EGYPTIAN ACADEMIC JOURNAL OF
BIOLOGICAL SCIENCES
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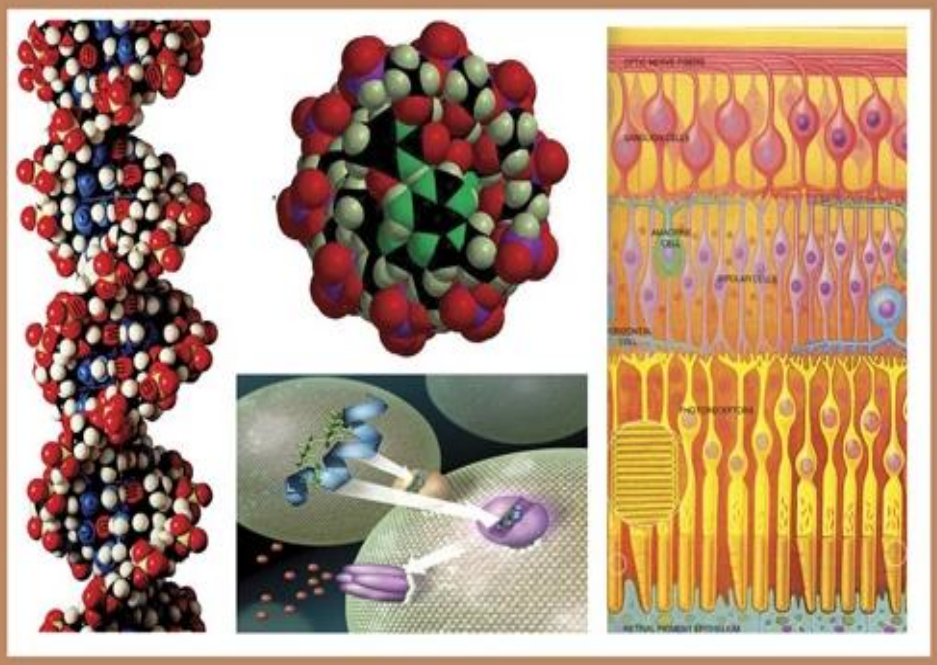
PHYSIOLOGY & MOLECULARBIOLOGY



ISSN
2090-0767

WWW.EAJBS.EG.NET

Vol. 14 No. 2 (2022)

Citation: *Egypt.Acad.J.Biolog.Sci. (C.Physiology and Molecular biology) Vol. 14(2) pp471-479 (2022)*
DOI: 10.21608/EAJBSC.2022.281806

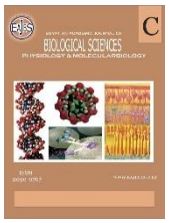
Egypt. Acad. J. Biolog. Sci., 14(2):471-479 (2022)

Egyptian Academic Journal of Biological Sciences

C. Physiology & Molecular Biology

ISSN 2090-0767

www.eajbsc.journals.ekb.eg



Indicators of Oxidative Stress and Antioxidant Defense in Diabetic Nephropathy

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ARTICLE INFO

Article History

Received:3/9/2022

Accepted:14/12/2022

Available:19/12/2022

Keywords:

Diabetic nephropathy; Oxidative stress; Free-cholesterol-LDL-TBARS; Antioxidant defense.

ABSTRACT

Diabetes mellitus is a chronic metabolic disorder, which can cause damage to multiple organs; one of the long-term effects of diabetes is diabetic nephropathy; oxidative stress is thought to play a role in the development of diabetic nephropathy, as it can induce an inflammatory response and damage cells by disrupting their redox homeostasis; The study's goal was to assess the levels of oxidative damage and antioxidant protection in diabetic nephropathy patients. During routine medical examinations, 105 patients and 101 controls were selected; in serum, the reaction of thiobarbituric acid with malondialdehyde can be quantified by measuring the intensity of a pink color produced at 95°C. The same method is used to assess the degree of oxidation of free-cholesterol-LDL; total plasma antioxidant status was determined by a colorimetric enzymatic peroxidase method. Erythrocyte superoxide dismutase activity was determined by xanthine oxidase, catalase and glutathione peroxidase activities were measured by a colorimetric method; ascorbic acid was measured in an acidic solution using a colorimetric technique at a wavelength of 465 nanometers. Retinol and tocopherol were isolated from serum by liquid-liquid extraction and immediately injected into a high-performance liquid chromatography system. The results show that patients had 1.77 times higher serum TBARS, 2.77 times higher free-cholesterol-LDL-TBARS, 1.43 times lower total antioxidant status, 1.30 times lower superoxide dismutase activity, 1.19 times lower catalase activity, 1.28 times lower glutathione peroxidase activity, 1.83 times lower α-tocopherol, 1.33 times lower ascorbic acid, and 1. 15 times lower retinol than controls; the results of the present research also demonstrate that significant differences were observed between patients and controls in blood urea (p=.013), serum creatinine (p=.007), uric acid (p=.015), albumin (p=.018), and glomerular filtration rate (GFR) (p=.006). In diabetic nephropathy, there is a negative correlation between glomerular filtration rate and oxidant status parameters, while there is a positive correlation with antioxidant status indicators.

INTRODUCTION

At present, the prevalence of diabetes mellitus is escalating at an alarming rate, becoming a worldwide epidemic; according to the International Diabetes Association, approximately 483 million adults have been diagnosed with diabetes, and estimations suggest that this number could nearly double by 2035 (Fekadu *et al*., 2019). Algeria is one of the countries with a high prevalence of diabetes; recent estimates indicate that approximately 4.5 million people in Algeria are living with diabetes (WHO, 2016). Research has demonstrated that diabetes mellitus can be associated with serious and potentially fatal chronic vascular complications, leading to early disability and mortality in affected individuals (Dal Canto *et al*., 2019).

Diabetic nephropathy is a common complication of diabetes, with an estimated prevalence ranging from 25 to 40% of individuals with diabetes (García-Martín *et al*., 2020). In diabetic nephropathy, a variety of structural and functional changes occur in the renal tissues, including albuminuria, glomerular scarring, tubulointerstitial fibrosis, and a progressive decline in renal functional activity that can result in end-stage renal failure (Wagnew *et al*., 2018).

It is well known that mitochondrial dysfunction plays a role in the onset and progression of diabetic nephropathy (Wei & Szeto, 2019). Mitochondria are known to be the major sources of reactive oxygen species and can lead to oxidative stress responses (Mailloux, 2020). Currently, the primary molecular mechanisms associated with oxidative stress in diabetic mellitus have been identified and are related to glucose and lipid metabolism; reactive oxygen species can interact with cellular components such as lipids, proteins, and DNA, leading to their modification. (Su *et al*., 2020). There is currently little evidence regarding the activity of oxidative stress responses in diabetic nephropathy subjects based on their stage of chronic kidney disease. The aim of the study was to evaluate the levels of oxidative damage and antioxidant protection in patients with diabetic nephropathy.

MATERIALS AND METHODS

After obtaining authorization from the institution's board of directors, patients aged between 18 and 60 years of both genders, who had diabetes mellitus and showed up at the outpatient internal medicine clinics of the Meslem Tayeb and Abdellah Ali Boukeroucha Hospital (Mascara, Algeria) between March 2020 and June 2022, were enrolled in this study. The type 2 diabetes mellitus was determined according to the criteria set by the World Health Organization (WHO, 2016): a fasting glycemia level of ≥ 7.00 mmol/L, an HbA1c level of ≥ 6.5%, and a glomerular filtration rate (GFR) of ≥ 60 ml/min; additionally, healthy subjects who came for a routine checkup were also included in the study as the control group. People with malignant conditions, as well as those taking thiazides, furosemide, acetylsalicylic acid, or lipid-lowering drugs, were excluded from the study.

The patients' height, age, waist circumference, weight, and systolic and diastolic blood pressure were recorded from their medical records. Body mass index (BMI) was calculated by dividing weight (in kilograms) by the square of height (in meters). Blood pressure was measured as the arithmetic mean of blood pressure readings from consecutive clinic visits for both arms.

Levels of fasting glucose (FG), blood urea, serum creatinine, hemoglobin A1c (HbA1c), albumin, and serum uric acid were obtained from the institutional database and reported. Glomerular filtration rate (GFR) was calculated using an abbreviated version of the Modification of Diet in Renal Disease (MDRD) equation: GFR (ml/min/1.73 m^2) = 186.3 × (serum creatinine)-1.154 × $(age)^{-0.203}$ (×0.742 in women) (Levey *et al*., 2003).

At a temperature of 95°C, the reaction of thiobarbituric acid (TBARS) with malondialdehyde can be quantified by

measuring the hue of the pink coloration produced at a wavelength of 532 nanometers. Concentrations of TBARS in serum can be determined using a calibration curve formed by the thermal conversion of 1,1,3-tetraethoxypropane (Djordjevic *et al.*, 2018). Selective precipitation of low-density lipoprotein (LDL) by amphiphilic polymers in the presence of Calcium (Ca^{2+}) has been successfully achieved, followed by redissolution of the precipitate in an alkaline medium. To evaluate the degree of oxidation of free-cholesterol-LDL, the same method used for serum was applied (Thant *et al*., 2018).

Total plasma antioxidant status (Sat) was determined by a colorimetric enzymatic peroxidase method (Randox, Antrim, UK). Erythrocyte superoxide dismutase (SOD) activity was determined by a colorimetric enzymatic method using xanthine oxidase (Ransod, Randox, Antrim, UK), and catalase (Cat) and glutathione peroxidase (GPX) activities were measured by a colorimetric method (Ransel, Randox, Antrim, UK).

The concentration of α-tocopherol in the plasma sample was determined using high-performance liquid chromatography (HPLC) on the Kontron system after hexane extraction. The chromatographic conditions included the use of a 3.9 × 150 mm symmetryTM C18 column (Waters, Milford, MA, USA) with an injection volume of 20 μL, a mobile phase of 100% methanol, and a flow rate of 0.9 ml/min, resulting in a retention time of 25 minutes. The concentration of ascorbic acid was quantitatively determined using a colorimetric method at a wavelength of 465 nanometers in an acid solution after the plasma sample had been stabilized with metaphosphoric acid. After performing adequate sample preparation, Vitamins A were extracted from serum using a liquid-liquid extraction (LLE) technique. The samples were then directly injected into a High-Performance Liquid Chromatography (HPLC) system equipped with a diode-array detector (DAD) for chromatographic separation. The entire process was completed within seven minutes.

Statistical Analysis:

Data were analyzed by SPSS software (SPSS 15.0; IBM Inc., Chicago, IL, USA). The distribution of the variables in study groups was conducted by Kolmogorov–Smirnov test. Homogenously distributed variables were expressed as mean ±standard deviation and compared with oneway ANOVA test. Non-homogenously distributed variables were expressed as median (minimum-maximum) and compared with Kruskal–Wallis test. Independent Student's t-test was used for comparing mean values between the two groups (patients *vs* controls). A p-value lower than 0.05 was considered statistically significant with a 95% confidence interval (95% CI). A Pearson's analysis was used to find out the correlation between FPG, HbA1c, fasting glycaemia, serum TBARS, free-cholesterol-LDL-TBARS, TAS, SOD, GPx, α-tocopherol, ascorbic acid.

RESULTS

Following the exclusion of subjects who did not meet the inclusion criteria, a total of 105 subjects with diabetic nephropathy were recruited into the study. The average age of the patients and controls was groups 55 ± 3.16 and 51 ± 5.35 years, respectively (p=.958).

Statistically significant differences were observed between the two study groups in terms of systolic blood pressure (p=.036), waist circumference (p=.004), body weight (p=.005), body mass index (BMI) (p=.019), fasting blood glucose (p=.003), glycated hemoglobin (HbA1c) (p=.001), blood urea (p=.013), serum creatinine (p=.007), uric acid (p=.015), albumin (p=.018), and glomerular filtration rate (GFR) (p<.006). The general characteristics and laboratory data of the study groups are provided in Tables 1 and 2.

Table 1. Medical history of the study population.

Characteristics	DN (n = 105)	Controls (n = 101)	*P-value
	X ± SD Median (Min-Max)		
Age (years)	55 ± 3.16	51 ± 5.35	0.958
Gender ratio (Men/Women)	52 / 53	50 / 51	0.002
Fasting glycaemia (mmol/L)	1.95 (0.79-5.08)	0.91 (0.77-0.98)	0.003
HbA1c (%)	10.03 (7.12-16.04)	5.51 (5.27-5.69)	0.001
Anthropometry			
Body weight (Kg)	85 (65.3-121.7)	78. 4 (59.1-86.8)	0.005
Height (m)	1.69 ± 0.07	1.73 ± 0.08	0.827
BMI (Kg/m^2)	30.8 (22.3-49.4)	27.6 (19.9-32.7)	0.019
WC (cm)	107 ± 10	88 ± 09	0.004
Blood pressure			
SBP (mm Hg)	140 (110-170)	110 (90-130)	0.036
DBP (mm Hg)	80 (60-100)	75 (65-85)	0.625

*P-value: significant difference between patients and controls using independent sample Student's t-test; DN: diabetic nephropathy; X: average; SD: standard deviation; Min: minimum; Max: maximum; HbA1c: glycated haemoglobin; BMI: Body mass index; WC: Waist circumference; SBP: Systolic blood pressure; DBP: Diastolic blood pressure.

Table 2 reports the oxidant and antioxidant status of the patients and controls. Logistic regression analysis revealed statistically significant ($P < 0.05$) differences between the two groups, with patients having 1.77 times higher serum TBARS, 2.77 times higher free-cholesterol-LDL-TBARS, 1.43 times lower total antioxidant status, 1.30 times lower superoxide dismutase activity, 1.19 times lower catalase activity, 1.28 times lower glutathion peroxidase activity, 1.83 times lower α-tocopherol, 1.33 times lower ascorbic acid, and 1.15 times lower retinol than the control subjects.

Table 2. Serum lipids, renal function tests and inflammatory markers of the studied population.

Characteristics	DN (n = 105)	Controls (n = 101)	*P-value
	X ± SD Median (Min-Max)		
Renal function tests			
Urea (mmol/L)	0.35 ± 0.11	0.25 ± 0.08	0.013
Uric acid (µmol/L)	0.06 ± 0.01	0.04 ± 0.01	0.015
Creatinine (µmol/L)	0.99 ± 0.25	0.70 ± 0.09	0.007
GFR (ml/min)	80 (25–117)	105 (95–111)	0.006
Albumin (g/L)	30.94 ± 5.13	59.17 ± 4.83	0.018
Oxidant status			
Serum TBARS (µmol/l)	31.1 ± 8.12	17.60 ± 6.08	0.019
Free-cholesterol-LDL-TBARS (µmol/l)	13.2 ± 4.05	4.76 ± 1.8	0.005
Antioxidant status			
TAS (mmol/l)	1.76 ± 0.79	2.52 ± 0.52	0.016
SOD (UI/l Hb)	149.91 ± 54.10	193.11 ± 63.76	0.003
Catalase (UI/l Hb)	2.87 ± 0.11	3.41 ± 0.16	
GPx (UI/l blood)	3266.28 ± 372.14	4194.05 ± 408.64	0.031
α-tocopherol (µmol/l)	17.37 ± 6.53	31.83 ± 5.92	0.004
Ascorbic acid (µmol/l)	41.06 ± 11.42	54.58 ± 9.19	0.033
Retinol (µmol/l)	0,42 (0,37-0,44)	0,45 (0,41-0,67)	0.047

*P-value: significant difference between patients and controls using independent sample Student's t-test; DN: diabetic nephropathy; X: average; SD: standard deviation; Min: minimum; Max: maximum; GFR: Glomerular filtration rate; free-cholesterol-LDL-TBARS: free-cholesterol-LDL-TBARS -associated thiobarbituric acid-reactive substances; TAS: total antioxidant status; SOD: superoxide dismutase activity; GPx: glutathion peroxidase activity.

A Pearson's correlation analysis revealed that glomerular filtration rate was significantly and positively correlated with total antioxidant status (r = 0.38, p=.021), superoxide dismutase activity (r = 0.34, p=.025), glutathion peroxidase activity (r = 0.29, p=.027), α-tocopherol (r = 0.41, p=.019), Ascorbic acid (r = 0.33, p=.025) levels. On the other hand, GFR was significantly and inversely correlated with glycated haemoglobin (r = -0.57, p=.011), Fasting glycaemia (r = -0.49, p=.013), serum TBARS (r = -0.44, p=.015), free-cholesterol-LDL-TBARS (r = -0.61, p=.001). Table 3 shows the correlation of glomerular filtration rate with other study parameters.

Table 3. Correlation of glomerular filtration rate with study parameters.

Characteristics	HbA1c	Fasting glycaemia	Serum TBARS	free-cholesterol-LDL-TBARS	TAS	SOD	GPx	α-tocopherol	Ascorbic acid
GFR									
r	-0.57	-0.49	-0.44	-0.61	0.38	0.34	0.29	0.41	0.33
P	0.011	0.013	0.015	0.001	0.021	0.025	0.027	0.019	0.025

GFR: Glomerular filtration rate; free-cholesterol-LDL-TBARS: free-cholesterol-LDL-associated thiobarbituric acid-reactive substances; TAS: total antioxidant status; SOD: superoxide dismutase activity; GPx: glutathion peroxidase activity; HbA1c: glycated haemoglobin.

DISCUSSION

Results of oxidative stress testing showed almost exclusively unidirectional changes in patients. Lipoperoxidation products were increased at all stages of lipoperoxidation. Oxidative stress has been shown to be a unifying factor of the major pathways associated with the development and progression of diabetic complications (Iacobini *et al.*, 2021, Sahajpal *et al.*, 2019). With regard to diabetic nephropathy, several pathways have been proposed that lead to increased production of advanced glycation end products (Kang *et al.*, 2020, Mahajan *et al.*, 2019). Excessive amounts of reactive oxygen species can alter the activity of protein kinase C, mitogen-activated protein kinases, various cytokines, and transcription factors, which could lead to increased expression of extracellular matrix genes, resulting in fibrosis and end-stage renal disease (Papachristoforou *et al.*, 2020, Sagoo *et al.*, 2019).

The activation of the renin-angiotensin system in diabetic nephropathy has been linked to further damage caused by reactive oxygen species. These species, which have a high reactogenic capacity, often react with polyunsaturated fatty acids, which are the primary components of membrane phospholipids. This reaction leads to the emergence of new oxidation chains and passive permeability channels for various ions and water in areas with high lipoperoxidation activity (Feng *et al.*, 2020, Malek *et al.*, 2019).

Excessive amounts of most lipoperoxidation products are highly toxic and can damage cell structural components such as lipoproteins, proteins, enzymes, and nucleic acids (Recknagel *et al.*, 2020, Taso *et al.*, 2019). Lipid hydroperoxides can inhibit DNA synthesis, induce apoptosis, inhibit cell proliferation, maturation and growth, and induce mutational changes (Su *et al.*, 2019). Other products of lipoperoxidation are aldehydes and ketones, which play an important role in the synthesis of prostaglandins and some steroids. Dialdehydes can interact with free groups of membrane compounds to form the end products of lipoperoxidation, the accumulation of which can destabilize membranes and lead to cell destruction (Endo *et al.*, 2022, Iuchi *et al.*, 2021).

The results of this study showed an

increase in toxic products (thiobarbituric acid reactive substances) in patients, which can be considered an indication of worsening disease. This is consistent with previous studies reporting an increase in lipoperoxidation products with increasing albuminuria (Vodošek *et al*., 2020, Chou *et al*., 2017).

These changes typically occur when the antioxidant system is unable to effectively neutralize the toxic effects of reactive oxygen species. In our study, we observed lower levels of total antioxidant status, superoxide dismutase activity, catalase activity, glutathion peroxidase activity, α-tocopherol, ascorbic acid, and retinol in patients compared to the control group. Glutathion peroxidase is found in the cytosol and mitochondria, and it degrades hydroperoxides and utilizes most of the phospholipids and fatty acid peroxides through glutathione oxidation (Wang *et al*., 2022, Altuhafi *et al*., 2021). Glutathione peroxidase is an important intracellular antioxidant that plays a role in the biochemical transformation of vitamins and other substances, the regulation of thiol-disulfide balance, nucleic acid synthesis, eicosanoid metabolism, and the conversion of dicarbonyl compounds to hydro acids (Sarıkaya *et al*., 2020, de Vega *et al*., 2016).

Correlation analysis revealed a relation between oxidative stress and lipoperoxidation products, highlighting the role of these components in the deterioration of renal structures, which increases in severity as the disease progresses (Okuyan *et al*., 2021, Rajeshwari *et al*., 2019).

In the future, it would be beneficial to further investigate the relationship between oxidative stress parameters and indicators of renal damage and to identify the most important indicators in order to develop strategies to prevent the progression of renal damage in diabetic nephropathy.

CONCLUSION

It can be stated that in diabetic nephropathy, regardless of the level of glomerular filtration rate, oxidative damage to the main structural components of the cell (lipids and proteins) is recorded. These abnormalities are present even in the early stages of the pathology when there are no pronounced changes in the functioning of the renal structures. This confirms the idea that even in the early stages of the disease there are conditions for the activation of reactive oxygen species synthesis and the development of diabetic complications, which can be used to develop potential strategies for the prevention and treatment of the development of diabetic nephropathy.

Conflict of Interest.

The authors declare no apparent or potential conflicts of interest related to the content of this article. All authors made significant contributions to the study and article preparation, and approved the final version of the article before publication.

Acknowledgement

The authors want to thank the staff of the department of general medicine of Youcef Damardji hospital (Tiaret) and Meslem Tayeb hospital (Mascara), especially the medical supervisor and staff analysis laboratory for their valuable assistance in this study

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