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Determination of Urinary Enzymes as a Marker of Early Renal Damage in Diabetic Patients

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Diagnosis of diabetic nephropathy in the early stages is very important since there are no clinical signs or symptoms. Urinary N-acetyl-β-D-glucosaminidase (NAG) excretion has been recommended as a tubular dysfunction marker that elevates before other markers, such as microalbuminuria and a decrease in creatinine clearance. In this study, we compared excretion of urinary enzymes with other markers that are used routinely in diabetic nephropathy assessment. Urinary NAG, lactate dehydrogenase (LDH), alkaline phosphatase (AP) activities, urea, creatinine, and albumin, with levels of serum glucose and creatinine and whole blood glycosylated hemoglobin (HbA1c) were measured in 32 diabetes mellitus patients and 25 healthy subjects (controls). Notably, urinary NAG, AP, LDH excretion, and microalbuminuria in the diabetic patients group were significantly increased compared to those in the control groups ($P < 0.001$, $P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively). Meanwhile, our results showed that the urinary NAG excretion had the highest sensitivity and specificity (100% and 87.5%, respectively) compared to other markers. We showed that measuring urinary NAG excretion could be useful for the assessment of renal failure in diabetes mellitus patients and confirmed the use of NAG as a routine screening test. J. Clin. Lab. Anal. 21:413–417, 2007.

Key words: diabetic nephropathy; urinary enzymes; urinary NAG excretion; microalbuminuria

INTRODUCTION

Currently, in clinical diagnostic practice for renal parenchymal tubular impairment, assessment of urinary enzymes is used (1–3). One of the most important frequently evaluated urinary enzymes is N-acetyl-beta-D-glucosaminidase (NAG; EC: 3.2.1.30), which is a hydrolytic lysosomal enzyme with high molecular weight and very low physiological activity (4). It originates principally in proximal tubules and normally cannot pass through the glomerular filtration (5). NAG has been reported to be a very sensitive and reliable marker of renal failure (5,6). Therefore, estimation of this biomarker is being done in various conditions involved with renal injury or dysfunction. Some of the uses of urinary NAG include: nephritic syndrome, nephrotoxic drugs, urinary tract infection, heavy metal poisoning, kidney transplants, vesicoureteral reflux, and diabetes mellitus (6).

Diabetes mellitus is a world health problem, affecting all age groups (7). Renal damage is a serious major microvascular diabetic complication leading to the death of diabetic patients (8). Thus, diagnosis of diabetic nephropathy in an earlier stage would be critical and would help to reduce morbidity and mortality. The routine classical evaluation of diabetic nephropathy includes: appearance of microalbuminuria, decrease in creatinine clearance, and increase in serum creatinine (8).

Clinical studies have demonstrated that quantitative assessment of NAG, together with other urinary enzymes such as alkaline phosphatase (AP; EC:

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3.1.3.1), a phosphohydrolase enzyme attached to the cell wall by glycosyl phosphatidyl inositol anchors, and lactate dehydrogenase (LDH; EC: 1.1.1.27), a key enzyme in energy metabolism located in the cell cytoplasm, are more prominent even before the appearance of microalbuminuria (9–11). Therefore, in this study, we determined the urinary enzymes activities and laboratory routine markers in patients with diabetes mellitus and healthy subjects.

MATERIALS AND METHODS

Patients

A total of 32 outpatients (20 men and 12 women, aged 56.7 ± 1.7 years) who had been previously diagnosed as diabetics (World Health Organization [WHO] criteria), were randomly recruited from the Alborz hospital laboratory, Karaj, Iran. All patients in this study had no medical history record of hypertension, urinary tract diseases, ischemic heart disease, cerebrovascular disease, use of nephrotoxic drugs, smoking, renal parenchymal disease, and infections over the previous months. A total of 25 healthy subjects (15 men and 10 women, aged 55.2 ± 3.6 years) were randomly selected as a control group from those who came to the hospital for routine medical checkups without inflammatory states or illness and abnormalities in lipids and carbohydrate metabolism.

Subjects' rights have been protected by an appropriate institutional reviewed board and informed consent was granted.

Sample Collection

Urine and blood specimens were obtained from the subjects of both groups. A 12-hr urine was collected in the morning to minimize diurnal variation in protein excretion. Since AP is stable only for 4 hr after urine collection and also LDH is not stable in the refrigerator, all tests were measured within 7 hr of sample collection except NAG determination, for which urine samples were frozen and stored at -30°C for 1 month. Venous blood samples were collected after a fasting state into two test tubes. The first, without any anticoagulant, to obtain serum; and the second, containing K3 ethylene diamine tetraacetic acid (EDTA), to obtain whole blood. Urine and blood samples were centrifuged (2,000 *g* for 10 min) immediately after collection.

Chemicals

All chemicals were of the highest quality available. ρ-nitrophenol(PNP) and ρ-nitrophenyl-N-acetyl-β-D-glucosamine (PNP-NAG) were obtained from Sigma Chemical Co. (St. Louis, MO). All other required chemicals were purchased from Merck GmbH (Darmstadt, Germany).

Determination of Biochemical Parameters

Serum glucose, serum and urinary creatinine, and urinary urea, AP, LDH, and microalbuminuria were measured by using commercially available assay kits (Pars Azmun Co. Ltd, Tehran, Iran) by GOD-PAP, Jaffe, urease-glutamate dehydrogenase (GLDH), diacylglycerol kinase (DGKG), DGKG, and immunoturbidimetry methods, respectively. All of these tests were measured with the Hitachi 902 autoanalyzer (Hitachi Ltd., Tokyo, Japan).

Glycosylated hemoglobin (HbA1c) was measured in whole blood by using the high-performance liquid chromatography (HPLC) method (Model D-10; Bio-Rad Laboratories, CA). HbA1c in nondiabetic, good glycemic control, and poor glycemic control were set to be $<6\%$, $<7\%$, and $>8\%$, respectively. NAG activity in urine was measured at 37°C, according to a described method (12) with PNP-NAG as substrate and measured using a photometer (Model ECOM-E 6125; Eppendorf, Inc., Hamburg, Germany) at 405 nm.

To correct for variations in urine flow, the urinary enzymes activities (U/L) were normalized to urinary creatinine concentration (g/L) and given as U/g creatinine. Creatinine clearance (CCr; mL/min) was calculated with reference to age, sex, and serum and urine creatinine using the standard formula method. Normoalbuminuria and microalbuminuria were defined as below 30 mg/g creatinine and 30–300 mg/g creatinine, respectively.

Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). The differences between the two groups were calculated with Student's unpaired *t*-test and the nonparametric by Mann-Whitney U-tests. A P value of 0.05 denoted the presence of a statistically significant difference. Sensitivity and specificity of serum creatinine, creatinine clearance, and urinary NAG, AP, and LDH were calculated according to the method of Salem et al. (13). Briefly; the cutoff value of serum creatinine, creatinine clearance, urinary NAG, AP, and LDH excretion, and microalbuminuria were calculated using control mean+2SD.

RESULTS

Clinical and Biochemical Characteristics of Groups

No significant differences were determined in gender and age between groups. A statistically significant increase in diabetic patients was found in fasting blood glucose, %HbA1c, urinary NAG, AP, and LDH excretion, and microalbumin compared with control

TABLE 1. Clinical and biochemical characteristics of diabetic patients and control groups[†]

		Diabetic patients		
	Controls (n = 25)	All patients (n = 32)	Microalbuminuria (n = 8)	Normoalbuminuria (n = 24)
Gender (M/F)	15/10	20/12	5/3	15/8
Age (years)	55.2±3.6	56.7±1.7	53.1±2.2	58.2±1.9
Fasting blood glucose (mg/dL)	84.9±2.8	190.8±10.5[c]	–	–
Serum creatinine (mg/dL)	1.08±0.07	1.18±0.02	1.22±0.03	1.11±0.03
HbA1c (%)	5.02±0.13	8.3±0.2[c]	8.4±0.4	8.1±0.3
Urine creatinine (g/L)	0.338±0.03	0.305±0.045	–	–
Creatinine clearance (mL/min)	91.8±5	69.8±6.4[a]	58.4±4.6	70.6±7
NAG (U/g Cr)	1.6 6±0.09	5.49±0.4[c]	7.6±0.6*	4.96±0.5
AP (U/g Cr)	8.08±1.1	15.4±2.6[a]	17.6±3.7	15.1±2.8
LDH (U/g Cr)	11.02±2.6	25.4±4.4[b]	35.4±7.1	22±5.2
Microalbuminuria (mg/g Cr)	9.6±1.6	16.7±2.9[a]	41.1±5.9**	10.3±1.2

[†]All data are presented as mean±SEM. A P value of 0.05 denotes the presence of a statistically significant difference.
[a]Significant difference between diabetic patient group and control group, $P<0.05$.
[b]Significant difference between diabetic patient group and control group, $P<0.01$.
[c]Significant difference between diabetic patient group and control group, $P<0.001$.
*Significant difference between diabetic patients with microalbuminuria and diabetic patients with normoalbuminuria, $P<0.05$.
**Significant difference between diabetic patients with microalbuminuria and diabetic patients with normoalbuminuria, $P<0.001$.

groups ($P<0.001$, $P<0.001$, $P<0.001$, $P<0.05$, $P<0.01$, and $P<0.05$, respectively). Also a statistically significant decreased in diabetic patients was found in creatinine clearance compared with control groups ($P<0.05$). In the diabetic patients group with microalbuminuria a statistically significant increase was found in urinary NAG excretion and microalbumin compared with the diabetic patients group with normoalbuminuria ($P<0.05$, and $P<0.001$, respectively) (Table 1).

Biochemical Parameters in Diabetic Patients

In diabetic patients with poor metabolic control (HbA1C >8%), a statistically significant increase in urinary NAG, AP, LDH, and microalbumin were found compared with the diabetic patients with good metabolic control ($P<0.001$, $P<0.01$, $P<0.01$, and $P<0.001$, respectively). Also, a statistically significant decrease in diabetic patients with poor metabolic control was found in creatinine clearance compared with the diabetic patients with good metabolic control ($P<0.001$) (Table 2).

TABLE 2. Association between HbA1c levels and other biochemical parameters[†]

	Good glycemic control (HbA1c<7%)	Poor glycemic control (HbA1c >8%)
Fasting blood glucose (mg/dL)	181±19.1	209.8±13.3
Serum creatinine (mg/dL)	1.05±0.04	1.15±0.04
Creatinine clearance (mL/min)	95.1±21.4	59.4±3.5**
NAG (U/g Cr)	5.3±0.65	8.1±0.4**
AP (U/g Cr)	7.7±1.5	25.8±4.8*
LDH (U/g Cr)	11.5±5.6	26.2±2.6*
Microalbuminuria (mg/g Cr)	11.8±2.5	40.1±13.1**

[†]All data are presented as mean±SEM. A P value of 0.05 denotes the presence of a statistically significant difference.
*Significant difference between diabetic patients with good glycemic control and diabetic patients with poor glycemic control, $P<0.01$.
**Significant difference between diabetic patients with good glycemic control and diabetic patients with poor glycemic control, $P<0.001$.

Diagnostic Validity of Biochemical Parameters

In the diabetic patients group, sensitivity for serum creatinine, creatinine clearance, urinary NAG, AP, and LDH excretion, and microalbumin were 25%, 50%, 100%, 87.5%, 62.5%, and 25%, respectively. By comparison, in the diabetic patients group, specificity for serum creatinine, creatinine clearance, urinary NAG, AP, and LDH, and microalbumin excretion were 24.9%, 58.3%, 83.3%, 58.3%, 58.3%, and 75%, respectively. The urinary NAG excretion had the highest sensitivity and specificity (100% and 87.5%, respectively) and serum creatinine had the lowest sensitivity and specificity (25% and 24.9%, respectively) (Table 3).

DISCUSSION

According to the WHO final report, diabetes mellitus can affect more than 171 million people worldwide, and

TABLE 3. Diagnostic validity of serum creatinine, clearance creatinine, urinary NAG, AP, LDH, and microalbumin

	Cutoff value	Sensitivity (%)	Specificity (%)
Serum creatinine (mg/dL)	1.78	25	24.9
Creatinine clearance (mL/min)	41.8	50	58.3
NAG (U/g Cr)	2.56	100	83.3
AP (U/g Cr)	19.8	87.5	58.3
LDH (U/g Cr)	37.02	62.5	58.3
Microalbuminuria (mg/g Cr)	30–300	25	75

will rise to 366 million by 2030 (14). This disease is a global public health problem and its complications in most diabetic patients had no reliable therapy, therefore preventing or delaying diabetic complications is of interest to reduce morbidity and mortality (7). It is well established that several known risk factors, such as age, age of disease onset, abnormal metabolic environment, hyperglycemia, hypertension, protein overload, disease duration, and smoking are important in diabetic complications (7).

Diabetic complications include macrovascular and microvascular disorders. Macrovascular disorders are coronary artery disease, cerebrovascular disease, and peripheral vascular disease; and microvascular disorders are nephropathy, retinopathy, and neuropathy (8,15). Renal damage is an important and serious diabetic microvascular complication, and is the leading cause of end-stage renal disease (16). Consequently, early diagnosis of nephropathy is very critical (17). In clinical trials, decrease in creatinine clearance, increase in serum creatinine, and especially appearances of microalbuminuria are keys for diagnosis and treatment of diabetic nephropathy (8,18). But these markers are insensitive, unreliable, nonspecific, and there is a time delay between renal injury and detection (19). Thus, if other biomarkers are found with improved specificity and sensitivity, this could reverse or prevent the onset of renal damage.

For several years, many studies, including in vitro and in vivo, have demonstrated that excreted urinary enzymes are useful biomarkers for evaluation and diagnosis of tubular dysfunction or injury, especially NAG (9–11,20–23). Hence, tubular damage most likely precedes glomerular damage so urinary enzyme excretion can be used (9).

Our study showed that urinary NAG, AP, and LDH were excreted higher in diabetic patients compared with control groups ($P<0.001$, $P<0.05$, and $P<0.01$, respectively). This data agrees with Uslu et al. (9), which demonstrated an increase in urinary NAG, AP, LDH, and serum cystatin C in diabetic patients. Increase in NAG excretion has been reported by several authors in diabetic patients (11,13,24).

Moreover, in our diabetic patients with microalbuminuria, urinary NAG excretion increased compared to diabetic patients with normoalbuminuria ($P<0.001$). On the other hand, all of the urinary excretions were elevated in diabetic patients with poor metabolic control (HbA1c $>8\%$), compared to diabetic patients with good metabolic control (see Table 2). Hyperglycemia shown before by others leads to enhanced reactive oxygen species production and as a result tubular cell damage and abnormal urinary enzyme excretion develops (22). Also, hyperglycemia is a risk factor for diabetic nephropathy and in patients with overt nephropathy, the mean level of HbA1c is correlated with the loss of renal function (13). Several studies have shown that good metabolic control is beneficial in slowing the progression of nephropathy in diabetic patients (13,25–27).

In our study, urinary NAG excretion showed the highest sensitivity and specificity (100% and 87.5%, respectively) as compared to sensitivity and specificity of serum creatinine, creatinine clearance, and microalbuminuria (25% and 24.9%, 50% and 58.3%, and 25% and 75%, respectively). Our data agree with other studies (9,13).

In conclusion, this study suggests that urinary NAG excretion with other urinary enzymes in diabetic patients are a proper biomarker for screening of diabetic renal dysfunction.

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