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Original Article

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Plasma Apelin Levels and Thiol/Disulfide Balance in Patients with Type 2 Diabetes Mellitus

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ABSTRACT

Objective: The main objective of this research is to investigate the relationship between the plasma apelin concentrations, oxidative stress biomarkers (dynamic thiol/disulfide balance), and albuminuria in patients with type 2 diabetes mellitus.
Methods: The study was carried out with 87 patients with type 2 diabetes mellitus and 24 age- and sex-matched healthy control group. Serum apelin concentrations were studied using the enzyme-linked immunosorbent assay method. Colorimetric method was used to determine native and total thiol levels. Concentrations of spot urine albumin and creatinine were measured to calculate the albumin creatinine ratio (mg/g).
Results: Serum apelin concentrations were significantly lower in patients with type 2 diabetes mellitus compared to the control group ($P < .001$). Native and total thiol ratios were also significantly lower in diabetic patients compared to healthy patients ($P < .001$). The calculated disulfide levels of the patients and control groups were similar ($P = .182$). A negative and significant correlation was detected between serum apelin concentrations, and glucose and hemoglobin A1c levels in diabetic patients ($r = -0.272$, $P = .004$, $r = -0.280$, $P = .003$, respectively). A negative and significant correlation was also observed between native and total thiol levels and albumin ($r = -0.338$, $P = .001$, $r = -0.328$, $P = .001$, respectively).
Conclusion: We found significantly lowered serum apelin concentrations and native and total thiol levels in patients with type 2 diabetes mellitus. An association was also observed between serum apelin concentrations and glycemic control. The role of apelin and thiol/disulfide balance in diabetic kidney disease requires more detailed studies.
Keywords: Diabetes mellitus, oxidative stress, dynamic thiol/disulfide balance, apelin, diabetic kidney disease, albuminuria

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INTRODUCTION

The occurrence of diabetes mellitus (DM) has been increasing over the years in many developed and developing countries. Diabetes mellitus is a chronic metabolic disease and has devastating chronic microvascular and macrovascular complications. Correlating with the increasing number of patients with DM, chronic complications of DM are also seen more frequently. A microvascular complication of DM results in diabetic kidney disease (DKD). The characterization of DKD was accomplished by monitoring morphologic and structural changes in the kidney, comprising of the enlargement of the matrix and damage of the filtration barrier on the glomerular basement membrane.[1] Clinically, the existence of albuminuria can be accepted as an early indicator of the development of DKD.[2] Currently, strict glycemic and blood pressure control, low-protein diet, lipid-lowering drugs, and suppression of the renin–angiotensin system are among the current treatment strategies for DKD.[3,4] Although the progress of DKD could be slowed down with these treatments, the effects of the disease and fatality remain very high. Therefore, most DKD patients can have the last stage of kidney disease. Therefore, a comprehensive study is

required to explain the molecular mechanisms of DKD and its progression.

Inflammation and oxidative stress have crucial roles in the origin and development of DKD. Exacerbating glucose oxidation and formation of mitochondrial reactive oxygen species (ROS) due to the occurrence of hyperglycemia result in increased oxidative stress. This problem generates DNA damage and accelerated apoptosis.[5] The primary target of ROS produced in the cell exposed to oxidative stress is thiol groups. Thiols are found in amino acids that are in the structure of proteins and contain sulfur in their composition. Thiol groups form reversible disulfide bonds after ROS exposure. In the presence of antioxidants, the disulfide bonds are reduced back to thiol groups, and thus disulfide balance is achieved.[6]

Apelin is an adipokine secreted from adipose tissue and is the endogenous ligand of the transmembrane G protein on the cell surface.[7] It has regulatory effects on glucose and lipid metabolism and insulin secretion. Apelin and its receptor APJ show pleiotropic effects. They inhibit oxidative stress and prevent cardiovascular diseases.[8]

Administration of apelin to diabetic mice resulted in a decrease of kidney and glomerular hypertrophy in addition to kidney inflammation and oxidative stress and partly reduced albuminuria in an experimental study.[9] There are very little data on serum apelin concentrations in diabetic patients and the results are conflicting.[10] The main objective of this research was to investigate the relationship between serum apelin concentrations, oxidative stress biomarkers (dynamic thiol/disulfide balance), and albuminuria in patients with type 2 DM.

METHODS

The study was carried out with 87 patients with type 2 DM and 24 age- and sex-matched healthy control group. Patients who are under 18 years, pregnant women, patients with acute and chronic infection, chronic inflammatory disease, hematological disease, advanced degree of chronic kidney disease (glomerular filtration rate < 30 mL/min/1.73 m^2), and a diagnosis of malignancy were excluded from the study. Demographic data were obtained from the hospital database system. This study was carried out with the permission of Hatay Mustafa Kemal University Ethics Committee which confirmed the study protocol (protocol number: 2019/14). Written permissions from all patients were obtained before they participated in the study.

MAIN POINTS

- Whole blood hemoglobin, serum glucose levels, and hemoglobin A1c (HbA1c) percentages were significantly high in patients with type 2 diabetes mellitus (DM).
- Serum apelin concentrations were significantly lower in type 2 DM patients.
- A significant negative relationship was observed between serum apelin concentrations and glucose and HbA1c levels.
- A significant positive correlation was found between native and total thiol levels and estimated glomerular filtration rate and hemoglobin levels.
- Native and total thiol levels were found to be low in diabetic patients.

Fasting morning blood samples were collected in biochemistry and lithium heparin tubes from the patient and control groups separately. Serum and plasma samples were centrifuged at 1500 ×*g* for 10 minutes. After that, all samples were stored at −80°C. Estimated glomerular filtration rate (eGFR) levels were calculated according to the creatinine-based Chronic Kidney Disease Epidemiology Collaboration (CKD–EPI) formula. Albumin and creatinine assessments were accomplished with the collection of first-morning urine specimen from each patient. Concentrations of albumin and creatinine were measured, and the albumin creatinine ratio (mg/g) was calculated from the spot urine albumin concentration divided by the corresponding spot urine albumin creatinine concentration. Albuminuria levels <30 mg/day are accepted as normal albuminuria, 30-300 mg/day as microalbuminuria, and >300 mg/day as macroalbuminuria.

Measurement of plasma apelin levels was assayed by the enzyme-linked immunosorbent assay (ELISA) using commercially available kits (USCN catalog number: CED066Hu, Wuhan, China). Optical density on ELISA kit plates was read with a spectrophotometer at 450 nm wavelength. A newly developed spectrophotometric method was employed to analyze native and total thiol ratios.[11] Briefly, thiol groups were generated by reducing disulfide bonds by sodium borohydride reducing agent. Formaldehyde was added to remove unreacted sodium borohydride to prevent the reduction of DTNB [5,5′-dithiobis-(2-nitrobenzoic) acid]. Reduced and native thiol functional groups were determined spectrophotometrically at 700/415 nm after the reaction with DTNB. The measurement was made on the Siemens Advia 1800 Biochemistry Device (Siemens, Germany). Disulfide levels were calculated by the following equation: disulfide levels (μmol/L) = (total thiol − native thiol)/2.

Statistics

Statistical Package for Social Sciences 22.0 statistical package program (IBM Corp.; Armonk, NY, USA) was used to analyze obtained data. Descriptive statistics for numerical variables were given as mean, frequency (%), standard deviation, and minimum and maximum. Normal distribution of the groups was determined using the Shapiro–Wilk test. Student's *t*-test was used for the quantitative comparison. Correlation analysis was achieved using Pearson's correlation test. Numeric values were tested with the chi-square test. The statistical significance level was accepted as $P < .05$.

RESULTS

Forty-six (52.7%) men and 41 (46.1%) women with type 2 DM were included in the study. The control group consisted of 13 healthy males (54.2%) and 11 (%) healthy females (45.8%). The average age was 61.83 (minimum: 27, maximum: 85) in the diabetic group and 58.13 (minimum: 45, maximum: 74) in control subjects. No important differences in age and gender were observed between groups. Table 1 summarizes the biochemical data for both groups. As expected, hemoglobin, serum glucose, and HbA1c levels were significantly high in patients with type 2 DM. In contrast, eGFR and serum albumin levels were significantly low in patients with type 2 DM. A total of 32 patients (36.8%) had normoalbuminuria, 30 patients had microalbuminuria (34.5%), and 25 patients (28.7 %) had macroalbuminuira in patients with type 2 DM. All patients had normoalbuminuria in the control group.

Serum thiol, disulfide, and apelin concentrations are presented in Table 2. Serum apelin concentrations were significantly low in patients with type 2 DM in comparison with the control subjects (P < .001). Similarly, serum native and total thiol levels were found to be significantly low in diabetic patients in contrast with the control subjects (P < .001). Similar disulfide ratios were observed between patients with type 2 DM and control subjects (P = .182).

Table 1 . Laboratory Parameters of the Patients and Control Subjects

Parameters	Patients with DM (n = 87)	Control Subjects (n = 24)	*P*
WBC (10^3µ/L)	7.97 ± 1.8	7.5 ± 1.5	.262
Hgb (g/dL)	13.08 ± 1.7	14.57 ± 1.6	**<.001**
PLT (10^3µ/L)	271.1 ± 63.3	236.7 ± 66.9	**.022**
Glucose (mg/dL)	175.1 ± 76.5	89.9 ± 9.5	**<.001**
Sodium (mmol/L)	138.8 ± 2.19	140.4 ± 2.22	**.002**
Potassium (mmol/L)	4.8 ± 0.39	4.5 ± 0.17	**.007**
Calcium (mg/dL)	9.4 ± 0.59	9.6 ± 0.45	.116
eGFR (mL/min/1.73 m^2)	71 ± 26.2	96.4 ± 8.1	**<.001**
Phosphorus (mg/dL)	3.58 ± 0.5	3.42 ± 0.46	.348
Uric acid (mg/dL)	6.07 ± 2.12	5.38 ± 1.2	.132
ALT (U/L)	24.1 ± 12.7	22.5 ± 9.97	.579
AST (U/L)	21.8 ± 10.7	21.9 ± 6.4	.947
Albumin (g/dL)	4.36 ± 0.28	4.57 ± 0.21	**<.001**
PTH (pg/dL)	76.08 ± 58.5	73.05 ± 26.7	.807
Ferritin (ng/mL)	76.01 ± 99.5	115 ± 113	.102
CRP (mg/L)	7.1 ± 9.7	5.03 ± 4.12	.317
HbA1c (%)	8.03 ± 2	5.64 ± 0.4	**<.001**

ALT, alanine aminotransaminase; AST, aspartate aminotransaminase; CRP, C-reactive protein; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; Hgb, hemoglobin; PLT, platelet; PTH, parathyroid hormone; WBC, white blood cell.
P < .05 means there is a statistically significant difference.

Table 2. Serum Thiol, Disulfide, and Apelin Concentrations

	Patients with DM (n = 87)	Control Subjects (n = 24)	*P*
Total thiol (µmol/L)	543.9 ± 108.9 Min-max:323.8-786.9	691.8 ± 125.9 Min-max: 342.7-1026.6	**<.001**
Native thiol (µmol/L)	429.4 ± 86.4 Min-max: 229.4-648.4	556.8 ± 114.8 Min-max: 225.9-871,9	**<.001**
Disulfide (µmol/L)	57.2 ± 34.5 Min-max: 13.2-204.7	67.4 ± 25.6 Min-max: 28.2-154.6	.182
Apelin (ng/L)	93.2 ± 18.6 Min-max: 33.8-126.9	116.1 ± 15 Min-max: 70.5-133.9	**<.001**

DM, diabetes mellitus; min-max, minimum-maximum.
P < .05 means there is a statistically significant difference.

A significant and negative relationship was observed between serum apelin levels and glucose and HbA1c and age in patients with type 2 DM patients. However, a positive and not statistically significant correlation was detected between serum apelin concentrations and eGFR (Table 3). A significant and negative moderate correlation was also observed between native and total thiol levels and albuminuria and age. A significant positive correlation was detected between native and total thiol levels and eGFR and hemoglobin levels (Table 3). But, no correlation was detected between serum apelin concentrations and albuminuria and total thiol and native thiol levels. Significant correlations between native and total thiol ratios and serum glucose and HbA1c levels were also

Table 3. Correlation Analysis of Laboratory Parameters in Patients with Type 2 DM

		Total Thiol (µmol/L)	Native Thiol (µmol/L)	Disulfide (µmol/L)	Apelin (ng/L)
Age (years)	*r*	**−0.295**	**−0.268**	−0.137	**−0.199**
	P	**.002**	**.004**	.150	**.037**
Hemoglobin (g/dL)	*r*	**0.450**	**0.463**	0.125	0.079
	P	**.001**	**.001**	.193	.414
Glucose (mg/dL)	*r*	−0.179	−0.159	−0.089	**−0.272**
	P	.062	.098	.354	**.004**
HbA1c (%)	*r*	−0.164	−0.187	−0.015	**−0.280**
	P	.085	.049	.873	**.003**
Albuminuria (mg/day)	*r*	**−0.328**	**−0.338**	−0.089	−0.053
	P	**.001**	**.001**	.355	.586
eGFR (mL/min/1.73 m^2)	*r*	**0.501**	**0.485**	0.188	**0.272**
	P	**.001**	**.001**	.048	**.004**

DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; HbA1c; hemoglobin A1c.
P < .05 means there is a statistically significant difference.

observed, but no correlations were detected for other laboratory parameters.

DISCUSSION

In the present study, serum apelin concentrations and native and total thiol levels were significantly lower in type 2 DM patients compared with the control subjects. A significant and negative correlation was observed between serum apelin concentrations and glucose and HbA1c levels and age in patients with type 2 DM. In contrast, a positive correlation was found between serum apelin concentrations and eGFR. However, there was no correlation between serum apelin concentrations and albuminuria, and oxidative stress parameters were observed. Moreover, a significant and negative moderate correlation was observed between native and total thiol levels and albuminuria and age. A significant positive correlation was also found between native and total thiol ratios and eGFR and hemoglobin levels in patients with type 2 DM.

Diabetes mellitus is the main reason for the end-stage kidney disease.[12] Diabetic kidney disease is one of the microvascular complications of DM, with slowly increasing albuminuria and a gradual decrease in kidney function.[13] Many patients with DM are at stage 1-3 chronic kidney disease. Unfortunately, most patients die before reaching to end-stage, mainly because of cardiovascular diseases. Many factors such as hyperglycemia, hypertension, obesity, sedentary lifestyle, genetics, smoking, and advancing age contribute to the development of DKD. Moreover, DM also reduces GFR and albuminuria which are the main risk factors for cardiovascular events and death.[14] Diabetic kidney disease is a progressive disease, and in the UK Prospective Diabetes Study, progression rates of newly diagnosed patients with type 2 DM in the stages of normoalbuminuria, microalbuminuria, macroalbuminuria, and kidney failure were found to be 2%-3% per year.[15] Therefore, the strategy of screening for early albuminuria and understanding the pathophysiology of DKD are important steps in the management of DKD.

Renin–angiotensin–aldosterone system shows a critical role in the pathophysiology of DKD. Expression of angiotensin II and angiotensin type I receptors in the kidneys of diabetic mice treated with apelin was found to be not affected. However, renal expression of APJ, which is localized in the glomeruli and blood vessels of the kidneys, decreases in diabetic mice, but its renal expression increases after apelin treatment.[9]

Oxidative stress and inflammation were found to be closely related to the development of DKD.[3,16,17] In a previous experimental study, the administration of apelin to diabetic mice reduced glomerular hypertrophy and albuminuria as well as kidney inflammation and oxidative stress.[8] In the present study, we found significantly reduced serum apelin concentrations in patients with type 2 DM, indicating the role of apelin and APJ system in the pathogenesis of DKD.

The progressive nature and pathogenesis of injury to endothelial and tubulointerstitial cells, podocytes and mesangial cells, and the role of podocyte injury in glomerular dysfunction seen in DKD is critical.[18] Due to the contribution of apelin to angiogenesis, the formation of new blood vessels and the increase in the permeability of microstructured vessels in nephrons are thought to have the greatest effect on the progression of DKD.[19] Apelin may have a role in podocyte autophagy. Podocyte count decreases in the early stages of diabetes, and the number decreases further as albuminuria increases. Podocyte count is inversely proportional to the degree of albuminuria.[20]

In the literature, there is limited data about the apelin/APJ system in the pathogenesis of kidney diseases particularly in DKD. There are some studies demonstrating that apelin levels increase in DKD and exacerbate podocyte damage.[21,22] Boucher et al showed that plasma apelin concentrations increased significantly in different obesity mouse models, while there was no increase in apelin plasma concentrations in the non-hyperinsulinemic obese mouse. Additionally, Cavallo et al[23] showed that diabetic obese patients had significantly higher apelin levels than non-diabetic obese patients. They hypothesize that increased apelin levels were directly related to the presence of diabetes rather than obesity itself. These findings suggest that insulin may affect serum apelin levels.[24] In another study conducted with type 1 DM patients, serum apelin levels were found to be higher compared to healthy controls.[25] They concluded that the relationship between serum apelin levels and body mass index may be limited. Also, they think that adiposity may not be the main determinant in some cases and different mechanisms may be involved in the regulation of apelin blood concentrations. In contrast to these previous studies, we found significantly decreased serum apelin concentrations in patients with type 2 DM. Moreover, a positive but not statistically significant correlation was observed between serum apelin concentrations and eGFR. However, no correlation was detected between serum apelin concentrations and albuminuria. In other studies conducted in our country, serum apelin concentrations in patients with type 2 DM were found to be lower than in healthy controls, similar to our study.[26,27]

The reason for the low apelin levels in type 2 DM can be explained by the regulation of insulin sensitivity, stimulation of glucose utilization, and increased adipogenesis in different tissues related to diabetes.[28] Increased serum apelin levels in type 2 DM patients may be related to glucose homeostasis.[23,29] In the same line with previous studies, a significant and negative correlation was observed between serum apelin concentrations and glucose and HbA1c levels in the present study.

Diabetes-induced hyperglycemia causes the overproduction of ROS in mitochondria in 3 ways: activation of the polyol and hexosamine pathway, increased formation of the advanced glycation end product, and expression of its receptor that contributes to ROS production by activation of protein kinase C

isoforms.[5] This oxidant state in the cell causes tissue damage. In addition, evidence from several studies indicates that enhanced oxidative stress may create the pathogenesis of DKD and development of end-stage kidney disease.[30] The oxidation of thiol groups of proteins is thought to be an early indicator of protein oxidation. If proteins are exposed to large amounts of glucose for a long time, glucose binds rapidly to non-enzymatic protein groups, and glycosylated proteins undergo autoxidation to produce free radicals. Thiol-containing proteins are known to be very important redox processes, and low redox potentials indicate the existence of high oxidative loads.[31] Sulfhydryl groups (reduced thiol, –SH) groups, both intracellular and extracellular, in free form or bound to proteins, play a crucial role in preserving the antioxidant condition of the body.[32] Thiol groups are known to be the main antioxidants in body fluids, reduce reactive free radicals, and thus protect biomolecules. It has been shown that the –SH group concentration in the serum of type 2 DM patients is significantly decreased compared with the healthy samples.[32] Possibly, increased free radical formation in DM could be the main reason for the decreased thiol levels. Since thiol functional groups are the main antioxidant ingredient of body fluids, the oxidation of thiol groups can majorly contribute to the oxidative damage of biomolecules in type 2 DM patients. In a recent study, it was shown that thiols in the urine of type 2 DM patients decreased, suggesting that this may be due to the increase in oxidation of –SH groups in the serum due to oxidative stress.[33]

It has been shown that hyperglycemia disrupted the balance in thiol/disulfide homeostasis, thiol levels decreased gradually, and contributed to the progression of DKD, and disulfide concentration was not influenced by the existence of nephropathy.[34] In this study, total and native thiol levels were found to be significantly low compared to healthy subjects. However, we could not find an association between serum apelin concentrations and total and native thiol levels. In another previous study, it has been reported that dynamic thiol/disulfide homeostasis was a decrease in the thiol levels due to hyperglycemia and chronic inflammation, resulting in an increase in disulfide levels.[35] However, the correlation of thiol/disulfide homeostasis parameters with glucose levels showed a close relationship between hyperglycemia and oxidative stress, as previously described. But, we could not show a statistically significant difference between glucose and HbA1c levels and thiol/disulfide levels. It has also been shown that low native thiol levels could be due to the high proteinuria in the group of prediabetes.[36] Similarly, native thiol showed a significant and positively high correlation with eGFR. On the other hand, native thiol showed a significant and negative correlation with albuminuria.

CONCLUSION

In conclusion, significantly lower serum apelin concentrations and native and total thiol levels were observed in patients with type 2 DM. A significant and negative correlation was detected between serum apelin concentrations and glucose and HbA1c levels and age in patients with type 2 DM. However, no association was found between serum apelin concentrations and albuminuria and total and native thiol levels. Further studies should be planned on the role of the apelin/APJ system in the pathogenesis of DKD.Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Hatay Mustafa Kemal University (Date: 2019, Number: 14).

Informed Consent: Written informed consent was obtained from all patientswho participated in this study.

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