〈Original Article〉

Oxidative stress and DNA damage in diabetic nephropathy

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Summary Type 2 diabetics show a prevalence rate of 40% for diabetic nephropathy (DN). DN is a devastating complication of diabetes mellitus and a major cause of end stage renal failure. Oxidative stress has been known to play an important role in the development and progression of DN. The present study has been undertaken to estimate the oxidative & nitrosative stress by measuring the levels of malondialdehyde (MDA) and nitrite/nitrate respectively and to evaluate the oxidative DNA damage by Comet assay in DN patients in comparison with healthy controls. The study population consisted of 110 patients with Type 2 DN and an equal number of age and sex matched healthy controls. Lipid profiles were significantly different in DN patients compared to controls ($p<0.01$). Mean (±SD) of MDA, nitrite/nitrate levels and DNA damage in the patients were significantly higher at $p<0.01$ compared to controls.

Key words: Diabetic nephropathy, DNA damage, Malondialdehyde, Nitrosative stress, Oxidative stress.

1. Introduction

DN is a leading cause of end stage renal failure worldwide. As the prevalence of diabetes has risen to epidemic proportions. DN has become one of the most challenging health problem. There is considerable evidence that hyperglycemia represents the main cause of complication of diabetes mellitus and oxidative stress resulting from increased generation of ROS play a major role in the development of DN[1, 2].

Oxidative degradation of lipids is referred as a lipidperoxidation and one of the most abundant carbonyl products of lipidperoxidation is malondialdehyde (MDA), whose formation is accelerated by oxidative stress[3]. Lipidperoxidation also induces endothelial damage and inflammatory response, impairs vasodilatation and activates macrophages[4]. It itself generates more free radicals and ROS thereby increasing the potential for renal injury.

Oxidative stress leads to protein, lipid, and DNA modifications that cause cellular dysfunction and contribute to the pathogenesis of DN[5]. It has also been suggested that damage most likely occurs when the endogenous antioxidant network and DNA repair systems are overwhelmed[6].

NO has multiple functions in the vasculature, act as a vasodilator, anti-inflammatory, anti-thrombotic and proliferative activities. Abnormalities and avail-

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ability of NO production in the kidney play a role in pathophysiology of the development and progression of diabetic nephropathy[7]. Hyperglycemia plays a major role in NO activation[8]. The increased NO activity in the kidney may contribute to the glomerular hypertension, hyperfiltration, and microalbuminuria[7]. The present study has been undertaken to assess the oxidative & nitrosative stress by estimating the levels of malondialdehyde & nitrate/nitrite and to evaluate the oxidative DNA damage in the peripheral lymphocytes of DN patients by Comet assay (Single gel electrophoresis).

2. Subjects and Methods

The study population consisted of 110 patients recruited from Nizam Institute of Medical Sciences, Hyderabad with Type 2 DN and an equal number of age and sex matched healthy controls without history of recent infections and any other systemic disorders. Written consent from all patients and institutional ethical committee clearance was also obtained. The body mass index was calculated by dividing weight (kg) by the square of the height (m^2), systolic and diastolic blood pressure was recorded by standard mercury sphygmomanometer.

The reagents used were of analytical reagent grade obtained from Merck (Mumbai, India), HiMedia Pvt.Laboratories (Mumbai, India) and Qualigens (Mumbai, India). The kits were obtained from Glaxo (Mumbai, India).

Tests for blood sugar levels, total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), and triglycerides (TG) were conducted by using commercially available kits. Low-density lipoprotein cholesterol (LDL-C) were calculated according to the Friedewalds equation: (LDL-C) = total cholesterol (TC) - (HDL-C + TG/5). Estimation of plasma MDA was carried out following the method of Gavino et al (1981)[9].

Serum NO_2^-/NO_3^- levels present in the reaction mixture were determined by using Griess reagent (a 1:1 mixture of 1% sulfanilamide in 5% H_3PO_4 and 0.1 % N-(1-napthyl)-ethylene-diamine) by the method of Lepoivre et al (1990)[10].

The protocol for the single-cell gel electrophoresis (SCGE) technique as given by Singh et al (1988)[11] has been performed. DNA migration length and scoring of comets and normal cells were carried out under a binocular microscope at 40X using an ocular micrometer. Fifty cells per slide were examined and hundred cells per individual were scored under binocular microscope. A quantitative value for DNA damage of each cell was calculated as the difference between length of the comet and diameter of the comet head. Pooled data from two slides were used for the Comet assay, since no significant difference was found between duplicate slides.

Statistical analysis was performed using SAS version 9, (SAS Institute Inc, Cary, NC, USA) and the

Table 1 The demographic and clinical data in the control and patient groups

Variables	Controls (n = 110)	DN patients (n = 110)
Female/male	48/62	34/76
Mean age ± SD (yrs)	55.68±15.46	57±8.1
Mean SBP (mmHg) ± SD	127.23±5.86	141±22.2
Mean DBP (mmHg) ± SD	80.89±3.22	82.9±9.91
Mean BMI ± SD	25.62±3.88	27±3.9
FBS (mg/dl)	97.0±5.86	142±60
PLBS (mg/dl)	125.7±5.36	223±70.6
Smoking (%)	–	52 (57.2)
Non vegetarians (%)	65 (71.5)	74 (81.4)

SD: standard deviation; DN: diabetic nephropathy; SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index

data was expressed as mean ± standard deviation. The Student's t-test was performed for lipid profiles, lipid-peroxidation (MDA), nitrite/nitrate and comet in order to determine the difference between the controls and DN patients. Multiple regression analyses were performed to assess the relationship between MDA and nitrite/nitrate and DNA damage.

3. Results

The demographic and clinical data of patients and controls is represented in Table 1. Number of males was more than the number of females in the present study. Fasting and post lunch blood sugar levels of patients were higher (142±60 and 223±70.6) when compared with controls (97.0±5.86 and 125.7±5.36). The risk factors for DN, blood pressure, obesity, smoking and non vegetarians were more prevalent in patients compared to controls.

Total cholesterol (162.51±4.56 vs. 181.11±51.59), triglycerides (147.76±4.57 vs. 163.35±74.54), LDL cholesterol (87.58±4.43 vs. 128.0±60.48) and VLDL (31.32±1.25 vs. 36.1±27.62) values were significantly higher ($p<0.01$) in patients compared to the control group, while HDL cholesterol (47.7±1.8 vs. 40.25±19.79) levels were lower in patients than controls ($p<0.01$) as shown in Figure 1. Plasma MDA levels (2.001±0.628 vs. 8.0±3.467) were found to be significantly high in the patients compared to the control group at $p<0.01$ as summarized in Figure 2. When serum nitrite/nitrate levels were compared with patient and control groups, nitrite/nitrate was tended to be higher in the patients (3.071±1.292) than the controls (1.80±0.555) with the difference being statistically significant at $p<0.01$ as shown in Figure 3. Comet tail lengths (oxidative DNA damage) of patients (22.74±5.055 vs. 11.01±1.683) was found to be significantly high at $p<0.01$ compared to controls as shown in Figure 4.

Stepwise multiple regression analysis carried out for all variables, which showed that MDA and nitrite/nitrate significantly influence the length of comet in DN patients as shown in Table 2. This was seen in the variation of patient comet values by the factor at 51.4% compared to the controls at 2.5% (i.e.

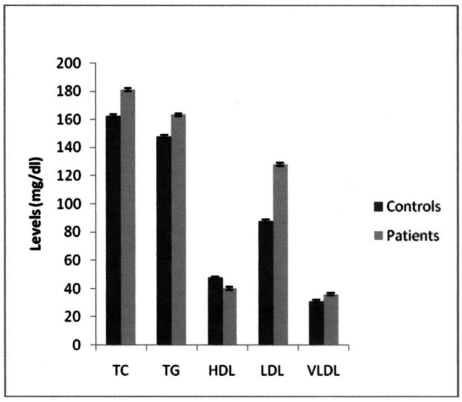

Fig. 1 Levels of lipid profiles in controls and DN patients.
TC: total cholesterol; TG: triglycerides; HDL: high-density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein

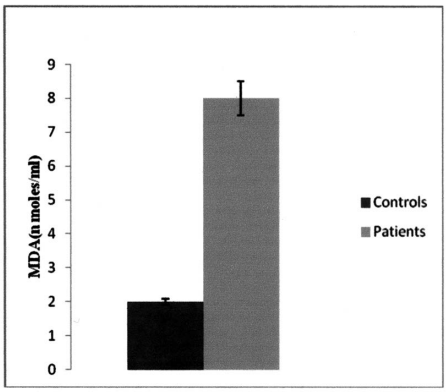

Fig. 2 Levels of MDA in controls and DN patients.
MDA: Malondialdehyde

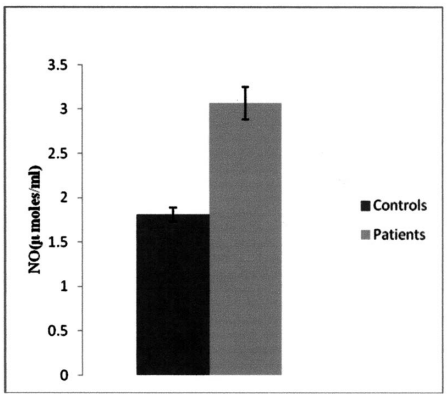

Fig. 3 Levels of NO in controls and DN patients.
NO: Nitrite/nitrate

Table 2 Multiple regression analysis of MDA and nitrite/nitrate on comet assay in the control and patient groups

Sujects	Variables	Unstandardised coefficients	Standardised coefficients	t	Significance	r^2
		Parameter	Standardised			
Patients	MDA	0.927	0.628	6.428	0.000	0.025
	NO	-0.769	-0.197	-2.013	0.049	
Controls	MDA	0.441	0.165	1.218	0.228	0.514
	NO	-0.105	-0.035	-0.255	0.800	

Dependent variable: COMET; MDA: Malondialdehyde; NO: Nitrite/nitrate.

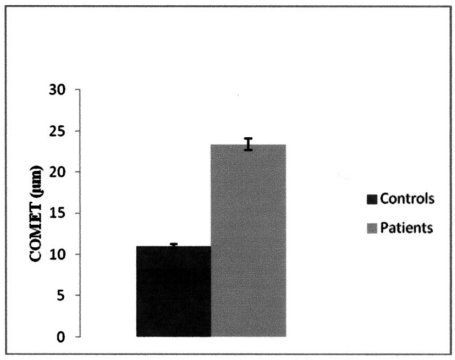

Fig. 4 DNA damage as assessed by comet assay in controls and DN patients.

R-square value), which was found to be significant at $p<0.01$. MDA was found to be associated with oxidative DNA damage (comet) in DN patients when compared to controls ($p<0.01$), and the increase in oxidative DNA damage (comet) was parallel to the increase in MDA, which was statistically significant ($p<0.0001$, $r = 0.628$). Stepwise multiple regression analysis also showed an association between nitrite/nitrate and DNA damage (comet) ($p<0.01$, $r = -0.197$). The influence of MDA was much greater than that of nitrite/nitrate in the patient group ($r=0.628$ for MDA vs. $r = -0.197$ for nitrite/nitrate).

4. Discussion

Diabetic nephropathy is a micro vascular complication of diabetes and a growing proportion of patients with ESRD (end-stage renal disease) in most parts of the world. The individuals with renal dysfunction have several risk factors, including duration of diabetes, age at diagnosis, race, systemic or glomerular hypertension, poor glycemic control, genetic predisposition to kidney disease, and dietary composition etc[12]. The present study mainly focuses on oxidative & nitrosative stress and its consequences to DNA damage in DN patients.

A number of studies supported the concept that exposure to a high blood sugar levels/hyperglycemic environment lead to DN often with co-exisisting obesity, metabolic syndrome, hypertension and hyperlipidemia[13].

One of the major risk factors for the development and progression of DN is dyslipidemia, clinical studies in patients with DN showed that lipid control can be associated with an additional effect of reduction in proteinuria. Lipids may induce both glomerular and tubulointerstitial injury through mediators such as cytokines, ROS and through hemodynamic changes[14]. A number of studies have shown that increase in triglycerides and LDL has also been linked to increased oxidative stress[15]. In the present study we have observed high levels of triglycerides, VLDL, LDL and low levels of HDL in DN compared to controls.

Increased oxidative stress is an important alteration in diabetes and its complications such as kidney disease[16]. Ha H and Lee HB (2001)[17] observed increased lipidperoxidation product (MDA) in proximal tubular epithelial cells, studies by Kedziora-Kornatowska KZ et al (1998)[18] observed increased lipid peroxidation in erythrocytes of patients with type 2 diabetic nephropathy. The results of the present study revealed that the mean values of MDA in DN patients were significantly higher than that of the

controls.

Over the last decade, a remarkable burst of evidence has accumulated, offering the new perspective that nitric oxide (NO) plays a pivotal role in DN. ROS and NO react in pathophysiological conditions, to generate dinitrogen trioxide and peroxynitrite the two toxic reactive nitrogen species that cause significant damage to cellular components (proteins, membranes, nucleic acid), leading to chromosomal alterations, subsequent cellular dysfunction and cellular death[19].

Sharma K et al (1995)[20] and Noh H et al (2002)[21], Chiarelli F et al (2000)[22] observed a positive correlation between serum NO levels, GFR, and albuminuria, suggesting a link between NO, glomerular hyperfiltration and microalbuminuria. Similarly we have also observed high levels of nitrite/nitrate in DN patients compared to healthy controls which is in accordance with earlier report.

High glucose increases ROS production in vascular cells and in renal cells including tubular epithelial cells and mesangial cells[23]. Increased formation of reactive oxygen species (ROS) contributes to endothelial dysfunction, vessel wall thickening, and lesion formation, thereby playing a crucial role in the progressive deterioration of vascular function and structure[24]. ROS and their byproducts are capable of causing oxidative damage which may be cytotoxic and may have some deleterious effects on DNA causing oxidative DNA damage, induces single- and double-strand breaks, abasic sites, and DNA cross-links[25]. Double-strand breaks are the toxic form of DNA damage, resulting in cell death, loss of heterozygosity, translocations, and chromosome loss may occur[26]. Simone S et al (2008)[27] reported the presence of leukocyte DNA damage by high performance liquid chromatography in diabetic nephropathy patients.

In the present study, we have also assessed the DNA damage in the peripheral lymphocytes of DN patients using comet assay as well as the markers of oxidative stress (MDA & nitrite/nitrate) and lipid profiles on a sample size (110) and confirmed the observations of earlier reports. Multiple regression analysis performed on all variables showed that MDA and NO significantly influenced the length of comet in patients (as evident by the R-square value [51.4% in patients vs. 2.5% in controls, Table 2]) and that the interactions of MDA and NO with ROS may lead to DNA damage. The study also revealed that oxidative DNA damage is strongly associated with MDA (r=0.628) when compared to NO (-0.197) in patients and there is no association has been found in controls.

Our study reports that patients with DN had increased levels of oxidative stress markers (MDA, Nitric oxide and DNA damage) and altered lipid profiles. The extent of damage caused by free radicals may be reduced through lifestyle modifications and dietary intervention such as increased intake of fresh fruits, vegetables and antioxidant supplements which might result in halting the progression of DN. Thus increased oxidative, nitrosative stress and somatic DNA damage markers may also act as prognostic predictors and potential targets for therapeutic strategies in DN for early management and prevention of the disease in at risk individuals.

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