulting in short-term metabolic changes in lipid and protein metabolism and in long-term irreversible vascular and connective tissue changes (1). The incidence of infections also increase in DM. Some of these infections are more likely to have a complicated course in individuals with or without diabetes. Concerning cellular innate immunity most studies show decreased functions (chemotaxis, phagocytosis, and killing) of polymorphonuclear leukocyte cells (PMNs) and monocytes/ macrophages with diabetes compared to cells of controls. Furthermore, some microorganisms become more virulent in a high glucose environment. Another mechanism which can lead to the increased prevalence of infections in person with diabetes is an increased adherence of microorganisms to cells with diabetes compared to cells without diabetes (2). Candida spp. are commensal microorganisms that are part of the microflora of different body sites. *C. albicans* infection is frequently found in people with diabetes compared to people without diabetes. In immunosuppressed

individuals whose condition may have been caused by diabetes mellitus, Candida spp. can express different virulence factors and may consequently become pathogenic (3, 4, 5). Besides that increase susceptibility to infection, diabetes mellitus is also associated with the accelerated development of vascular disease and there is evidence that platelets actively contribute to this process. Platelets of patients with DM are characterized by dysregulation of several signaling pathways, leading to a hyperreactive phenotype with enhanced adhesion, aggregation, and activation (6, 7).

Tissue factor (TF) is a prothrombotic transmembrane protein, expressed by both vascular and non-vascular cells, including monocytes, and platelets (8, 9). TF expression by endothelial cells is up-regulated in the presence of low-grade inflammation, frequently found associated with Type II DM. In contrast, vascular smooth muscle cells, which become exposed after plaque rupture, constitutively express TF and this expression is further enhanced after stimulation with cytokines. Patients with Type II DM have higher levels of circulating TF, as well as increased monocyte TF mRNA, which are directly modulated by both glucose and insulin, with an additive effect. TF levels may also be influenced through the formation of advanced glycation end products, which activate NF-kB leading to TF production (8, 9,10).

As *C.albicans* is the most frequently used microorganism for the studies of leukocyte cell functions, in the present study the effect of hyperglycemia and *in vitro* effect of *C.albicans* on the TF activity of PMNs, mononuclear leukocytes (MNs) and platelet cells within two hours was determined.

MATERIAL AND METHODS

Blood Samples

Fresh human whole blood samples were taken from Şişli Etfal Training and Research Hospital. All the investigations were performed in both a fasting state were conducted between 8:30 to 9:30 AM. The subjects with type II DM were instructed to hold their morning oral diabetes medications prior to their blood draw. Whole blood samples were drawn from either healthy volunteers or volunteers with diabetes using evacuated tubes containing EDTA anticoagulant. A hundred samples (fifty samples for group with diabetes and fifty samples for healthy group) were used in this study. Half part of these blood samples was used for leukocyte isolation (PMNs and MNs) and the other half part was used for the platelet isolation. Experiments were approved by the Marmara University School of Medicine Ethical Committee.

HbA1c Determination

HbA1c was tested at Şişli Etfal Trainning and Research Hospital by using Variant II Turbo Hb testing system (Bio Rad, UK).

Whole Blood Hemoglobin, Hematocrit Determination and Leukocyte, Platelet Count

Hemoglobin, hematocrit levels and leukocyte and platelet counts were measured by using Roche Sysmex XT-2000i whole blood count analyzer.

Leukocyte cell isolation

PMNs and MNs were isolated from whole blood containing EDTA of healthy donors using the technique of Boyum (11). Briefly, dextran sedimentation (6% Dextran '70'; Sigma) of the whole blood was followed by Ficoll (Sigma) gradient centrifugation of the leukocyte-rich supernatant. The MNs that are found at the interface between the plasma and the Ficoll-Paque layer were recovered from the interface and subjected to a short washing step with PBS by centrifugation. Then cells suspended in 1 ml of PBS.

The bottom layer that contains mostly PMNs was transferred to centrifuge tube. Contaminating erythrocytes were lysed by suspension of the PMNs pellet in distilled water for 30 s, followed by the addition of hypertonic saline (1.8% NaCl) to correct the tonicity of the solution. This suspension was centrifuged at 160xg for 5 min. The suspended cells were washed twice in PBS and suspended with 1 ml PBS.

The viability of both PMNs and MNs was determined by trypan blue (Sigma) exclusion criteria and was found to be 95% (12).

Platelet Isolation

Venous blood samples was centrifuged at 72xg for 15 minutes. The upper layer was taken and centrifuged at 1006xg for 5 minutes. The upper phase was platelet poor plasma and the bottom phase was platelet rich plasma. Upper phase was discarded. 0.5 mL distilled water was added to the bottom layer and vortexed. Immediately 0.5 mL NaCl (%1.8) was added and centrifuged at 160xg for 4 minutes. Upper liquid was discarded and washing step was repeated. Platelet cells were suspended with 0.5 mL PBS and centrifuged at 160xg for 5 minutes. Washing step can be repeated when needed. Cells were then suspended with 1 mL PBS (13).

Leukocyte Count

Venous blood sample was suck up into a leukocyte pipette, identified with a white pearl, to mark 1.0, then Turk's solution (1 mL glacial acetic acid+1 mL Genetian violet + 98 mL water) to mark 11.0. Pipette was shaken

vigorously and counting chamber was filled. The counts were taken in all squares of the chamber. Counting was carried out under low magnification (x 40) (14).

Leukocyte count: Total chamber count x 1000 = Leukocytes/mm^3

Platelet Count

Venous blood sample was suck up into an erythrocyte pipette to mark 1.0, then ammonium oxalate solution (1%) was suck up to mark 101 and was shaken for 5 minutes. First few drops were discarded then one of the counting chambers was filled and was placed for 30 minutes in a moist chamber. Platelet cells was counted twice in all 5 large squares of the counting chamber, using a phase-contrast microscope at a magnification x40 (15).

Platelet Count = Number of platelets counted x dilution factor (100) x volume factor (10) = Platelets/mm^3

Preparation of *C.albicans*

ATCC 10231 strain of *C.Albicans* was obtained from Marmara University, Faculty of Dentistry, Microbiology Department. This strain was passaged and incubated on Sabouraud dextrose agar at 37^0 C for 24 hours. From breeding colonies *C.albicans* suspension was prepared by using PBS solution. There was 10^8 blastoconidia per mL in the suspension. 9 mL PBS solution was added to 1 mL of this suspension. There was 10^7 *C. albicans* cells in the suspension at the end of the dilution.

Incubation of Leukocyte and Platelet cells with *C.albicans*

PMNS, MNS and platelet cells that were isolated from both healthy volunteers and volunteers with diabetes were mixed with *C. albicans* cells. In the mixture the number of the PMNs, MNs and platelet cells equal to the *C. albicans* cells (for 1 mL 10^6 *C. albicans* cells, there was 10^6 leukocyte or platelet cells).

Tissue Factor Activity

Tissue factor activity of PMNs, MNs and platelet cells was evaluated according to Quick's one stage method using normal plasma. This was performed by mixing 0.1 mL cell suspension with 0.1 mL of plasma, with the clotting reaction being started on addition of 0.02 M $CaCl_2$. All reagents were in the reaction temperature (37ºC) before admixture. Tissue factor activity was expressed as seconds. Shortened clot formation time shows increased TF activity (16).

Changing of Tissue Factor Activity of PMNs, MNs and Platelet Cells Over Time

The cells which were isolated from either healthy volunteers or volunteers with diabetes were checked for viability and were counted. Then tissue factor activity of the PMNs, MNs and platelet cells was determined at 0, 10^{th}, 20 th, 30 th, 60 th, 90 th and 120 th minutes.

Table1: Hemoglobin and hematocrite levels and leukocyte and platelet counts

	Healthy (n=50)	Diabetic (n=50)	p (t-test)
Hemoglobin (% g)	13.04 ± 1.23	12.99 ± 1.86	0.8322
Hematocrite (%)	39.81 ± 4.45	39.38 ± 4.77	0.5684
Leukocyte count (mm^{-3})	6703 ± 1348	7081 ± 1141	0.2155
Platelet count (mm^{-3})	248900 ± 66580	247200 ± 61460	0.8897

Changing of Tissue Factor Activity of PMNs, MNs and Platelet Cells Over Time following incubation with *C. albicans*

The cells which were isolated from either healthy volunteers or volunteers with diabetes were checked for viability and were counted. *C. albicans* suspension was added to the cells at time zero with an equal number of cells (10^6). Tissue factor activity of PMNs, MNs and platelet cells which were added *to C.albicans* suspension was determined at 0, 10^{th}, 20^{th}, 30^{th}, 60^{th}, 90^{th} and 120^{th} minutes.

Statistics

All data were expressed as mean values ± SD. Statistical analysis was carried out using Graph Pad Prism 3.0 (Graph Pad Software, San Diego,CA, USA). Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $p < 0.05$ were regarded as significant.

RESULTS

The comparison of whole blood hemoglobin, hematocrit levels and leukocyte and platelet counts of healthy and individuals with diabetes

There was no significant difference between the healthy individuals and individuals with diabetes when compared according to their hemoglobin, hematocrit levels and leukocyte and platelet counts (Table 1).

HbA1c values of individuals with diabetes

In healthy individuals HbA1c levels were between 4-6 % g. Mean HbA1c value of individuals with diabetes was 8.00±1.37 % g.

Tissue Factor Activity of Platelets, PMNs and MNs cells

Tissue factor activity of platelets, MNS and PMNS cells

Table 2: Time dependent effect of *C.albicans* to the tissue factor activity of platelets which were isolated from healthy and diabetic individuals

TF activity (sec)	HP (n=50)	CHP (n=50)	DP (n=50)	CDP (n=50)	P (Anova)
0 (Beginning)	170.6 ± 10.31	175.3 ± 12.99^{++}	208.3 ± 26.28**	173.5 ± 21.92^{+}	0.0202
10th minute	183.8 ± 9.02	173.9 ± 12.81^{+++}	252.1 ± 29.91**	138.3 ± 18.68^{+++} *** $^{\#\#}$	0.0001
20 th minute	148 ± 6,35	168.9 ± 13.64^{++}	246.1 ± 52.67***	120.0 ± 12.77^{+++} $^{\#\#}$	0.0001
30th minute	178.1 ± 7.30	166.4 ± 10.32^{+++}	261.6 ± 3.39***	102.4 ± 11.98^{+++} *** $^{\#\#\#}$	0.0001
60th minute	179.6 ± 13.68	163.3 ± 10.87^{+++}	266.4 ± 46.13	94.13 ± 12.41^{+++} ***	0.0001
90 th minute	170.1 ± 11.44	158.4 ± 10.20^{+++}	283.6 ± 32.41***	80.88 ± 7.20^{+++} *** $^{\#\#\#}$	0.0001
120th minute	158.3 ± 8.92	154.6 ± 8.28 $^{+++}$	315.9 ± 8.23***	45.00 ± 0.31^{+++} *** $^{\#\#\#}$	0.0001
p (Anova)	0.0001	0.0051	0.0001	0.0001	

Values are given as Mean ± SD, **HP**: Healthy platelet , **CHP:** *C. albicans* added healthy platelet, **DP:** Diabetic platelet, **CDP:** *C. albicans* added diabetic platelet. (**): p<0.01, (***) : p<0.001 compared to healthy group,
($^{+}$) : p<0.05, ($^{++}$) : p<0.01, ($^{+++}$) : p<0.001 compared to diabetic group, ($^{\#\#}$) : p<0.001, ($^{\#\#\#}$) : p<0.001 compared to *C. albicans* added healthy group

Table 3: Time dependent effect of *C.albicans* to the tissue factor activity of MNs which were isolated from healthy and diabetic individuals

TF activity (sec)	HM (n=50)	CHM (n=50)	DM (n=50)	CDM (n=50)	P (Anova)
0 (Beginning)	213.3 ± 20.41	196.6 ± 19.94^{++}	231.5 ± 19.35	237.4 ± 23.27$^{\#\#}$	0.0044
10th minute	270.8 ± 0.81	199.0 ± 18.15*** $^{++}$	236.9 ± 16.49**	255.3 ± 24.53$^{\#\#\#}$	0.0001
20 th minute	252.5 ± 2.21	193.3 ± 17.38^{***++}	241.1 ± 22.04	283.8 ± 39.59^{+} $^{\#\#\#}$	0.0080
30th minute	184.0 ± 14.10	188.4 ± 13.87^{+++}	230.5 ± 19.27***	284.3 ± 25.88$^{***+++\#\#\#}$	0.0002
60th minute	239. ± 26.16	188.1 ± 13.17*** $^{++}$	226.0 ± 20.14	273.6 ± 22.77$^{***+++\#\#\#}$	0.0001
90 th minute	234.3 ± 22.16	186.4 ± 13.43*** $^{+++}$	266.0 ± 18.38**	264.1 ± 24.62* $^{\#\#\#}$	0.0001
120th minute	254.1 ± 23.81	185.5 ± 12.97*** $^{+++}$	244.9 ± 19.64	257.4 ± 27.70$^{\#\#\#}$	0.0004
P (Anova)	0.0001	0.5049	0.0036	0.0133	

Values are given as Mean ± SD, **HM**: Healthy MNs, **CHM:** *C. albicans* added healthy MNs, **DM:** Diabetic MNs, **CDM:** *C. albicans* added diabetic MNs. (*) : p<0.05, (**) : p<0.01, (***) : p<0.001 compared to healthy group, ($^{++}$) : p<0.01, ($^{+++}$) : p<0.001 compared to diabetic group ($^{\#\#}$) : p<0.01, ($^{\#\#\#}$) : p<0.001 compared to *C.albicans* added healthy group

Table 4: Time dependent effect of *C.albicans* to the tissue factor activity of PMNs which were isolated from healthy and diabetic individuals

TF activity (sec)	HPM (n=50)	CHPM (n=50)	DPM (n=50)	CDPM (n=50)	P (Anova)
0 (Beginning)	164 ± 12.64	139.4 ± 10.36**	131.9 ± 19.47**	97.88 ± 11.43*** $^{+++}$ $^{\#\#\#}$	0.0001
10th minute	228.8 ± 14.19	133.4 ± 11.04***	134.9 ± 18.54***	114.3 ± 13.33*** $^{++}$ $^{\#\#}$	0.0001
20 th minute	212.5 ± 15.33	132.8 ± 12.36***	132.0 ± 16.78***	107.5 ± 10.18*** $^{++}$ $^{\#\#}$	0.0001
30th minute	216.1 ± 14.41	126.8 ± 11.16***	149.1 ± 21.72^{++}	121.5 ± 11.63^{***++}	0.0002
60th minute	194.8 ±12.87	118.8 ± 9.94*** $^{+}$	139.5 ± 20.56***	154.0 ± 12.92*** $^{\#\#\#}$	0.0001
90 th minute	189.4 ± 11.84	102.8 ± 7.46*** $^{+++}$	157.0 ± 22.16**	150.1 ± 17.02*** $^{+++}$ $^{\#\#\#}$	0.0001
120th minute	237.0 ± 15.13	94.00 ± 9.212^{***+++}	157.8 ± 22.23***	158.1 ± 16.59*** $^{\#\#\#}$	0.0001
p (Anova)	0.0001	0.0001	0.0685	0.0001	

Values are given as Mean ± SD, **HPM**: Healthy PMNs, **CHPM:** *C. albicans* added healthy PMNs, **DPM:** Diabetic PMNs, **CDPM:** *C. albicans* added diabetic PMNs. (**) : p<0.01, (***) : p<0.001 compared to healthy group, ($^{+}$) : p<0.05, ($^{++}$) : p<0.01, ($^{+++}$) : p<0.001 compared to diabetic group, ($^{\#\#}$) : p<0.01, ($^{\#\#\#}$) : p<0.001 compared to *C.albicans* added healthy group

were presented at Table 2, Table 3 and Table 4 respectively. Shortened clot formation time shows increased TF activity.

DISCUSSION

The present study was undertaken to establish the TF activity difference between individuals with diabetes and healthy individual's PMNs, MNs and platelet cells that were stimulated with *C.albicans in vitro*. It was also investigated the time dependent change of TF activity of these cells as for monitoring the activation of these cells. TF is the prime initiator of blood coagulation, and precise regulation of its production and activation is vital for normal hemostasis (17, 18). Patients with Type II diabetes have elevated plasma TF antigen and TF procoagulant activity (9, 10). As *C.albicans* infections more often in type II DM patients than those without DM, in the present study the change of TF activity in *C. albicans* stimulated PMNs, MNs and platelet cells was investigated both in healthy and states with diabetes after 10, 20, 30, 60, 90 and 120 minutes incubation.

As tissue factor (TF) is a prothrombotic transmembrane protein, expressed by both vascular and non-vascular cells, including monocytes, and platelets, it is important to evaluate tissue factor activity of main blood cells in different states. Platelets of patients with DM are characterized by dysregulation of several signaling pathways, leading to a hyperreactive phenotype with enhanced adhesion, aggregation, and activation (6, 7). Thus, platelets are reported to respond more frequently to sub-threshold stimuli, becoming consumed more rapidly which results in an accelerated thrombopoiesis of more reactive platelets.

In the present study, TF activity of platelets in DM patients showed time dependent decrease. This decrease shows the TF activity loss of platelets *in vitro* in DM patients. Addition of *C.albicans* to the platelets with diabetes increased tissue factor activity from 10 th minutes to 120 minutes after contamination. As activated platelets release FVa, which is thought to be important in the initial phase of thrombin generation and in the feedback activation of FVII by FXa (18, 19), this TF increase of platelets contaminated with *C. albicans* may reflect to the clot formation in type II DM patients. Ostreud revealed the persistence of controversy of the presence, synthesis and functional activity of TF in platelets (18) **.** He explained this by the findings of Panes et al (20) that[25] O. Panes, V. Matus, C.G. Sáez, T. Quiroga, J. Pereira and D. Mezzano, Human platelets synthesize and express functional tissue factor, *Blood* 109 (12) (2007), pp. 5242–5250. Full Text via CrossRef | View Record in Scopus | Cited By in Scopus (57)(20) claims the presence of TF in quiescent platelets and their enhanced activation. Furthermore, neo-synthesis of TF by platelets was reported in resting platelets as well as activated. Mezzano and Panes also revealed that von Willebrand Factor (vWF) plus Ristocetin might induce TF activity in platelets in short time and that cell membranes needed to be intact (not lysed) in order to detect TF activity (19, 21). They said that the TF activity was independent of adding FVII and was not affected by anti-TFPI. In the present study, *C.albicans* might induce the expression of TF from the platelets on individuals with diabetes. The reason for these events in platelet cells may be contributed to the toxic microenvironment due to hyperglycemia or to intrinsic platelet abnormalities.

In general, circulating blood cells do not express TF in healthy individuals (22, 23), although very low levels of TF antigen have been detected in a small subset of CD14-positive monocytes (24, 25). Patients with type II DM have higher levels of circulating TF, as well as increased monocyte TF mRNA, which are directly modulated by both glucose and insulin, with an additive effect (9, 26, 27). Bach et al found that in blood of healthy individuals, only 1.5% of the circulating monocytes are positive for CD14 and express TF. However, after stimulating freshly isolated monocytes for 10 minutes with the Ca ionophore A23187, significant TF activity could be detected (28), particularly in cells from so-called high responders. Osterud (18) describes data generated in his own laboratory and from other groups on the ability of various blood cell types to express TF in health and disease. According to his findings, the only blood cells capable of synthesizing TF in humans are monocytes. A variety of disease conditions activate monocytes to express TF. In the present study TF activity of healthy MNs cells significantly increased 10, 20, 60, 90 and 120 minutes later than the *C.albicans* stimulation. This stimulation was not seen in the TF activity of MNs cells with diabetes. Conversely, TF activity decreased in MNs cells with diabetes after 10, 30 and 90 minutes incubation time when compared to healthy ones. This decrease continued at 20, 30, 60 and 90 minutes later than the *C.albicans* addition to the monocyte cells. As a conclusion, *C.albicans* did not stimulate the tissue factor activity of MNs cells with diabetes. We think that the reason for this unresponsiveness both in some time intervals and in *C.albicans* stimulation, is the damaged monocyte cell functions seen in type II DM. Similar to our results Vambergue et al (29) found the decrease of monocyte TF activity in patients with Type II patients compared to controls.

Interestingly, in contrast to platelet and MNs cells, PMNs cells with diabetes showed higher TF activity

compared to healthy PMNs cells without *C.albicans* stimulation. Additionally *C.albicans* stimulation increased this TF activity of PMNS cells both in healthy individuals and individuals with diabetes and at the beginning of the experiment and 10, 20, 30, 90 minutes later than the stimulation. With these findings, we focused our attention to the question which factor in platelet, MNs and PMNs cells with diabetes is responsible for the TF activity differences mentioned above. According to some studies , neither neutrophils nor eosinophils express TF but can acquire TF by binding monocyte-derived microparticles (MPs) (30). Although human neutrophils most probably do not synthesize TF, under pathophysiological conditions featuring aberrant profiles of circulating MPs, such as severe sepsis, diabetes mellitus or unstable angina, the circulating neutrophils may acquire functional TF via binding interactions with the MPs. When TF-rich MPs are exposed to neutrophils, they become instantly attached to these cells. Leukocyte-derived, TF-positive MPs may bind to various cell types, such as platelets, granulocytes and endothelial cells (30). We think that in the present study, the main reason for increased TF activity pf PMNs with diabetes might be the MPs which were transferred to PMNs cells as MPs can be transferred from one cell to another.

CONCLUSION

In vitro addition of *C.albicans* effects TF activity of blood cells according to the nature of these cells. As the blood cells are the source of active tissue factor, it is important to specify their role in diabetes based fungal infections for clarifying the advanced mechanisms between diabetes and its complications. The findings of this study will also contribute to the treatment of diabetic patients with or without candida infection in terms of tissue factor.

Candida albicans Tip II Diabetes Mellitus'ta kan hücrelerinin doku faktörü aktivitesini etkiler

ÖZET

Hemostatik mekanizmadaki bozukluklar diyabetik komplikasyonların ve kardiyovasküler olayların gelişmesinde rol oynar. *C.albicans* enfeksiyonu diyabetik hastalarda diyabetli olmayan kişilere göre daha sık oluşur. Bu çalışmada, *C.albicans'*ın sağlıklı ve diyabetli kişilerin kanlarından izole edilen polimorfonükleer lökositler (PMNs), mono nükleer lökositler (MNs) ve trombositler üzerine *invitro* etkisi doku faktörü aktivitesi açısından incelenmiştir. İzole edilen kan hücreleri canlılık testleri yapıldıktan sonra sayılmıştır. *C.albicans* süspansiyonu hücrelere eklenmiştir. Eşit hacimde fosfat tampon da *C.albicans* yerine yine hücrelere eklenmiştir. Doku faktörü aktivitesi Quick testi ile tayin edilmiştir. Hücrelerin doku faktörü aktivitesi, *C.albicans* eklenmesi ile değişmiştir. Ayrıca diyabetli kişilerden izole edilen PMNs, MNs ve trombosit hücrelerinin doku faktörü aktivitesi de anlamlı olarak değişmiştir. Bu çalışmanın bulguları candida enfeksiyonu olan veya olmayan diyabetli kişilerin tedavisine doku faktörü açısından katkıda bulunacaktır.

Anahtar Kelimeler: Doku faktörü aktivitesi, diyabet, *Candida albicans,* lökosit, trombosit

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