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Original Article

A study of oxidative stress, antioxidant status and lipid profile in diabetic patient in the western region of Nepal

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Abstract

Aims and Objectives: Diabetes mellitus (DM) is often termed as a disease of premature aging. Several studies have indicated lopsided redox balance due to pro oxidant environment as one of the important etiological factors. Some recent researches also indicate a causal relationship with oxidative stress (OS). So far, no study has been undertaken on this aspect in Nepali populations. We, therefore, aimed this maiden study in Nepali population to examine redox balance by measuring OS and antioxidant status along with lipid profile in 37 patients of DM type- 2 and 30 matched normal subjects.

Methodology: Thirty seven patients of DM type-2 without any complications (mean age= 57.6± 10.6 years) and 30 normal subjects (mean age= 55.8 ± 14.8 years) were included in this study. Body Mass Index (BMI) and Waist/Hip (W/H) ratio were measured. Fasting blood sample was collected for the analysis of total antioxidant activity (TAA), plasma and urinary thiobarbituric acid reactive substances (TBARS) and lipid profile by standard procedures in both the groups. The statistical analysis was done with SPSS 10 version.

Results: Total cholesterol, triglyceride, VLDL-cholesterol, LDL-cholesterol, plasma and urinary TBARS were significantly raised whereas, plasma TAA was significantly reduced in DM type-2 patients as compared to controls. The comparison of old and fresh cases revealed that though TAA was lower and PTBARS and UTBARS were higher in patients but did not attain the level of significance. W/H ratio is significantly higher in patients compared to normal subjects. But, no significant correlation of BMI and W/H with lipid profile is observed in both control and patients.

Conclusion: Oxidative stress is raised in type 2 DM patients. This along with deranged lipid profile and decreased antioxidant status could be the risk factors in the development of complications associated with DM.

Key words: oxidative stress, antioxidant, lipid profile, diabetes mellitus, Nepal.

DM type-2 is a multicausal disease which develops slowly and in a stepwise order[1-3]. Initially, it commences with insulin resistance, which progress gradually with the passage of time. Secondary hyperinsulinism develops to counter it, but it too at one point of time fails to maintain glucose homeostasis resulting in glucose intolerance. Eventually, insulin resistance continues to progress, β-cells fail to cope up with requirement and insulin secretion goes down progressively. Although blood insulin level may remain normal or high yet hyperglycaemia may develop and may be accompanied with glycosuria. Systemically, these perturbations are accompanied with changes in a variety of biochemical processes and are exacerbated by overweight and obesity, altered lipid profile, degree of hyperglycaemia, smoking and/or genetic profile. In these composite risk factors, gathering evidence suggests that reactive oxygen species (ROS) intervene at different points to initiate and promote DM type-2[3-5].

A very recent and excellent study has shown that ROS have a causal relationship with insulin resistance which is a cardinal feature of DM type-2, through their cell culture as well as leptin deficient ob/ob mouse model studies[6]. It also demonstrated that ROS progressively aggravated it. In brief, persistent hyperglycaemia, auto-oxidation of glucose, obesity, glycation of proteins, activation of NADPH oxidase, nitric oxide synthase and xanthine oxidase cumulatively contribute to free radical pool in DM[5-7].

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Free radicals have proclivity to attack unsaturated fatty acids, LDL and cholesterol, as such their enlarged pool, as is often seen in DM type-2, produces undesirable oxidized products contributing to initiation and progression of the disease along with complications [7,8]. In fact there are several animal and human studies temptingly suggesting that ROS also stimulate and hasten the complications in DM type-2 [4,9,10,11].

The modern concept of medicine envisages that a subtle balance of redox homeostasis is necessary to maintain normal health and to avoid disease and that in this redox homeostasis free radicals and antioxidants play a crucial role, in many diseases especially age related ones. To our knowledge, no work has so far been done in this regard on Nepali population and relationship, if any, with BMI and W/H ratio, which are currently used as anthropometric markers for measuring overweight and obesity except some studies published from our laboratory. This maiden study addresses oxidant: antioxidant balance along with lipid profile in freshly detected and old cases of DM type-2.

Materials and methods

This study was carried out in the Department of Clinical Biochemistry, Manipal Teaching Hospital, Pokhara, Nepal. Our study group included 37 patients of controlled DM type-2 patients without any complications (diagnostic criteria prescribed by the American Diabetes Association) [12] (males=23 and females=14) with a mean age of 57.6±10.6 years and 30 age and sex matched healthy controls (males=17 and females=13) with a mean age of 55.8 ± 14.8 years. The diabetic patients were normotensive, without secondary causes of hyperglycaemia and were under treatment with oral hypoglycaemic agents. In order to study the relation of the disease duration with oxidative stress, we categorized DM patients into two categories, i) fresh case (duration ≤ 1year) and old case (duration > 1year). Detailed present and past history of the patients was collected on pre-tested proforma which included name, age, sex, dietary habit, family history, smoking and drinking habit, socio-economic status, community and occupation along with their consent for the study. Body Mass Index (BMI) and Waist/Hip (W/H) ratio were measured in both the groups by standard procedure. Six ml of fasting blood was collected from each subject by venipuncture and transferred to EDTA containing vial. The sample was centrifuged for 10mins at 3000rpm. The plasma was used for the analyses of total antioxidant activity (TAA) by ferric reducing antioxidant power assay (FRAP)[13], plasma thiobarbituric acid reactive substances (TBARS)[14] and lipid profile (total cholesterol (TC)[15], triglycerides (TG)[16], HDL-cholesterol, LDL-cholesterol and VLDL-cholesterol[17]. To determine the urinary excretion of TBARS, about 30ml of morning urine sample was collected without preservative in clean dry container for the analysis of urinary TBARS.

Analytical reagents for estimation of TAA and TBARS were purchased from Central Drug House Pvt Ltd., Bombay-Delhi India and fresh reagents were prepared in laboratory prior to estimation. Lipid profile were done by kits (Randox Laboratories Ltd, United Kingdom) and processed through semi-autoanalyzer, Microlab-300, Netherland. The results are reported as mean± SD. The statistical analysis was done with SPSS 10 version software. Independent sample 't' test, one way ANOVA and Pearson's correlation coefficient were calculated and for all determinants, $p<0.05$ was considered significant.

Results

The results of the present study are described in Tables 1-6. It can be seen from Table 1 that the mean ages, BMI, W/H ratio of the normal subjects were 55.8±14.8 years, 23.6±3.9 Kg/M^2 and 0.94±0.50 respectively. The respective figures for DM type-2 patients were 57.6±10.6 years, 24.8±4.0 Kg/M^2 and 1.00±0.22 respectively. Among patients total cholesterol ($p<0.001$), triglycerides ($p<0.01$), VLDL-cholesterol ($p<0.01$) and LDL-cholesterol ($p<0.001$) were significantly high compared to controls but there was no difference in HDL-cholesterol between them. Notably, there was a significant increase in both plasma TBARS ($p<0.001$) and urinary TBARS ($p<0.05$), with a significant decrease in TAA ($p<0.01$) in patients. No significant difference was noted in aforesaid parameters between fresh cases and old cases (Table 2). However, TAA was relatively lower with higher level of plasma and urinary TBARS in the old cases. Probably, a discernable trend can be obtained with a larger series. In another exercise, the patients were divided on the basis of serum cholesterol level. Incidentally, hypercholesterolic patients had significantly lower TAA levels ($p<0.001$) but TBARS levels did not show any significant difference (Table 3). Though there is no significant increase in BMI, there is significant increase in W/H ratio ($p<0.01$) in patients, however no deducible trend was visible in the normal subjects and patients when categorized on the basis of cholesterol level (Table-4) or BMI (Table-5) or W/H ratio (Table-6). The scattered significances seem to be fortuitous.

Table 1: Lipid profile, oxidative stress and antioxidant status in controls and type 2-DM patients

Parameters	Control (N=30) Mean + SD	Type 2 DM (N=37) Mean + SD
Age (years)	50.80±14.84	57.59±10.60
BMI (Kg/ m^2)	23.6± 3.9	24.8± 4.0
W/H ratio	0.94±0.50	1.00±0.22[b]
TC (mg/dl)	155±25	196±45 [c]
TG (mg/dl)	123±36	169±68 [b]
HDL (mg/dl)	45±8	47±8
VLDL (mg/dl)	25±7	34±14b
LDL (mg/dl)	85±20	116±38 [c]
STAA (µmol/ml)	726±200	582±148 [b]
PTBARS (nmol/l)	2.41±0.61	3.55±1.04 [c]
UTBARS (nmol/l)	3.84±1.63	4.82±1.76 [a]

Independent sample-t test: p<0.05=a, p<0.01=b and p<0.001=c

Table 2: Biochemical parameters in fresh cases and old cases of type-2 DM patients

Parameters	Fresh Cases (N= 05) Mean ± SD	Old Cases (N= 32) Mean ± SD
TG (mg/dl)	153 ± 67	166 ± 73
TC (mg/dl)	201 ± 40	195 ± 46
HDL (mg/dl)	52 ± 8	46 ± 8
VLDL (mg/dl)	32 ± 15	34 ± 14
LDL (mg/dl)	118 ± 27	115 ± 40
TAA (nmol/ml)	676 ± 146	567 ± 146
Plasma TBARS (µmol/l)	3.08 ± 0.68	3.62 ± 1.07
Urinary TBARS (µmol/l)	4.32 ± 1.84	4.90 ± 1.76

Independent sample-t test: p<0.05=a, p<0.01=b and p<0.001=c

Table 3: Comparison between hypercholesterolemic and normocholesterolemic group of type-2 DM patients

Parameters	Hypercholesterolemic (N= 19) Mean ± SD	Normocholesterolemic (N= 18) Mean ± SD
TG (mg/dl)	192 ± 83	135 ± 44 [a]
HDL (mg/dl)	49 ± 8	46 ± 8
VLDL (mg/dl)	41 ± 14	27 ± 9 [c]
LDL (mg/dl)	141 ± 32	88 ± 22 [c]
TAA (nmol/ml)	524 ± 131	642 ± 144 [a]
Plasma TBARS (µmol/l)	3.27 ± 0.81	3.84 ± 1.19
Urinary TBARS (µmol/l)	4.16 ± 1.42	5.05 ± 2.08

Independent sample-t test: p<0.05=a, p<0.01=b and p<0.001=c

Table 4: Percentage distribution of control and patients at different ranges of total cholesterol, HDL-C and LDL-C

Description	Control n=30			Type 2-DM n=37		
	%	BMI	W/H ratio	%	BMI	W/H ratio
Cholesterol						
<200mg/dl	96.7%	23.5±0.9	0.93±0.09	45.94%	25.5±4.8	1.03±0.06
200-239mg/dl	3.3%	25.5	0.97	40.54%	24.1±2.4	0.99±0.05
>240mg/dl	-	-	-	13.51%	24.0±4.9	0.96±0.05
HDL-C						
<40mg/dl	30%	24.3±4.6	0.97±0.11	13.51%	23.7±3.9	1.02±0.05
40-60mg/dl	63.3%	23.3±3.8	0.91±0.08	78.37%	25.3±4.0	1.0±0.06
>60mg/dl	6.7%	23.8±1.6	0.98±0.04	8.10%	21.4±1.9	0.99±0.09
LDL-C						
<130mg/dl	93.3%	23.8±3.9	0.94±0.09	72.97%	25.3±4.1	1.01±0.06
130-159mg/dl	6.6%	18.6	0.85	13.51%	22.5±2.8	0.99±0.8
160-189mg/dl	-	-	-	13.51%	24.4±4.7	0.97±0.05

Table 5: Lipid profile, oxidative stress and total antioxidant activity in normal subjects and type 2-DM patients based on their BMI

Parameter	Controls			Type 2-DM patients		
BMI (Kg/m^2)	**<18.5 (2)**	**18.5-25 (17)**	**>25 (11)**	**<18.5 (3)**	**18.5-25 (17)**	**>25 (17)**
TC (mg/dl)	154±24	150±20	160±32	119±13	211±39	194±40[b]
TG (mg/dl)	131±4	113±37	135±29	122±13	174±84	162±63
HDL (mg/dl)	46±9	45±8	43±8	41±6	51±9	44±6[b]
VLDL (mg/dl)	26±8	22±7	27±6	24±3	35±17	35±10
LDL (mg/dl)	82±22	84±19	87±21	61±29	127±33	114±37[b]
TAA (nmol/ml)	588±110	685±161	814±239	467±75	541±130	642±155[a]
TBARS (µmol/l)	2.92±0.28	2.50±0.60	2.18±0.61	5.75±0.91	3.53±0.88	3.17±0.71[c]
UTBARS (µmol/l)	3.37±0.37	3.94±1.82	3.77±1.53	6.02±1.79	4.57±1.87	4.86±1.64

One way ANOVA test done in controls and type 2-DM separately: p<0.05=a, p<0.01=b and p<0.001=c

Table 6: Biochemical parameters in normal subjects and type-2 DM patients on the basis of waist/hip ratio

Parameters	Waist/ Hip Ratio					
	Controls			type-2 DM patients		
	Males		Females	Males		Females
	≤ 0.95	**> 0.95**	**> 0.80**	**≤ 0.95**	**> 0.95**	**> 0.80**
TC (mg/dl)	152±16	151±21	159±32	226±10	189±44	196±50
TG(mg/dl)	120 ± 50	132±20	117±33	220 ±12	160± 56	155 ± 95
HDL(mg/dl)	44 ± 6	44 ± 9	47± 9	53± 5	48± 9	44 ± 7
VLDL (mg/dl)	24 ± 10	26 ± 6	23 ± 7	45 ± 3	32 ± 11	30 ± 12
LDL (mg/dl)	84 ± 12	82 ± 19	89 ± 25	129± 10	110 ± 34	122 ± 49
TAA(nmol/ml)	705±148	820±279	665± 125	545±120	623±153	535 ±140
PlasmaTBARS (µmol/l)	2.48±0.53	2.28±0.59	2.48±0.69	3.51±0.89	3.39±0.91	3.77±1.25
Urinary TBARS (µmol/l)	3.10±1.32	3.52±1.40	4.48±1.80	3.72 ±0.64	4.76±1.92	5.22±1.68

Independent sample-t test: p<0.05=a, p<0.01=b and p<0.001=c

Discussion

Ever since Sato et al[18] reported increased level of TBARS in plasma, i.e. raised level of OS in both NIDDM and IDDM patients, abounding literature have been gathered to implicate OS in the aetiopathogenesis of DM and further development of various complications. These observations are further supported by *in vitro* studies. Houstis et al [6] demonstrated that ROS, through insulin resistance, may lead to DM. Jain [19] demonstrated that hyperglycaemia stimulated the lipid peroxidation of RBC and Kannan and Jain [20] later showed that it raised OS *in vitro* cells, an African monkey cell line. Our mean data on patients support this assumption. The oxidative stress was significantly higher in patient group (Plasma TBARS = 3.55±1.04 µmol/L) than controls (Plasma TBARS = 2.41±0.61 µmol/L). This was further reconfirmed by significantly increased excretion of TBARS in urine of patients (TBARS = 4.82±1.76 µmol/L) than controls (TBARS = 3.84±1.63 µmol/L). Animal studies have shown that kidneys tend to eliminate the lipid peroxidation products to avoid their toxic effects [21]. Contrary to our observations and those of others, there are several studies which did not find raised OS in either DM type-2 or type-1 patients [22]. An excellent animal study in this regard is of Midaoui and Champlain [23] who examined OS in two models of rats, one representing type 2 DM and the other type 1 DM. Notably, they observed that hyperglycaemia alone did not induce OS unless accompanied by insulin resistance; thereby, implying that the involvement of ROS is selectively related to insulin resistance [6]. It is clearly discernible that OS is increased in patients of our study, however, because we have considered the diagnosed diabetic patients, we are unable to specify whether the increased OS in patients is due to insulin resistance or hyperglycaemia or both. Recent studies by Monnier et al [24] demonstrated that glucose fluctuations during postprandial periods activated oxidative stress even more specifically than sustained chronic hyperglycaemia. Hence, we cannot make absolute assumption that there is no hyperglycaemia induced oxidative stress in the patients though we have included controlled diabetic patients. Thus, well designed study is needed to get a better insight about this matter.

Others have argued that hyperglycaemia provokes ROS production through multiple routes especially through glucose auto-oxidation and AGE formation pathways. If hyperglycaemia is not controlled, enhanced generation of ROS can lead to enhanced lipid, protein and nucleic acid peroxidation, leucocyte adhesion, foam cell formation, TNF-α expression, endothelin, TXA-2 expression and nitric oxide synthesis. These perturbations can lead to complications if blood glucose is not controlled. The disease may worsen further as peroxidation adducts may have additive effects. For example raised MDA level itself is quite toxic in several ways: a) it stabilizes cross linking of collagen and allows further glycation b) this in turn aggravates glycated collagen to initiate further lipid oxidation and release more MDA, thus establishing a vicious cycle of lipid peroxidation c) lipid peroxides in general enhance prostaglandin synthesis which becomes another source of free radicals and lastly enhanced nitric oxide production is a well known risk factor for atherosclerotic complications [5,7,8]. These postulates though not tested in this series, are supported by our observations on 5 freshly detected and 32 old cases of patients. Though, statistically not significant, both plasma and urinary TBARS tended to be higher in old patients (Plasma TBARS = 3.62 ± 1.07 µmol/L and urine TBARS = 4.90±1.76 µmol/L) compared to fresh cases (Plasma TBARS = 3.08±0.68 µmol/L and urine TBARS = 4.32±1.84 µmol/L).

Since redox imbalance has been incriminated in DM, it is natural to expect that antioxidant defence system may be involved to counter-balance the pro-oxidant environment. Indeed, there is a body of evidence suggesting that antioxidant components become weak probably due to extra utilization and these antioxidants have therapeutic value[7,25,26]. Even supplemental value of antioxidant has been advocated. So far, techniques to raise endogenous antioxidants *in vivo* are ineffective; as such one has to depend on dietary antioxidants including the nutrient ones. Presently, it is realized that measurement of total antioxidant activity is 'much better index of antioxidant strength than the measurement of individual antioxidant as they work' in tandem. For this purpose FRAP (ferric reducing ability of plasma) procedure, used here in, is considered to be one of the best available procedures as it excludes enzymatic antioxidants, reduced glutathione and other thiol group compounds [13]. Our data revealed that mean TAA was significantly lower in patient group compared to controls; that it tended to be lower in old cases (TAA= 567± 146µmol/L) than freshly detected cases (TAA= 676 ± 146µmol/L); and that even freshly detected cases had lower levels than the normal subjects(TAA= 726 ± 200µmol/L). All these data taken together point out that patients should consume more antioxidants in an effort to balance the redox status. This was further supported by significant inverse relationship between TAA and TBARS in normal subjects ($r = -0.339$, $p<0.01$) which though still inversely related was disrupted in this disease ($r = -0.133$, p= not significant).

Overweight and obesity are alarming problems in affluent countries and are knocking at the doors of developing countries with progressive movement towards advancement. Both of them are risk factors in DM type 2 and hasten cardiovascular complications mainly due to altered lipid profile. In this regard, abdominal adiposity is considered to be a good test to determine the risk in both overweight and obese persons [27, 28]. Imaging techniques, though ideal for this purpose, are impractical due to high cost and methodological problems. Alternatively BMI, W/H ratio and waist circumference (WC) have widely been used for this purpose. In our study we have measured BMI (kg/m^2) and W/H ratio and examined their relationship with lipid profile and other parameters. Our result depicts alarming picture i.e. diabetic patients have significantly increased W/H ratio along with Total cholesterol, VLDL and LDL cholesterol and triglycerides. BMI is also raised in patients but is statistically not significant. HDL-cholesterol was also comparable with controls. Unlike the study conducted by Gupta et al **[29]**, patients and controls classified into three groups on the basis of BMI: underweight (<18.5), normal weight (18.5-25) and overweight & obese (> 25) and W/H ratio: with cut-off value 0.95 for male and 0.80 for female [28] did not show significant relationship with regard to lipid profile. Therefore, we support the opinion that poor nutrition, ethnicity and geographical conditions are important determinants of fat content of body tissues and abdominal adiposity and that total fat content of body may not necessarily reflect similar distribution of lipid components in different compartments in developed, developing and underdeveloped populations [28, 30].

Numerous literatures have shown that obesity is related to decreased antioxidants [31] and increased oxidative stress [32]. On the contrary, our result showed increased TAA and decreased TBARS with BMI in both controls and patients but was significant only in case of patients. It suggests that better antioxidant status, most likely was due to better nutrition and in all probability tries to keep OS low in related to patients. W/H ratio also did not exhibit any correlation to plasma TBARS or urine TBARS.

Conclusion

Our data suggest that there is raised oxidative stress, decreased antioxidant status, increased W/H ratio and deranged lipid profile in type 2 DM patients. Numerous literatures have supported that these factors are associated with diabetes related complications. Thus, in our population also, these factors could be the cause of diabetes associated complications.

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