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Evaluation of oxidative stress biomarkers and inflammation in pathogenesis of diabetes and diabetic nephropathy

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In this study, we tried to understand better the association of oxidative stress and inflammation with type-2 diabetes and diabetic nephropathy to provide a basis for investigating improved diagnostic possibilities, treatment and prevention of the disease. Blood samples were collected from 498 West Indian individuals aged 42–72 years. Differences in the level of oxidative stress markers (MDA, GSH, SOD and CAT) and inflammation (TNF-α and IL1-α) was determined in patients (type 2 diabetic and diabetic nephropathy) and control participants. Patients with nephropathy showed significantly decreased SOD, GSH levels, but significantly increased MDA and CAT activity compared to DM patients as well as controls. Significant higher level of expression of TNF-α and IL1-α ($P< 0.05$) was observed in DM and DN patients as compared to controls. The results suggest that oxidative stress and inflammation are triggered in patients of type 2 diabetes and diabetic nephropathy possibly due to hyperglycemia.

Keywords: Albuminuria, Hypertension, Insulin-resistance, Oxidative stress

Type 2 Diabetes mellitus (DM) has become a major global health problem. It is one of the serious devastating diseases found in endemic proportion and the frequency of DM patients is expected to go even higher in the future. Long term hyperglycemia in DM results in DN and if DM is not treated on time[1]. DN is a progressive complication of DM which takes several years for development. It includes unusual functional clinical abnormalities of the kidney such as eminent creatinine, urea, albuminuria, reduced glomerular filtration rate, hypertension and fluid retention rate[2]. DN may lead to severe complications like End Stage Renal Disease (ESRD) and patients of DN will have to undergo hemodialysis. The frequency of reported ESRD was 4.0-4.3% with type-1 diabetes mellitus and 4.5-4.7% with DM. The pathogenesis of DN is complicated and comprises many factors like time-interval of DM, deprived glucose control, oxidative stress, high blood pressure and hyper tri-glyceridemia[3].

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Abbreviations: BMI, Body mass index; CAT, Catalase; DBP, Diastolic blood pressure; DM, Type 2 diabetes; DN, Diabetic nephropathy; GSH, Glutathione; HbA1c, Glycosylated hemoglobin; IL1-α, Interleukin 1-α; MDA, Malondealdehyde; NDN, Non diabetic nephropathy; SBP, Systolic blood pressure; SOD, Superoxide dismutase; TNF-α, Tumor necrosis factor-α

Oxidative stress can be defined as disruption flanked by pro- and anti-oxidant factors that fallout in tissue damage. It is found that many pathological conditions are result of increase in oxidative stress and it is believed that it functions as a major pathogenic factor in many of these conditions. Oxidative stress is suggested to be an essential factor in the development and progression of DM as well as in the development of diabetic complications like DN[4-7]. The principal mechanism in the onset of DM are still complex because hyperglycemia may be both root source and outcome of increased oxidative stress[8,9]. There has been a recent notion that inflammation and activation of the innate immune system are closely involved in the pathogenesis of DN[10,11]. Oxi-flammation (*i.e.* oxidative stress and inflammation) influence multiple cellular responses in different organ system are associated with chronic inflammatory mechanism[12,13]. The constructive feedback cycle concerning persistence inflammation, oxidative stress, and development of insulin-resistance contribute to several diabetes-linked consequences such as cardiovascular diseases, kidney diseases and several types of cancer[14]. Various studies proved noteworthy role of inflammatory molecules like chemokines, cytokines and adhesion molecules in the pathogenesis of DN[15,16].

In spite of the improvement in our knowledge about the pathogenesis of DN, the intricate mechanisms leading from long term hyperglycemic condition to the development of DN is yet not completely known. As hyperglycemic condition is well-known to trigger both oxidative stress and inflammatory pathways, the connections of these two pathways are recommended to act in the hyperglycemia mediated renal damage. These findings have stimulated the exploration for an intent biomarker of oxidative stress and inflammation which might be clinically useful in patients with DM and DN. This study aims to enhance the understanding of the link between oxidative stress, inflammation and DN. To achieve this, differences in the level of biochemical markers of oxidative stress and inflammation were determined in patients suffering from DM and DN.

Methods

Selection of cases

This study was carried out in Indian participants specially residing towards western region. They were further divided into four groups: 1) healthy control (n=153), 2) DM (n=128), 3) DN (n=102) and 4) NDN (n=115). Diagnosis of diabetes mellitus was based on the recommendation of American Diabetes Association (ADA). Duration of DM was considered as the time from which the patient was diagnosed with DM. Nephropathy was diagnosed on the basis of persistent GFR <80 mL/min per 1.73 m2. Other baseline criteria included urinary protein excretion >0.05 g/kg per 24 h with hypoalbuminaemia <25 g/L in diabetic and non-diabetic. This study was approved by institutional ethical committee of G.J. Patel Ayurvedic College, Vallabh Vidyanagar, Gujarat. The purpose of this study was properly explained to every participant and informed consent forms were signed before carrying out the study.

Estimation of oxidative stress markers

The blood samples were collected from each participant in EDTA coated Vacutainer tube. After centrifugation at 4000 rpm for 8 min, the plasma and buffy coat were removed to collect the red blood cells (RBC).

Glutathione (GSH) estimation

Estimation of GSH from blood samples was carried out using slightly modified Khyanriam and Prasad method[17]. 100ul of blood, 0.5 mL water and 1 mL precipitating solution (glacial metaphosphoric acid 1.7 g, sodium EDTA 0.2 g, NaCl 30 g in 100 mL of water) were added and mixed well for 10 min of incubation at room temperature. The reaction mixture was centrifuged (4000 g for 10 min). To the 400 µL of clear supernatant, 1 mL of 0.3 mol/L phosphate solution and 200 µL DTNB solution were added. Blank and sample reaction mixtures were read against 415 nm in spectrophotometer.

Malondialdehyde (MDA) estimation

MDA was estimated by using modified method of Mossa *et al*.[18]. 250 microliters (µL) of serum and 250 µL of 50% trichloroacetic acid (TCA) were added mixed until the proteins precipitated. Samples were centrifuged at 9000 rpm for 3 min. To the 150 µL of supernatant 150 µL of thiobarbituric acid (TBA) and samples were incubated in boiling water bath for 20 min. The colored product due to reaction of TBA with MDA was measured spectrophotometrically at 530 nm.

Estimation of CAT activity

CAT activity was determined using modified procedure of Goth *et al*.[19]. 0.5 mL of serum and 2 mL of substrate solution (60 pmol/mL hydrogen peroxide (H_2O_2) in 50 mM/L sodium-potassium phosphate buffer, pH 7.4) were incubated at room temperature for 1 min. The reaction was stopped with 0.7 mL of ammonium molybdate and the yellow colored complex formed was measured at 400 nm against blank.

Estimation of Superoxide dismutase (SOD) activity

The SOD activity was estimated using modified method of Madesh and Balasubramanian .[20]. To 0.5 mL of serum 1.5 mL of reaction mixture was added 1.5 mL of (0.052 mM) sodium pyrophosphate buffer, 0.2 mL of (185 mM) phenazine methosulphate, 0.5 mL of 300 mM nitro blue tetrazolium (NBT). Enzymatic reaction was initiated by adding 0.5 mL of NADH and stopped by adding 1.2 mL of glacial acetic acid. Colour product was measured at 560 nm using spectrophotometrically.

Determination of expression level of inflammatory markers (IL1-α and TNF-α) by qRT-PCR

Expression level of IL1-α and TNF-α in DM, DN and NDN was checked against control. Total RNA was extracted using Trizol method[21]. cDNA synthesis was performed using sample mix 13.5 µL (1 µL template RNA, 1 µL oligo dT and 11.5 µL MilliQ) and reaction mix 6.5 µL (1 µL reverse transcriptase, 0.5 µL riboblock, 1 µL dNTPs, 4 µL RT buffer) according to standard procedures. Expression analysis was performed with the help of quantitative real-time polymerase chain reaction (qRT-PCR) (Rotor gene 6000, Corbett research). The primers used were: human IL1-α forward: 5′-TTGGTGCACATGGCAAGTGGAACGACGCCC

TCAATCA AA-3′; reverse: 5′-GCACAGTCAAGG CTATTTTTCCAGGGCTC GACTCCTTCAT-3′ and human TNF-α forward: 5′-CGGTGGTGGGACTCGTA TGCAAGTAACAAGCCGGTAGCC-3′; reverse: 5′-CTGGTTGTCTTCCAGCTTCACATGGAAGACTCC TCCCTGGTA-3′. The values were normalized against the housekeeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Statistical evaluation

The data obtained were analyzed statistical using SPSS version 21.0. All data are presented as mean ± S.D. Statistical analysis was done by using student's *t*-test. The statistical *t*-test was considered significant with a *P*-value of 0.05 or less.

Results and Discussion

In the present study we carried out case-control studies, to understand the role of oxidative stress markers in diabetes and diabetic nephropathy in Indian population. The clinical details of control, DM, DN and NDN subjects are summarized in (Table 1). The mean age of the control subject (44±11.2) was non-significantly lower than the DM (52±7.3) or DN (58±12.5) or NDN (51±12.3). We found no significant difference in body mass index (BMI), systolic and diastolic blood pressure in all the three studied groups of patients in compare to control. However, glycosylated haemoglobin (HbA1c) in NDN patients and in DN were significantly ($P< 0.05$) higher than control subjects. Whereas, serum creatinine level of was significantly greater in NDN and DN ($P< 0.05$) as compared to DM and control.

Many experimental studies suggested the major role of oxidative stress in the pathogenesis of DM and DN. Increased production of ROS in diabetes may be one of the common mechanisms of diabetic vascular complications like DN. Therefore, evaluation of the oxidative stress production is essential for the prediction and prevention of DN[22]. The assessment of lipid peroxidation status is found to be evidence of cellular oxidative stress. Majority of membrane phospholipids are easily oxidized[23]. It results into the formation of lipid peroxides which are decomposed to aldehydes like MDA[24]. The present study revealed a significant increase in MDA production in patients' group compared to control as shown in (Table 2)

Table 1 — Clinical and biochemical characteristics of studied subjects

Variables	Control	DM	DN	NDN
Number (n)	153	128	102	115
Age (years)	44±11.2	52±7.3	58±12.5	51±12.3
BMI (kg/m²)	20.7±2.5	23.5±2.5	23.6±2.4	21.8±1.5
HbA1c (%)	4.39±0.9	7.12±1.2*	8.56±1.2*	5.22±1.2
Diabetic Duration (years)	-	5.6±3.3	12.13±4.0	-
SBP (mm Hg)	138.5±8.1	147.3±11.3	149.25±12.5	140.35±10.9
DBP (mm Hg)	91.33±5.3	95.54±7.3	96.22±8.1	92.01±7.1
Creatinine (mg/dL)	0.56±0.6	0.83±0.6	4.23±0.4*#	3.87±0.8*
Cholesterol (mg/dL)	162.48±5.3	180.83±6.3	191.76±6.3	181.38±5.1
LDL (mg/dL)	102.65±4.5	117.45±3.2	124.54±4.4	110.31±6.3

Data are expressed as mean ± SD; * $P< 0.05$ when compared to control, # $P< 0.05$ when compared to DM

Table 2 — Oxidative stress markers in control and patients group

Groups	MDA (μM/L)	% Change	GSH (mg/mL)	% Change	SOD (U/mL)	% Change	CAT (U/mL)	% Change
Control(143)	35.75±0.53	-	0.71±0.01	-	17.11±0.32	-	62.62±0.66	-
DM (103)	38.29±0.41	+107.10[a]	0.68±0.01	−95.77[a]	13.69±0.27	−80.01[a]	65.31±1.03	+104.29[a]
	$P< 0.05$[a]		$P> 0.05$[a]		$P< 0.05$[a]		$P< 0.05$[a]	
DN (102)	49.39±0.78	+138.15[a]	0.53±0.01	−74.64[a]	10.48±0.25	−61.25[a]	68.33±0.85	+109.11[a]
	$P< 0.05$[a]	+128.98[b]	$P< 0.05$[a]	−77.94[b]	$P< 0.05$[a]	−76.55[b]	$P< 0.05$[a]	+104.62[b]
	$P< 0.05$[b]		$P< 0.05$[b]		$P< 0.05$[b]		$P< 0.05$[b]	
NDN (108)	51.21±0.95	+145.27[a]	0.50±0.01	−70.42[a]	10.02±0.22	−58.56[a]	71.89±0.67	+114.80[a]
	$P< 0.05$[a]	+133.74[b]	$P< 0.05$[a]	−73.52[b]	$P< 0.05$[a]	−73.19[b]	$P< 0.05$[a]	+110.07[b]
	$P< 0.05$[b]	+103.68[c]	$P< 0.05$[b]	−94.33[c]	$P< 0.05$[b]	−95.61[c]	$P< 0.05$[b]	+105.21[c]
	$P> 0.05$[c]		$P> 0.05$[c]		$P> 0.05$[c]		$P< 0.05$[c]	

[a]compared with control; [b] compared with DM; [c] compared with DN

($P< 0.05$). Similar to our results, Ohtsuki *et al.*[25] presented the increased MDA level because of reduced activity of most of the antioxidant enzyme. Glutathione is thiol-containing tripeptides which in its reduced form (GSH) is present in living cells at high concentrations. When it reacts with ROS, it gets oxidized to glutathione radical which is regenerated to its reduced form through glutathione reductase activity. We found a significant decrease in GSH concentration in patients' group which may be due to conversion of reduced form to oxidized form (GSSH) by excessive production of reactive oxygen species.

SOD catalyzes the superoxide dismutase to H_2O_2 which is further diminished by the CAT and glutathione peroxidase activity[26,27]. The current study demonstrates a significant reduction in SOD activity in patients as compared to control. The auto oxidation of glucose leads to H_2O_2 formation, which inactivate SOD[28]. Decreased SOD activity could be due to ageing in DM patients and it may result in increase in the glycation of SOD. Ceballos *et al.*[29] denoted that during ageing process the steady state concentration of H_2O_2 is found to be higher which could increase peroxidation of PUFA in cell membrane. It leads to the lysis of erythrocytes resulting in the increase in the level of antioxidant enzymes like CAT in the extracellular fluid. In erythrocytes, H_2O_2 is mainly detoxified by activity of glutathione reductase and peroxidase. When these enzymes activity reduces because of poor availability of NADPH and GSH, the other enzyme (*i.e.* CAT) expresses and detoxifies the H_2O_2. Kumar *et al.*[30] in his studies has shown that decrease in activity of glutathione peroxidase, there is compensatory increase in the CAT activity. Inal *et al.*[31] also reported increased activity of CAT with age due to increase formation of H_2O_2.

Diabetic patients generally have less significant plasma antioxidant level with poor glycemic control compared to healthy individuals[32]. Oxidative stress in DM coexists with a decrease in the antioxidant status, which can further increase the harmful effects of free radicals[33]. Korkmaz *et al.*[34] investigated the status of markers of oxidative stress in DM and reported a significant reduced level of antioxidant power. On the basis of the result obtained they concluded that the higher glucose concentration escort to tissue injury by increasing oxidative stress. Similar to this study, we have found significant decrease in SOD activity in patients' groups compared to control group may be due to higher oxidative stress produced by higher glucose level (Fig. 1). Increased production of oxidants consequential from different biochemical pathways (*i.e.* glucose autoxidation, non-enzymatic glycation of proteins and activation of protein kinase C activated by hyperglycemia) may result in decreased SOD activity. Thus, elevated oxidative stress, as well as reduced antioxidant capacity may perhaps be connected to the complications in patients with diabetes such as DN[34].

The TNF- α and IL1-α expression profile was analyzed for all groups of patients and control by using Real time PCR (Fig. 2). The results showed significantly increased expression of TNF-α and IL1-α in patients' group compared to unaffected controls after normalization with GAPDH expression. It denotes that TNF-α and IL1-α play important role in disease progression by inducing inflammation. Navarro *et al.*[35,36] has reviewed the importance of TNF-α as therapeutic target for DN. Our expression studies data analysis indicates that there is significant increase in TNF-α transcript levels between control and patients group. At the time of stress or any injury cell releases IL-1, it stimulates various cell protective responses such as establishment of early transcription genes that uphold the cell survival and proliferation. In experimental models of DN, renal expression of IL-1 increases, which is related to

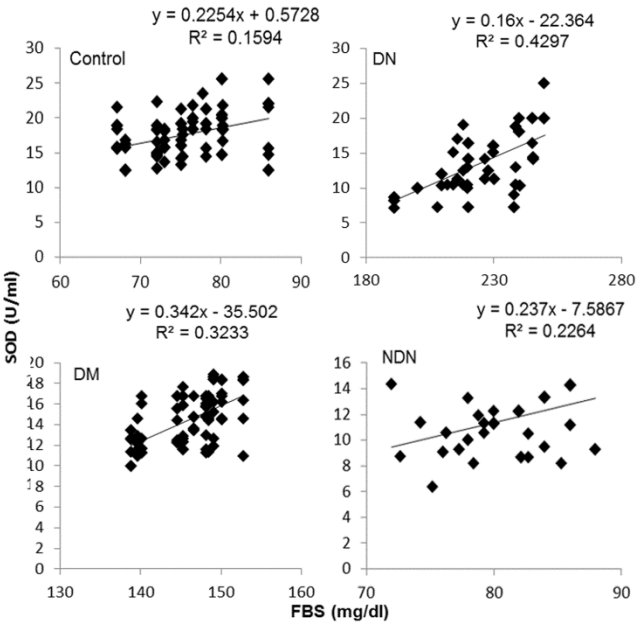

Fig. 1 — Correlation between FBS (fasting blood sugar) and SOD activity

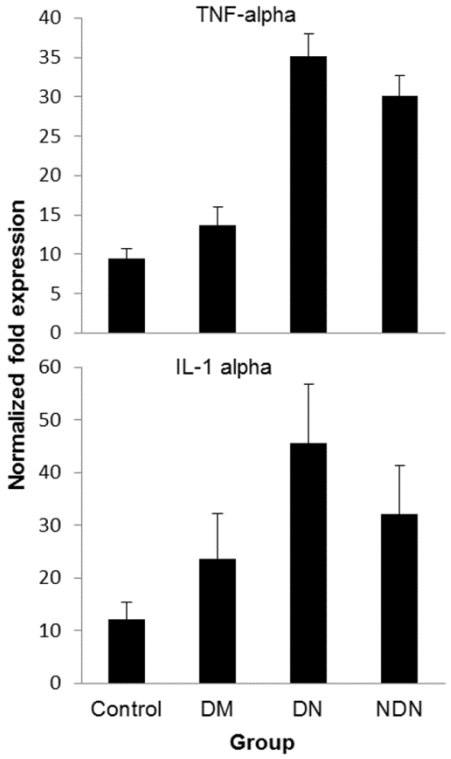

Fig. 2 — Expression profile of TNF-α and IL1-α in control and patients group

successive expression of chemotaxis factors and adhesion molecules[37-39].

Conclusion

The current study disclose that hyperglycemia produced marked oxidant impact as evidenced by the considerable increase in lipid peroxidation products (MDA) as well as decrease in antioxidants including SOD and GSH content. Patients with high values for inflammatory markers have shown increased oxidative stress which proves oxidative stress and inflammation to be the pivotal pathophysiological triggers in DM and DN. Amending these processes using restorative methods may restrict the occurrences of diabetic complications like DN.

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Conflict of Interest

All authors declare no conflict of interest.

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