

Urea and Oxidative Stress in Type 2 Diabetes

Afsaneh Morteza, Yaser Jenab, Arash Aghajani Nargesi, Zaniar Ghazizadeh, Mirataollah Salabati, Alireza Esteghamati and Manouchehr Nakhjavani*

Endocrinology and Metabolism Research Center (EMRC), Vali-Asr Hospital, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Introduction : Studies have shown altered protein metabolism in the presence of excess carbohydrate such as type 2 diabetes (T2DM). Protein metabolism is impaired in T2DM as a result of oxidative stress and insulin resistance. Here we aimed to study the correlation of serum urea as an indicator of protein metabolism with malondialdehyde (MDA) and superoxide dismutase (SOD), as indicators of oxidative stress in T2DM patients.

Methods : We performed a cross sectional study on 151 patients with T2DM and 45 healthy controls. We quantified fasting blood sugar (FBS), HbA1C, lipid profile, urea, MDA and SOD in the studied groups.

Results : Patients had a higher serum urea, FBS, HbA1C, triglyceride, cholesterol, LDL, MDA and SOD than controls. GFR and serum HDL levels were lower in patients. Diabetic men had a lower HDL and a higher albuminuria compared to diabetic women. There were no difference in any of the studied variables between men and women in control group. Serum urea levels were negatively correlated with MDA ($r = -0.70$, $p < 0.01$) and SOD ($r = -0.60$, $p < 0.01$) in men with type 2 diabetes. This was significant after multiple adjustments for HbA1C, GFR, albuminuria and duration of diabetes.

Discussion : We showed the negative correlation of serum urea levels with the markers of oxidative stress in T2DM men. It could be concluded that protein metabolism and urea formation is more severely influenced in diabetic men. This explains the negative correlation of urea with MDA and SOD, only in men with T2DM.

Keywords: MDA; SOD; Urea; Type 2 diabetes; Oxidative stress; Gender difference.

Introduction

In about 2000 years ago, Aretaeus the capudocian, the Greek physician, described diabetes as a condition with a "melting down of the flesh and limb into urine" [1]. With reference to type 1 diabetes, this is remarkably amazingly accurate observation. In type 2 diabetes reports have been inconsistent, that protein metabolism has been reported to be both unaffected and affected [2,3]. In 1993, "insulin resistance of protein metabolism", was firstly introduced in patients with type 2 diabetes [4,5]. It is defined as the defect in amino acid metabolism and suppression of protein breakdown which is correlated with insulin resistance [6]. Since then many studies have shown a negative nitrogen balance and loss of nitrogen from most organs in patients with type 2 diabetes [7,8]. Protein malnutrition is associated with an increased level of oxidative stress [9]. Protein restriction in rats with type 2 diabetes, cause an accelerated oxidative stress [10,11]. Children with kwashiorkor have a higher level of lipid preoxidation [12-14]. Consistently protein malnutrition in intra uterine growth retardation pregnancies is the leading cause of oxidative stress in these patients [15,16]. Insulin resistance of protein metabolism could be impaired as one of the causes of protein malnutrition [17]. It often precedes the onset of type 2 diabetes by many years. Studies have shown the role of insulin resistance in the induction of reactive oxygen species (ROS) and oxidative stress in type 2 diabetes [17-19]. Oxidative stress is a disturbance in the pro-oxidant, anti-oxidant balance in favor of the former, which leads to the potential damage [20]. Superoxide dismutases (SOD) are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. SOD levels are increased in the situations of oxidative stress [21]. Malondialdehyde (MDA) is the organic compound considered as a reactive species which occurs naturally and is a marker for oxidative stress [22]. Superoxide results into macromolecules alteration such as polyunsaturated fatty acids in membrane lipids. This results into the generation of MDA [22]. Both MDA and SOD are increased in patients with type 2 diabetes, due to increased oxidative stress [23].

Here we aimed to study the correlation of serum urea as an indicator

of protein metabolism with MDA and SOD as markers of oxidative stress in patients with type 2 diabetes.

Methods

We performed a cross sectional study on 151 patients with type 2 diabetes from the diabetes clinic of Vali Asr hospital affiliated with Tehran University of Medical Science plus 45 controls. Diabetes was diagnosed according to the criteria of the American Diabetes Association which is based on glycemia [24]. Exclusion criteria were smoking, pregnancy, creatinine > 1.5 mg/dl or GFR < 70 cc/min, glomerulonephritis, thyroid disorders, acute infections, stroke, diabetic ketoacidosis, non-ketonic hyperosmolar diabetes, congestive heart failure, use of antioxidant and hospital admission in recent 6 months. None of the participants were on hormone replacement therapy. Controls were healthy volunteers from the patients' concomitants or hospital staffs. Healthy controls were selected from those without any known disease including type 2 diabetes, hyperlipidemia, ischemic heart disease and malignancy. Demographic and anthropometric data including age, sex, duration of diabetes, height and weight in light clothing and blood pressure in sitting position were recorded. Blood pressure was re measured twice after 5 minutes average. The body mass index (BMI; Kg/m²) was calculated according to the Quetelet formula.

Diet was almost similar in composition in all the studied groups.

Corresponding author: Manouchehr Nakhjavani, MD, Professor of Endocrinology, Endocrinology and Metabolism Research Center (EMRC), Vali-Asr Hospital, Tehran University of Medical Sciences, Tehran, P.O. Box: 13145-784, Iran, Tel: (+9821)-8841791; Fax: (+9821)-64432466; E-mail: nakhjavanim@tums.ac.ir

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Patients and controls were instructed to consume standardized meals that contained 50% carbohydrates, 30% fat and 20% protein, for two weeks before the beginning of the study. All the patients were consulted by a nutritionist during the study. The glomerular filtration rate (GFR) was calculated using the Cockcroft-Gault formula [25]. All participants gave written informed consent before participation. The research was carried out according to the principles of the declaration of Helsinki; the local ethics review committee of Tehran University of Medical Science approved the study protocol.

Blood Samples

Blood samples were collected after 12 hours of fasting were centrifuged and kept at -70° C until analysis. Glucose measurements (intra-assay coefficient of variants [CV] 2.1%, inter-assay CV 2.6%) were carried out using the glucose oxidase method. Cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and triglycerides were determined using direct enzymatic methods (Parsazmun, Karaj, Iran). Urea was measured using colorimetric assay (Parsazmoon, Karaj, Iran). Creatinine was measured using calibrated Jaffe method (Parsazmoon, Karaj, Iran). Patients were instructed in the collection of timed 24-hour urine for measurement of urinary albumin excretion and were told to return on the morning after the end of the urine collection. Women were not examined during menstruation. All specimens were confirmed to be sterile by culture. Urinary albumin was measured by immunoturbidometry (DAKO, Denmark) in duplicate and the average of the 2 measurements was used for the analysis. Serum EC-SOD was assayed using a 2-step ELISA with a monoclonal antibody using the Cayman Chemical SOD assay kit (Cayman Chemicals, Ann Arbor, MI, USA). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Serum MDA levels were measured using the colorimetric method. After reaction of thiobarbituric acid with MDA, the reaction product was extracted in butanol. Separation of the organic phase was facilitated by centrifugation at 3,000 rpm for 10 minutes and its absorbance was determined spectrometrically at 530 nm (Cayman Chemicals, Ann Arbor, MI, USA).

	Patients with type 2 diabetes (n=151)	Healthy controls (n=45)	P value
Age (yrs)	58.9±0.7	59.0±1.0	NS
Female (n, %)	80 (52%)	40 (44%)	NS
BMI(kg/m2)	27.04±0.4	27.2±0.4	NS
Systolic blood pressure (mmHg)	143.5±6.7	125.2±1.5	NS
Diastolic blood pressure (mmHg)	84.3±0.8	78.4±1.2	<0.001
Urea (mg/dl)	50.2±1.9	27.5±1.3	<0.001
Fasting Blood Sugar (mg/dl)	173.7±5.0	88.6±1.0	<0.001
HbA1C (%)	8.8±0.2	4.8±0.1	<0.001
Triglyceride (mg/dl)	191.8±6.4	105.7±5.5	<0.001
Cholesterol (mg/dl)	194.4±4.1	205.4±3.4	<0.001
LDL-C (mg/dl)	88.8±2.2	113.2±1.7	<0.001
HDL-C (mg/dl)	31.7±0.5	52.0±1.6	<0.001
MDA (µmol/L)	3.67±0.09	2.47±0.13	<0.001
SOD (U/ml)	84.1±1.6	70.4±0.9	<0.001
GFR (ml/min)	73.0±1.9	89.2±2.7	<0.001
Log-albuminuria (mg/day)	2.006±0.079	-	-

Variables are expressed as mean ± standard error of mean (SEM). The column of "P value" is presented when comparing patients with type 2 diabetes and controls.

Table 1: The primary characteristics of patients with type 2 diabetes and controls.

	Patients with type 2 diabetes		Healthy Controls	
	Men (n=71)	Women (n=80)	Men (n=20)	Women (n=25)
Age (yrs)	60.4±1.04	59.4±0.8	59.6±1.72	58.5±1.25
Urea (mg/dl)	50.3±2.3	50.1±2.8	30.2±2.7	26.7±1.4
HbA1C (%)	8.9±0.2	8.7±0.1	4.8±0.1	4.9±0.1
Fasting Blood Sugar (mg/dl)	174.2±6.6	173.2±7.4	89.2±1.8	88.2±1.2
Triglyceride (mg/dl)	188.3±8.0	194.8±9.7	101.5±7.3	109.0±8.1
Cholesterol (mg/dl)	195.1±6.0	193.7±5.5	204.3±4.5	206.3±4.9
LDL-C (mg/dl)	89.9±3.1	87.8±3.2	117.3±1.7	109.9±2.6
HDL-C (mg/dl)	30.7±0.6*	32.7±0.7	49.0±2.1	54.3±2.3
MDA (µmol/L)	3.8±0.09	3.6±0.12	2.52±0.19	2.48±0.17
SOD (U/ml)	88.7±2.2	79.9±3.9	71.3±1.9	69.5±2.01
GFR (ml/min)	75.8±2.4	70.6±2.6	93.3±4.4	85.9±3.4
Log-albuminuria (mg/day)	2.24 ±0.109**	1.87±0.109	-	-

Variables are expressed as mean ± standard error of mean (SEM). *p<0.05; **p<0.01; ***p<0.001, when comparing men and women in diabetes and control groups

Table 2: Characteristics of the patients in the studied groups stratified according to gender.

Statistical Analysis

The statistical package SPSS 17 for windows (Chicago, Illinois, USA), was used for analysis. Variables distributed normally are presented as mean and standard error of mean (SEM). Amount of albuminuria was log-transformed and employed for further analysis. Independent sample test was used to compare variables between patients and controls as well as between men and women. Pearson's correlation test was employed to test the correlation of serum urea with other studied variables. Partial correlation was employed to test the correlation of urea with MDA and SOD after multiple adjustments for GFR, HbA1C, albuminuria and duration of diabetes.

Results

Characteristics of the patients and controls are presented in Table 1. The frequency of insulin therapy was (30/151; 20%) and the frequency of statin therapy was (70/151; 46%) in patients with type 2 diabetes. Patients had a higher serum urea, FBS, HbA1C, triglyceride, cholesterol, LDL, SOD, MDA and a lower HDL than controls (Table 1). We then stratified the studied groups according to gender. Diabetic men had a lower HDL and a higher albuminuria compared to diabetic women. There were no difference in any of the studied variables between men and women in control group (Table 2).

Serum urea levels were negatively correlated with MDA (r= -0.70, p<0.01) and SOD (r= -0.60, p<0.01) in men with type 2 diabetes (Figure 1), when there was not such a significant correlation among women and controls (Table 3). This was significant after multiple adjustments for HbA1C, GFR, albuminuria and duration of diabetes (MDA: r= -0.72, p<0.001; SOD: r= -0.59; p<0.001) using partial correlation. Serum urea levels were not correlated with any of the studied variables in men and women in the controls group (Table 3).

Discussion

Oxidative stress is the hallmark of type 2 diabetes. It precedes diabetes years before the diagnosis of diabetes and is also the culprit mechanism of disease such as metabolic syndrome or insulin resistance. Urea is one of the main indicators of protein metabolism and nitrogen balance in humans [26], which is recently found to have

some association with the markers of oxidative stress [27,28]. We also performed another study and consistent with the current findings, showed that urea is negatively correlated with the makers of metabolic syndrome such as LCAT enzyme (unpublished paper). This is the first report demonstrating the negative correlation of serum urea levels with markers of oxidative stress including MDA and SOD in men with type 2 diabetes. This was significant after multiple adjustments for HbA1c, GFR, albuminuria and duration of diabetes.

Mitochondria, described as a cellular power plants, are the site of reaction of oxidative phosphorylation in electron transport chain, which results in the formation of ATP [29,30]. In the situation of hyperglycemia the voltage across the mitochondrial membrane increases above the critical threshold for superoxide production. This will induce the production of reactive oxygen species [31]. Urea genesis is a biochemical cycle in the liver initiated in the mitochondria and completed in the cytosole [32]. The effect of mitochondrial hyperactivity with glucose oxidation on hepatic urea genesis has not been examined. Amino acids ingested from the foods which are not used for the synthesis of proteins are oxidized [33]. The oxidation pathway starts with transamination, the removal of the amino group by a transaminase [33]. All amino nitrogen from amino acid that undergoes transamination, are concentrated in L-Glutamate [32,33]. This is important because L-Glutamate is the only amino acid that undergoes oxidative deamination (release of ammonia) [33]. Release of ammonia from glutamate is then catalyzed by the hepatic L-Glutamate dehydrogenase (GDH), which is located in the mitochondria of the liver tissue [33]. GDH is allosterically inhibited by ATP, GTP and NADH and is activated by ADP [32,34]. Under the situation of caloric restriction and low blood glucose, the activity of GDH is raised in order to increase the amount of energy [33,35]. In diabetic cells with high glucose inside, there is more glucose being oxidized which in effect pushes more

electron donors into electron transport chain [36]. This results into the production of free radical hydrogen peroxide, superoxide and a decreased NAD/NADH ratio [36]. Under normal conditions, ROS are cleared from the cell by the action of SOD, catalase, or glutathione peroxidase. The main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, which results into the generation of MDA. Moreover SOD increases to protect cells against oxidative damage. Urea genesis decreases in hyperglycemia. This may explain the negative correlation between serum urea and markers of oxidative stress such as MDA, SOD in patients with type 2 diabetes (Figure 2).

In consistent with our findings, Dasarathy and collaborators showed that intralipid infusion in patients with non alcoholic steato hepatitis (NASH) increases plasma glutathione levels. Because plasma glutathione is mostly derived from the liver, this increase is a result of elevated oxidative stress caused by higher fatty acid oxidation in subjects with NASH. Likewise, intralipid infusion decreased urea genesis in these patients. They concluded that increased level of hepatic β -oxidation is associated with a decreased level of ureagenesis [37]. Interestingly Anderson et al, showed that the mitochondria in the arterial tissue of type 2 diabetes show a sharply decreased capacity of glutamate metabolism compared to non diabetic subjects [38]. It is shown that there is a reduced mitochondrial protein metabolism in patients with type 2 diabetes [39,40].

Why the negative correlation between serum urea and markers of oxidative stress is only observed in men with type 2 diabetes? We do not have a definite answer to this question, but previous studies have postulated a number of theories. Chevalier et al. showed sex difference on protein anabolic actions of insulin which is significantly greater in men [41]. Consistently Gougeon et al. showed that the kinetics of whole body proteins is elevated in hyperglycemic men. There is an insulin resistance protein metabolism in diabetic men, when this would not happen in diabetic women [2,42]. Pereira et al. [43] reported the results from an extensive study which defined whole-body protein metabolism in patients with type 2 diabetes. The study was designed to evaluate the basal, postabsorptive and insulin-stimulated amino acid and glucose metabolism, using infusion of labeled leucine in patients with type 2 diabetes and healthy controls. They showed that despite a similar basal whole-body leucine fluxes between patients and controls, total leucine

		SOD (U/ml)	MDA (μ mol/L)
Urea (mg/dl)	Women with type 2 diabetes	-0.02	-0.01
	Men with type 2 diabetes	-0.60***	-0.70***
	Women in control Group	-0.03	-0.37
	Men in control group	0.23	-0.05

*p<0.05; ** p<0.01

Table 3: Presenting the partial correlation coefficient (r) of serum urea with MDA and SOD in diabetic men, diabetic women, men and women in control group.

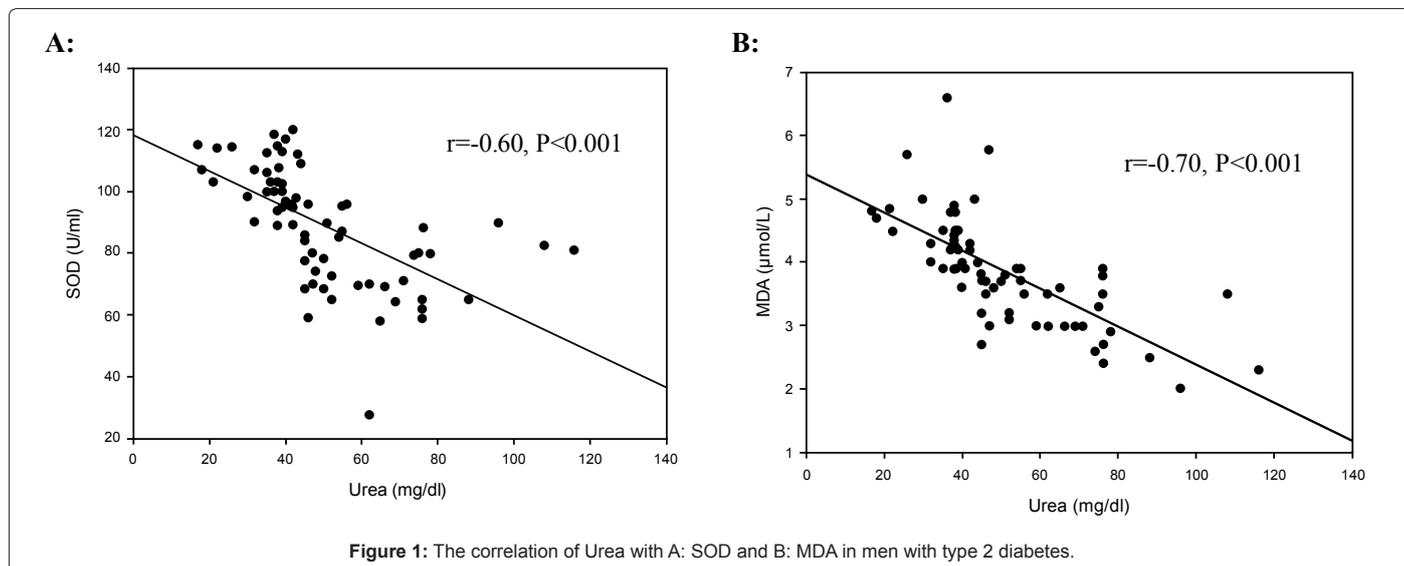


Figure 1: The correlation of Urea with A: SOD and B: MDA in men with type 2 diabetes.

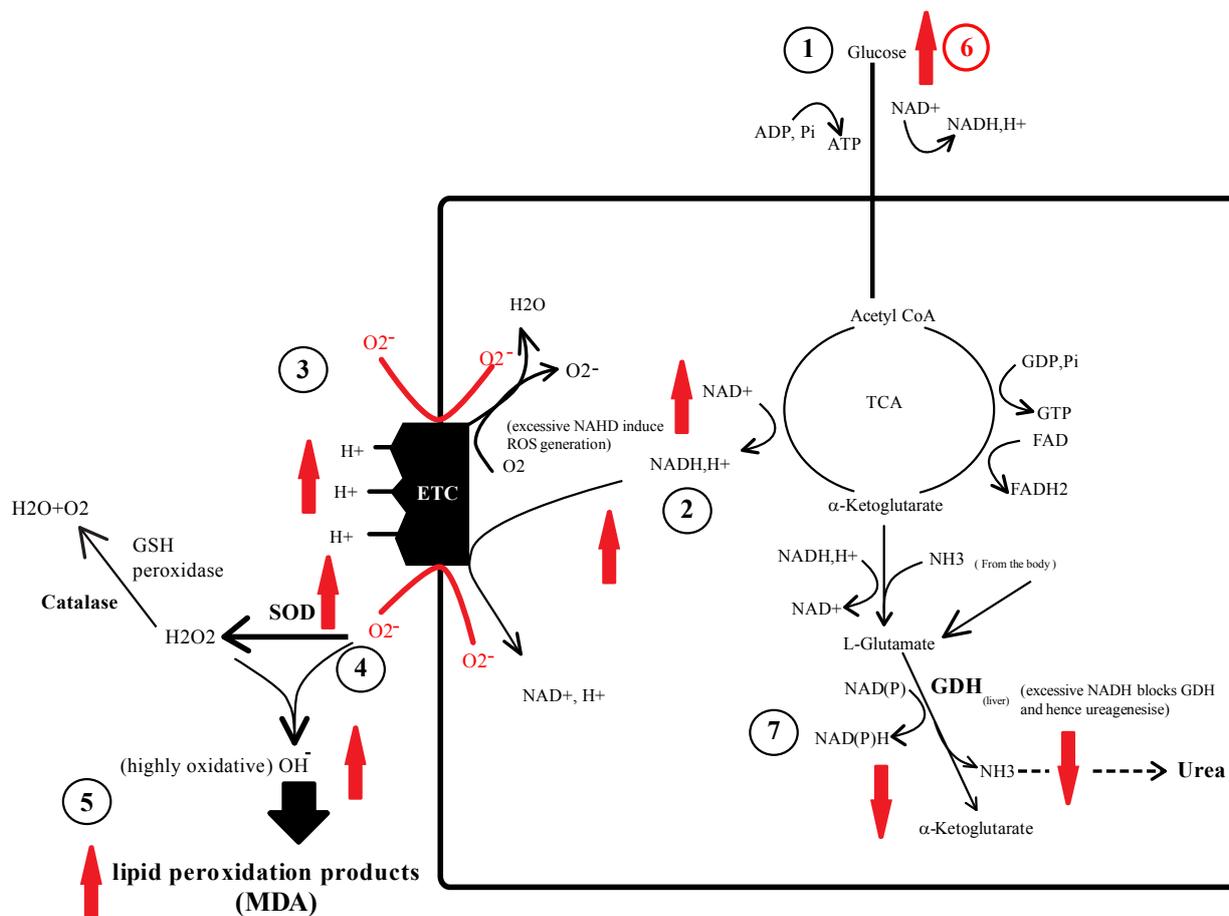


Figure 2: Effect of hyperglycemia on the production of reactive oxygen species and hepatic urea genesis.

1: Greater glucose oxidation and increased glucose uptake results into increased NADH and decreased NAD: NADH ratio. 2: Increased NADH results into the hyperactivity of electron transport chain and consequently 3: the generation of superoxide (O₂⁻). 4: Superoxide dismutases (SOD) catalyze the dismutation of superoxide (O₂⁻) into hydrogen peroxide. 5: abundant level of superoxide and hydrogen peroxide results into the generation of reactive oxygen species (ROS). Under normal conditions, ROS are cleared from the cell by the action of SOD, catalase, or glutathione peroxidase. 6: In hyperglycemia there is abundant level of NADH and ROS. The main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids. This results into the generation of malondialdehyde (MDA). Moreover SOD increased to protect cells against oxidative damage. Urea genesis decreases in hyperglycemia. 7: On the other hand release of ammonia from glutamate is catalyzed by the hepatic glutamate dehydrogenase (GDH), which is inhibited by NADH, ATP and GTP. Urea genesis results into NAD(P) reduction This may explain the negative correlation between serum urea with markers of oxidative stress such as MDA and SOD in patients with type 2 diabetes.

TCA: Tricarboxylic acid cycle; ROS: reactive oxygen species; ETC: electron transport chain; GSH: glutathione; MDA: malondialdehyde; SOD: superoxide dismutase; GDH: glutamate dehydrogenase.

flux and protein synthesis increased less in type 2 diabetic men, during the hyperinsulinemic, glucose and amino acid clamp. They concluded that hyperglycemic men have insulin resistance of protein metabolism [43]. Theoretically it could be concluded that protein metabolism and urea formation is more influenced in diabetic men. Likewise insulin resistance increase markers of oxidative stress. This may partially explain the correlation between serum urea with MDA and SOD, only in men with type 2 diabetes. Future prospective studies, using larger trials investigating male/female differences or a trial to ascertain the impact of statin treatment upon these inflammatory markers would help to bolster the potential causation role of these inflammatory markers as culprits in the atherosclerotic process.

The principal limitation of the present study is its cross sectional nature. We did not measure the nitrogen balance; however diet was

almost similar in composition in all the studied groups. Patients and controls were instructed to consume standardized meals that contained 50% carbohydrates, 30% fat and 20% protein, for two weeks before the beginning of the study. All the patients were consulted by a nutritionist during the study. On the other hand we took advantage of a relatively large sample size and close similarity between groups in most of the potentially confounding variables. In conclusion we showed the negative correlation between markers of oxidative stress and serum urea levels in men with type 2 diabetes. Though we cannot show the direction of causality, this finding paves the way for important future works aimed at determining the role of protein metabolism on markers of oxidative stress in patients with type 2 diabetes.

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