Oxidative damage indices for the assessment of subclinical diabetic macrovascular complications

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ABSTRACT

Subclinical cardiovascular disease (SCVD), including complications in diabetes, is associated with oxidative damage and precedes future cardiovascular disease (CVD). Hence, assessment and management of oxidative damage is imperative. This study investigates biomarkers associated with CVD, diabetes and oxidative stress in order to determine a set of indices that could be useful to assess oxidative damage in diabetic macrovascular pathogenesis. A total of 266 participants were selected and divided into seven groups (control, family history of diabetes, prediabetes, prediabetes with CVD, diabetes mellitus [DM], DM+CVD and CVD) based on clinical history/status. Blood glucose (BG) level, erythrocyte glutathione (GSH), malondialdehyde, methaemoglobin, D-dimer, homocysteine, blood viscosity and cholesterol profile were determined. Factorial MANOVA and independent univariate analyses were performed. Prevalence of significant biomarkers was assessed following a 3.5-year retrospective study. Multivariate analysis showed statistically significant differences between groups ($P<0.0001$) with *post hoc* tests identifying a statistically significant association for BG level ($P<0.0001$), GSH ($P<0.0001$), D-dimer ($P<0.02$) and total cholesterol ($P<0.0001$). Of the subjects who showed hyperglycaemia-associated progression in clinical and biochemistry status, 89% had low-level GSH and 44% had high-level D-dimer. Four individuals exhibited prediabetic status at some stage and would qualify for macrovascular disease intervention. The results of this study suggest that BG level, D-dimer, GSH and total cholesterol contribute significantly to a diabetic oxidative damage panel of markers that could assist in evidence-based pharmacological intervention with anti-aggregation and/or antioxidant agents against future CVD in diabetes.

KEY WORDS: Antioxidants.
Cardiovascular diseases.
Diabetes mellitus.

Introduction

Oxidative damage is a fundamental mechanism in human diseases such as diabetes mellitus (DM) and its macrovascular sequelae.[1,2] In the management of diabetic macrovascular complications, approaches include drug therapy as an option. One important target for medication is the preservation of normal oxidant–antioxidant balance.[3,4] Another medication intervention option or target is atherothrombogenesis with an anti-aggregation agent such as aspirin.[5]

The pharmacological side-effects associated with these agents have limited their use, making evidence-based prescription necessary. However, a diagnostic tool for evidence-based decision-making for antioxidant therapy is not yet available in clinical practice. Thus, it is necessary to explore suitable laboratory markers to determine oxidative damage.

Oxidative stress accelerates arterial thrombosis,[6] and blood flow factors, including viscosity and red cell aggregation, are predictors of this form of thrombosis.[7] Induction of erythrocyte oxidative stress (EOS) reduces erythrocyte membrane fluidity, which is associated with the increased potential for blood aggregation and clotting,[8] and enhanced viscosity, endothelial dysfunction and atherothrombosis.[9] Here, a simplified pathway from EOS to the vascular events is presented to show the points at which anti-aggregation agents and antioxidants are required to intervene against progression to atherothrombogenesis (Fig. 1).[10]

Atherothrombosis, endothelial dysfunction and blood viscosity comprise Virchow's triad, which predisposes to future cardiovascular disease (CVD).[11] Thus, there is a pathophysiological process involving a sequence of hyperglycaemia-induced EOS, reduced erythrocyte membrane fluidity, increased blood aggregation, increased viscosity and/or endothelial dysfunction and atherothrombosis. The last is a serious CVD condition that requires intervention.

Although the rise or fall in antioxidant level and an increase in peroxidation products constitute oxidative stress (OS),[12] concomitant demonstration of any of the effective vascular events is necessary to establish oxidative damage.[2] Therefore, in order to implicate OS and oxidative damage overtly in diabetes macrovascular pathogenesis, the following three questions need to be answered. Is hyperglycaemia present, demonstrated by elevated blood glucose (BG) level? Is OS present, demonstrated by loss of antioxidants (e.g., glutathione [GSH]) and/or high levels of oxidation products (e.g., malondialdehyde [MDA])?[13] Is there any vasculopathy indicated by high D-dimer, homocysteine or blood viscosity?

Given the fact that cardiovascular oxidative damage involves loss of antioxidants and concomitant evidence of

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vascular events,[2] the hypothesis examined in this study proposes that indices of oxidative damage include antioxidant and lipid peroxidation products as well as an indicator of vascular events. The study aims to establish a set of measurable parameters that would be useful as an oxidative stress panel (OSP) to assess EOS associated with both hyperglycaemia and vascular events, and thereby function as a laboratory determinant of oxidative damage associated with DM progression.

Materials and methods

A total of 266 volunteers from Albury (NSW) and Wodonga (Victoria) and surrounding areas were enlisted for evaluation of EOS in diabetes and its cardiovascular complications. The study was part of a PhD research project approved by the Charles Sturt University Ethics in Human Research Committee.

The participants included persons with diabetes, with or without cardiovascular complications, those with an established diagnosis of cardiovascular disease only, and persons without any evidence of disease. Participants were divided into seven groups: healthy controls (n=49), healthy individuals with a family history of diabetes (FH–DM, n=27), prediabetics (preDM, n=41), prediabetics with macrovascular disease (preDM+CVD, n=34), patients with diabetes mellitus (DM, n=30), DM+CVD (n=42) and patients with CVD (n=43). Classification was determined from the medical history obtained from a health questionnaire and from pathology reports. Information obtained included past results of BG level, electrocardiography (ECG), serum creatinine, urinary albumin excretion, established diagnosis of diabetes, CVD, kidney disease and/or other disease conditions. Family history of diabetes, body mass index (BMI), age, gender, smoking, alcohol consumption and medication were also recorded.

Participants were included if their BMI was within the range 20–30 kg/m,[2,14] daily consumption of standard alcoholic drinks was ≤2 for females and ≤4 for males,[15] and no other disease was reported or identified. Exclusion criteria included chronic diseases such as arthritis, depression, glaucoma, kidney disease or abdominal ulcer, and also ongoing medication (except for an established diagnosis of DM and/or CVD).

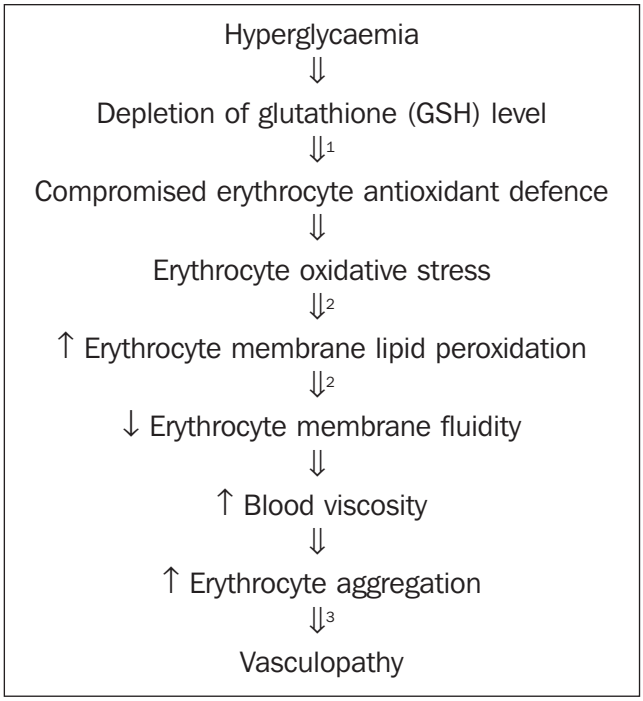

Fig. 1. Sequence of erythrocyte oxidative stress through to vasculopathy, illustrating points of action of relevant intervention therapies (1=GSH supplementation; 2=vitamin E and other co-antioxidant supplementation; 3=anti-aggregation therapy).[8,10]

Prediabetes in this study was defined as an established prediabetic status identified by general practice, or participants who were otherwise healthy (i.e., undiagnosed) but who presented with elevated BG level. In either case, fasting BG level ≥5.6–≤7.0 mmol/L or a random BG level 5.6–11.0 mmol/L were used.[16]

Participants were tested for BG level, erythrocyte GSH,

Table 1. Statistical characteristics of the groups studied.

	Parameter	Control	FH–DM	preDM	preDM+CVD	DM+CVD	DM	CVD
	Number (F/M)	49 (29/20)	27 (14/13)	41 (17/24)	34 (18/16)	42 (23/19)	30 (12/18)	43 (25/18)
	Age (years)	55	50	58	65	65	58	65
Oxidative stress	GSH (mg/dL)*	67±23	68±30	56±23	56±15	63±20	58±26	78±32
	MDA (nmol/mL)*	29±21	34±21	40±25	33±24	37±24	35±0.26	30±20
	MetHb (%)	0.75±0.56	0.59±0.31	0.72±0.41	0.76±0.50	0.79±46	0.81±0.36	0.66±0.35
Cholesterol	HDL (mmol/L)	1.42±0.45	1.27±0.31	1.22±0.33	1.40±0.68	1.21±0.38	1.33±0.49	1.48±0.61
	TC (mmol/L)	4.90±1.19	4.85±0.95	4.97±1.11	4.62±1.29	4.25±1.08	4.06±0.95	5.25±0.91
	TC:HDL ratio	3.66±1.19	4.07±1.12	4.40±1.31	4.33±2.09	3.71±1.27	3.34±1.12	3.96±1.17
Vascular event	D-dimer (µg/L)	154±2.30	177±2.3	175±3.0	279±3.0	252±2.3	264±3.0	247±200
	Hcy (µmol/L)	8.81±2.80	9.49±2.68	9.84±2.53	9.66±3.02	10.64±3.57	8.95±3.81	9.58±2.09
	V-low (mPas)†	81±32	79±42	98±37	84±39	96±47	84±32	102±36
Other factors	BGL (mmol/L)	4.50±0.71	4.77±0.59	5.63±0.88	5.79±1.35	7.39±2.91	7.23±2.81	4.67±0.36
	BP (mmHg)§	125±15	121±15	127±15	142±18	142±18	132±14	142±17

*Packed cell volume (erythrocyte); †V-low: viscosity at low shear rate; §systolic blood pressure. Hcy: homocysteine; TC: total cholesterol.

Table 2. Results of Fisher's LSD *post hoc* test for statistical differences between groups.

	Control	FH	preDM	preDM+CVD	DM+CVD	DM	CVD
Control		NS	0.05	0.02	NS	NS	NS
FH	NS		0.03	0.01	NS	0.04	NS
preDM	NS	NS		NS	NS	NS	0.0001*
preDM+CVD	0.007	NS	0.02		NS	NS	0.001*
DM+CVD	NS	NS	NS	NS		NS	0.02*
DM	0.003	0.04	0.006	NS	NS		0.001*
CVD	NS	NS	NS	NS	NS	0.04	

P values for GSH in unshaded cells; *P* values for D-dimer in shaded cells; NS: not significant.
*Statistical significance of GSH level lower in the CVD group.

MDA and methaemoglobin (metHb) as EOS indices, for plasma D-dimer, homocysteine and blood viscosity as a profile of associated vascular events, and for high-density lipoprotein (HDL)-cholesterol, total cholesterol (TC) and TC:HDL ratio as a cholesterol profile. Blood glucose level was measured using the Accu-Chek method. Erythrocyte GSH was determined by the 5,5-dithiobis-2-nitrobenzoic acid (DTNB) reaction method. Malondialdehyde and metHb were determined by the thiobarbituric acid reactive substances (TBARS) and cyanmethaemoglobin methods, respectively. Plasma D-dimer and homocysteine were determined by the MiniQuant (Biopool) and AxSYM (Abbott Laboratories) methods, respectively. Blood viscosity was measured using a Silenus viscometer. Cholesterol values were assayed using Cholestech LDX (Cholestech).

Factorial MANOVA was designed to investigate the multivariate statistical significance of changes in the EOS profile, the profile of vascular events and the conventional cholesterol profile at the different DM stages. Univariate post-hoc analysis was performed to determine which variables contributed to the statistical significance observed in the multivariate analysis. Analyses were performed using SPSS version 11.5 for Microsoft Windows.

To investigate the prevalence of significant biomarkers associated with diabetes pathogenesis, nine participants who showed progression in clinical or biochemical presentation over the 3.5-year study period were selected. Biochemistry results and other data were reviewed, with particular emphasis on emerging biomarkers that showed a positive correlation relative to the control group.

Results

The multivariate analysis based on observed means (Table 1) showed statistically significant differences between groups ($P<0.0001$). Univariate analyses showed statistically significantly differences between groups for GSH ($P<0.001$) and D-dimer ($P<0.0001$). Statistically significant differences were also observed between groups for TC and TC:HDL ratio.

With particular interest in antioxidant status and atherothrombosis, the results of a post-hoc analysis of erythrocyte GSH and plasma D-dimer are presented in Table 2. The results show statistically significant lower levels of GSH in the prediabetes group relative to the control group, CVD and FH-DM groups. Also highlighted is the fact that the hyperglycaemic groups (DM, DM+CVD, preDM and preDM+CVD) had statistically significant lower levels of GSH compared to the CVD group. There were no statistically significant differences between the normoglycaemic groups (control, CVD and FH-DM). Although there were no statistically significant differences between the hyperglycaemic groups (preDM, preDM+CVD, DM and DM+CVD), the DM+CVD group showed a slight increase in GSH compared to the other groups. Plasma D-dimer levels were higher in the DM group relative to the control, FH–DM and preDM groups, and in the preDM+CVD group relative to the control and preDM groups.

In each individual, all tested parameters that were abnormal relative to mean levels in the control group showed progression associated with elevated BG level, as shown in Table 3. Except where indicated, 'positive' is relative to the control group. Table 3 shows that all individuals who presented with disease progression and qualified for classification in the preDM group had abnormality in one or more of the oxidative stress indices, cholesterol profile and/or vasculopathy, in addition to the high initial BG level. Furthermore, when individuals with CVD were first identified to have high BG levels, abnormally low levels of erythrocyte GSH were also observed.

Eight out of the nine (89%) individuals who showed progression presented with low erythrocyte GSH levels. In addition, there were relatively high levels of D-dimer in four patients (44%), MDA in three patients (33%) and homocysteine in two patients (22%). High metHb and gross dyslipidaemia (including low HDL level, high TC and high TC:HDL ratio) were observed in an individual who already had a clinical diagnosis of CVD.

Discussion

Differences in GSH levels between the groups included in the present study are supported by similar results reported from a study of type 1 diabetics.[17] It is noteworthy, however, that the GSH levels were lower in the hyperglycaemic groups compared to the normoglycaemic groups. This may be further evidence for an association between low-level erythrocyte GSH and hyperglycaemia, which has been reported previously.[18–22] Based on the present results, it is likely that the observed oxidative stress is indicated by low levels of erythrocyte GSH in the prediabetes stage.

Plasma D-dimer level was significantly higher in the pre-

DM+CVD group compared to control group and highest in individuals with DM. It is also likely that vascular events indicative of macrovascular diabetic complications are observable prior to an established diagnosis of DM. This is consistent with the possibility that loss of GSH can compromise the erythrocyte's free-radical scavenging function,[19] which can lead to EOS and vascular events.[10] Present observations are also consistent with reports that erythrocyte GSH is significantly reduced in impaired glucose tolerance.[23,24]

Changes in the levels of OS biomarkers (GSH, MDA and metHb) were not linear or unidirectional with DM progression. This observation supports the report that antioxidant depletion is not proportional to the oxidative damage in DM.[20] Therefore, taking a decision on antioxidant status based on perceived oxidative damage is not recommended.

Erythrocyte MDA, metHb, plasma homocysteine, HDL and whole blood viscosity did not show statistical significance in multivariate analysis, although the concentration of MDA was higher in the DM groups compared to controls. In a pilot study, a significant difference between groups was noted, where the prediabetes group presented a significantly higher level of erythrocyte MDA and plasma homocysteine.[25]

Results from the present study lend additional support to an earlier report that showed elevated homocysteine levels in a subset of diabetes patients were likely to be associated with an increased risk of cardiovascular involvement and oxidative stress.[26] Therefore, its ongoing use as a marker for CVD in diabetes is warranted.

High BG level, associated with oxidative stress, predisposes individuals to future diabetes and its macrovascular sequelae.[27–30] In addition, current data demonstrate that GSH is most frequently associated with progression of diabetes and its macrovascular complications. This indicates that the incidence of depleted erythrocyte GSH in diabetic macrovascular pathogenesis and progression can be as high as 90%; thus, antioxidant deficiency of the erythrocyte may be a major factor in the development of diabetic complications.[18,19]

The present results indicate that changes in erythrocyte GSH and plasma D-dimer levels are statistically significant and, after adjusting for confounding factors, are a useful set of indices for oxidative damage associated with diabetes.

A set of indices is used for the assessment of subclinical CVD, which includes ankle-brachial index, carotid artery ultrasound and ECG, in addition to traditional risk factors.[5] However, these do not help in the identification of subclinical diabetic macrovascular complications, which is a challenge in prediabetes management.[31] This draws attention to the need for an aggressive diagnostic approach in asymptomatic diabetic individuals who have at most one conventional risk factor for macrovascular disease complications present.[32]

Currently, plasma D-dimer results are useful in excluding intravascular coagulation,[33] but it is not informative of either normal haemostasis or decreased fibrinolysis that could arise from hyperglycaemia-induced plasminogen activator inhibitor-1 activity. The importance for diagnostic pathology is that an oxidative stress panel, which includes BG level, erythrocyte GSH and D-dimer, creates more information and provides better interpretation of both negative and positive plasma D-dimer results.

For example, co-presentation of low plasma D-dimer level and high BG in a non-diabetic individual may be due to hyperglycaemia-induced hypofibrinolysis. A concomitant presentation of low erythrocyte GSH would indicate the likelihood of oxidative stress-induced hypercoagulation complicating the hypofibrinolysis, thereby providing evidence that a low or normal plasma D-dimer may not imply normal haemostasis.

In DM, the frontline cellular antioxidant is GSH.[29,34] The importance of a particular antioxidant therapy depends on the micro and macro environment at a specific time and also on the nature of the oxidant injury.[35] As the major oxidative injury arises from the depletion of cellular GSH, the option of glutathione supplementation in diabetes is worth consideration.

In the pharmacokinetics of vitamin E as an antioxidant, GSH is involved in its regeneration. Furthermore, GSH is a substrate of glutathione peroxidase and is involved indirectly in the process of recycling vitamin C – the antioxidant activity involved in the recycling of vitamin E.[34]

Table 3. Laboratory results at first and second screening visits in subjects with hyperglycaemia-associated disease progression.

Subject	First visit		Second visit	
	Former group	Positive results	Latter group	Positive results
1	FH–DM	Nil	preDM*	BGL, GSH
2	FH–DM	Total cholesterol, MDA	preDM*	Total cholesterol, BGL
3	CVD	BP, gross dyslipidaemia, homocysteine, methaemoglobin	preDM+CVD	BGL, GSH, gross dyslipidaemia
4	preDM*	BGL, GSH	preDM+CVD	BP, D-dimer, total cholesterol
5	preDM*	BGL, GSH, total cholesterol	preDM+CVD	BP, D-dimer
6	preDM+CVD	BGL, BP, GSH	DM+CVD	BGL, total cholesterol
7	preDM+CVD	BGL, BP, GSH	DM+CVD	BGL, BP, GSH, MDA
8	DM	BGL, BP, GSH, D-dimer, homocysteine, MDA	DM+CVD	BGL
9	DM	BGL	DM+CVD	BP, BGL, GSH, D-dimer

BGL: blood glucose level ≥5.6 mmol/L; BP: systolic blood pressure >140 mmHg; gross dyslipidaemia: HDL <1.0 mmol/L and total cholesterol >5.5 mmol/L; GSH: reduced erythrocyte glutathione; MDA: malondialdehyde.

*prediabetic state with abnormal levels >2 parameters.

When there is inadequate GSH, both vitamins E and C are inadequately regenerated. A pathophysiological effect of this is that unregenerated vitamin radicals will lead to deleterious pro-oxidant activity.[2] Thus, prevention of the pro-oxidant effects of the common antioxidant vitamins indicates the need for GSH supplementation.

It has been reported that enhancement of the renal glutathione system decreases arterial pressure markedly.[36] In light of this evidence of the effectiveness of GSH on vascular function, it is an advantage to investigate, via the oxidative stress panel used in the present study, whether or not the findings apply to diabetes management.

Blood glucose level, cholesterol profile, D-dimer and homocysteine have been adopted in clinical practice, although not as part of an oxidative damage panel. Different methods to determine erythrocyte GSH and MDA concentrations have been used in research, although a diversity of techniques has resulted in different ranges and units of value. For example, the present study showed a GSH range for the healthy control group that was higher (67±23 mg/dL) than the reference interval (24–37 mg/dL) provided by Lehmann and Henry.[37] These were not comparable because the former was based on erythrocyte membrane content, while the latter was based on whole blood. Present MDA results for the healthy control group were from adults and were much higher (29±21 nmol/mL) than the 1.30±0.04 nmol/mL reported for children.[38]

The present study provides reference values for healthy adult controls and people with a family history of diabetes, prediabetes and diabetes; however, it is limited by a small sample size in each group. Thus, it is recommended that appropriate reference values be determined from a larger data set.

In conclusion, BG level, erythrocyte GSH, erythrocyte MDA, plasma D-dimer and homocysteine constitute a useful oxidative stress panel. Furthermore, BG level, erythrocyte GSH and plasma D-dimer may constitute the minimum indices for an oxidative damage panel, while the addition of MDA, homocysteine and cholesterol profile would be advantageous. Given the prevailing involvement of oxidative stress in atherothrombosis and diabetic macrovascular pathogenesis, this proposed oxidative damage panel should be evaluated in clinical practice as a new diagnostic tool for the assessment and management of oxidative damage. □

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