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A preliminary evaluation of a novel method to monitor a triple antioxidant combination (vitamins E, C and α-lipoic acid) in diabetic volunteers using in vitro methaemoglobin formation☆

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Abstract

Eight otherwise healthy diabetic volunteers took a daily antioxidant supplement consisting of vitamin E (200 IU), vitamin C (250 mg) and α-lipoic acid (90 mg) for a period of 6 weeks. Diabetic dapsone hydroxylamine-mediated methaemoglobin formation and resistance to erythrocytic thiol depletion was compared with age and sex-matched non-diabetic subjects. At time zero, methaemoglobin formation in the non-diabetic subjects was greater at all four time points compared with that of the diabetic subjects. Resistance to glutathione depletion was initially greater in non-diabetic compared with diabetic samples. Half-way through the study (3 weeks), there were no differences between the two groups in methaemoglobin formation and thiol depletion in the diabetic samples was now lower than the non-diabetic samples at 10 and 20 min. At 6 weeks, diabetic erythrocytic thiol levels remained greater than those of non-diabetics. HbA_{1c} values were significantly reduced in the diabetic subjects at 6 weeks compared with time zero values. At 10 weeks, 4 weeks after the end of supplementation, the diabetic $HbA1_c$ values significantly increased to the point where they were not significantly different from the time zero values. Total antioxidant status measurement (TAS) indicated that diabetic plasma antioxidant capacity was significantly improved during antioxidant supplementation. Conversion of α-lipoic acid to dihydrolipoic acid (DHLA) in vivo led to potent interference in a standard fructosamine assay kit, negating its use in this study. This report suggests that triple antioxidant therapy in diabetic volunteers attenuates the in vitro experimental oxidative stress of methaemoglobin formation and reduces haemoglobin glycation in vivo.

Keywords: Methaemoglobin; Vitamins C and E; α-Lipoic acid; Dapsone hydroxylamine; Human

1. Introduction

Diabetic tissues are at risk of damage from three main threats: normal cellular reactive species production, hyperglycaemia-mediated reactive species formation and consequent attrition in antioxidant cellular defence mechanisms (Coleman, 2000). Although the precise details of how hyperglycaemia leads to tissue damage, organ failure and life-threatening vascular disease are still under investigation, it is clear that the reactivity of glucose leads to widespread non-enzymatic glycosylation which promotes deficits at virtually every stage of cellular metabolism (Bunn and Higgins, 1981; Baynes, 1991; Wautier et al., 1994; Baynes and Thorpe, 1999). Oxidant defence mechanisms in diabetics such as superoxide dismutase and the glutathione system are diminished in effectiveness by glycation, shortages in reducing power necessary for optimal enzyme function, as well as the high demand for reducing power caused by compensatory cellular responses to oxidative and carbonyl stresses present in diabetes (Coleman, 2000).

As complications effectively shorten diabetic lifespan by up to a decade (Skyler, 1996; Nathan et al., 1997) clinical efforts to control complications have been

☆ Dedicated to the memory of M.J. Winn, PhD.

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centred on maintaining precise glycaemic control, which is the main recommendation of large-scale trials in diabetic patients such as the: The Diabetes Control and Complications Trial (DCCT, 1993) and more recently the United Kingdom Prospective Diabetes Study (Stratton et al., 2000). In practice, many diabetics may well be unable to attain the standards of glycaemic control over many years which are necessary to significantly diminish their risks of developing some degree of complications. The use of antioxidants has been widely proposed to assist diabetics in avoiding complications, although studies to date have been less clinically effective than initially anticipated (Baynes and Thorpe, 1999; Coleman, 2001a). Agents such as ascorbate, α-lipoic acid and vitamin E have shown promise in diabetes (Ting et al., 1996; Borcea et al., 1999; Engelen et al., 2000) while other studies with some flavonoids have been unable to demonstrate such positive effects (Keenoy et al., 1999; Blostein-Fujii et al., 1999). Compounds such as α-lipoic acid have often been used at very high doses to ameliorate the effects of long-term severe complications, which may be biochemically beyond the limits of their effectiveness (Coleman et al., 2001a). Other problems include difficulties in demonstrating significant improvements in diabetic oxidant status during antioxidant therapy (Coleman, 2001b). Indeed, direct measurement of key parameters such as GSH concentrations have not always even shown consistent deficits in diabetes (Di Simplicio et al., 1995; Yoshida et al., 1995).

Therefore, in order to assess if a positive cellular impact is indeed occurring during antioxidant supplementation, it may be advantageous to explore dynamic indices of cellular resistance to oxidative stress, thus complementing other passive parameters of glycaemic control, such as HbA_{1c} and fructosamine as well as other parameters of antioxidant status, such as Total Antioxidant Status (TAS; Miller et al., 1993). Recent work has shown that in vitro xenobiotic-mediated methaemoglobin formation can be employed to delineate between the relative oxidative stress resistances of diabetic and non-diabetic erythrocytes (Coleman, 2000, 2001b). This preliminary report was designed firstly to evaluate the potential of in vitro methaemoglobin formation and consequent GSH depletion as a clinical tool to determine antioxidant effectiveness in diabetic subjects. Secondly, rather than employ high doses of single antioxidants, supplementation in this report was provided by modest doses of three major antioxidants, α-lipoic acid, ascorbate and vitamin E (Coleman, 2001a). Overall, the aim of this work was to evaluate whether a short 6 week course of these agents affected diabetic erythrocytic thiol levels during oxidative stress in vitro.

2. Materials and methods

2.1. Chemicals

Dapsone hydroxylamine (DDS-NHOH) was kindly supplied by Dr Malcolm Tingle (University of Auckland) and was greater than 98% pure. Vitamin E (100 IU in capsules), α-lipoic acid (30 mg capsules) and vitamin C (250 mg tablets) were manufactured and supplied by Holland and Barrett, Nuneaton, UK. Reagents for the glutathione assay were obtained from Sigma (Hydrochloric acid 20 mM, glutathione, 5-sulphosalycylic acid and 5,5′-dithiobis 2-nitrobenzoic acid). All other reagents, such as dimethyl sulphoxide were supplied by Sigma, UK. Glycosylated haemoglobin (HbA_{1c}) and fructosamine concentrations were assayed using Sigma Diagnostic Kits 441-B (now discontinued) and 465-A, respectively, while TAS was measured using a Randox 2332 kit (Randox Laboratories, Crumlin, UK), which is based on a previous method (Miller et al., 1993).

2.2. Blood sample preparation

Whole human venous blood samples were obtained from the volunteers (non-fasted) and a small aliquot allowed to clot, then centrifuged at $1121 \times g$ for 10 min to separate the serum, which was subsequently frozen at $-20\,^{\circ}C$ and later analysed for fructosamine concentrations. The remainder of the blood samples were anticoagulated with lithium heparin. A further small aliquot was refrigerated prior to HbA_{1c} assay the following day. The remainder was then centrifuged for 11 min at $1121 \times g$ and some plasma retained and stored at $-20\,^{\circ}C$ for TAS analysis. The erythrocytes were then washed with two equal volumes of 15 mM HEPES buffer containing 10 mM glucose, then made up to a 50% haematocrit.

2.3. Experimental design

Eight diabetic volunteers (four male, four female, mean insulin dosage, 0.72 ± 0.18 U/kg per day) were recruited for the study (aged 26 ± 4 years) and non-diabetic age and sex-matched (aged 26 ± 4 years) volunteers were also asked to provide blood samples for the study. The study received ethical approval from Aston University Human Research Committee and all subjects provided written informed consent. The diabetic volunteers were all healthy type I insulin-dependent individuals, not suffering from any complications and taking no concurrent medication. The non-diabetic volunteers did not take any medication and did not receive antioxidants during the study. After the removal of a time zero blood sample, the diabetic volunteers were issued with 6 weeks supply of antioxidants and asked to take 200 IU vitamin E, 250 mg ascorbic acid and 90 mg

α-lipoic acid with food to maximise absorption (Ceriello et al., 2001), daily for 6 weeks (42 days). At the end of 3, 6 and 10 weeks blood samples were drawn from the diabetic and non-diabetic volunteers as described above.

2.4. Experimental methods

Quadruplicate aliquots of washed blood (500 μl) from diabetic and non-diabetic volunteers in Eppendorf tubes were cooled to 4 °C on ice and 5 μl of a 15mM stock solution in acetone of DDS-NHOH was added to the aliquots, yielding a final concentration of 0.15 mM DDS-NHOH. Control tubes received 5 μl acetone. After mixing by gentle inversion, the tubes were placed in a water bath maintained at 37 °C and tubes were removed at 5, 10 15 and 20 min and replaced on ice. Methaemoglobin formation was measured using an IL-482 CO-oximeter (Instrumentation Laboratories, Warrington, UK). A 50 μl aliquot was removed from each tube for total thiol assay according to the GSH method of Anderson (1985)

2.5. Analysis interference

α-Lipoic acid is converted to dihydrolipoic acid (DHLA) in vivo (Biewenga et al., 1997) and this agent is known to interfere strongly with the thiol assay (Coleman and Baker, 2001). This assay therefore measures total thiol content and is not specific for GSH. In addition, although the proprietary literature states that GSH causes slight interference with the Sigma fructosamine assay, it was found in this study that the dithiol DHLA, but not α-lipoic acid itself, was potent enough to interfere with this assay at concentrations as low as 8 μM, but not at the level of 1 μM. Hence, fructosamine values that had been determined at 3 and 6 weeks in this study were discarded. Neither α-lipoic acid, nor DHLA were found to interfere with the HbA_{1c} assay.

2.6. Data analysis

Methaemoglobin formation was expressed as a percentage of the haemoglobin present in the sample and is listed graphically as mean ± S.D. Total thiols were expressed as a percentage of the non-diabetic values which were regarded as 100%. The thiol values are represented graphically as mean ± S.D. Statistical analysis was carried out using a one-way ANOVA and the Tukey–Kramer Multiple Comparisons Test.

3. Results

3.1. Methaemoglobin studies

At time zero, after the addition of DDS-NHOH to the samples, methaemoglobin formation at 37 °C in the non-diabetic subjects was significantly greater at all four time points compared with that of the diabetic subjects ($P < 0.01$; Fig. 1A). 3 weeks later, there were no significant differences between the two groups in methaemoglobin formation (Fig. 1B). At 6 weeks, the non-diabetic levels of haemoglobin oxidation were once again significantly greater compared with the those of the diabetic subjects (Fig. 2A). The differences between the groups were greater at 6 weeks, than at time zero ($P < 0.05$). At 10 weeks, 4 weeks after the subjects had completed their antioxidant courses, again, non-diabetic methaemoglobin levels were significantly greater than those of the diabetic subjects ($P < 0.001$; Fig. 2B),

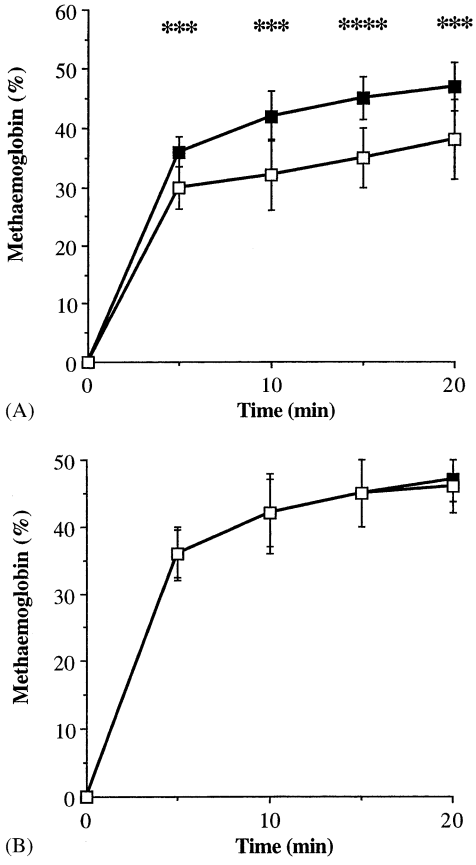

Fig. 1. Methaemoglobin (%) formation in vitro in non-diabetic (■) and diabetic (□) erythrocytes isolated from volunteers. (A) Time zero. (B) After 3 weeks of daily supplementation of the diabetics with vitamin C (250 mg), vitamin E (200 IU) and α-lipoic acid (90 mg). Values assayed in quadruplicate per subject, data represented as mean ± S.D., n = 4 per point; *; **; ***; **** denotes $P < 0.05$; 0.01; 0.001; 0.0001 for significant differences between the observations from the non-diabetic and diabetic volunteers blood.

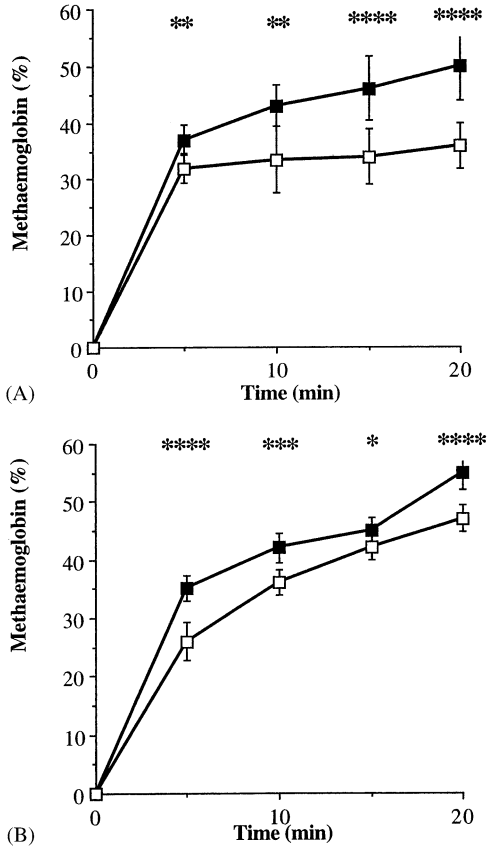

Fig. 2. Methaemoglobin (%) formation in vitro in non-diabetic (■) and diabetic (□) erythrocytes isolated from volunteers. (A) After 6 weeks of daily supplementation of the diabetics with vitamin C (250 mg), vitamin E (200 IU) and α-lipoic acid (90 mg). (B) 4 weeks after the end of supplementation. Values assayed in quadruplicate per subject, data represented as mean ± S.D., n = 4 per point; *; **; ***; **** denote $P < 0.05$; 0.01; 0.001; 0.0001 for significant differences between the observations from the non-diabetic and diabetic volunteers blood.

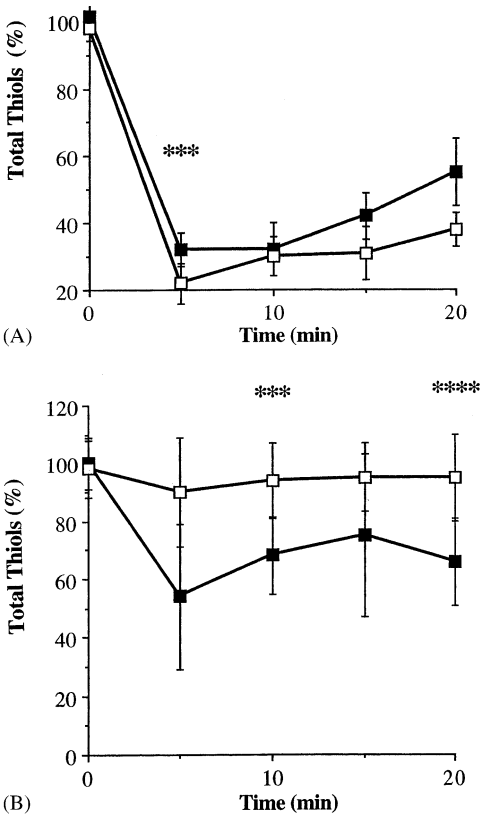

Fig. 3. Total thiol levels in vitro during dapsone hydroxylamine-mediated methaemoglobin formation in vitro in non-diabetic (■) and diabetic (□) erythrocytes isolated from volunteers. (A) Time zero. (B) After 3 weeks of daily supplementation of the diabetics with vitamin C (250 mg), vitamin E (200 IU) and α-lipoic acid (90 mg). Data expressed as a percentage of non-diabetic resting thiol levels. Values assayed in quadruplicate per subject, data represented as mean ± S.D., n = 4 per point; **; ***; denotes $P < 0.01$; 0.001; for significant differences between the observations from the non-diabetic and diabetic volunteer thiol levels.

although this difference was not significantly different from time zero.

3.2. Thiol studies

Control thiol levels at time 0, 3, 6 and 10 weeks showed no significant differences between diabetic and non-diabetic erythrocytes. Total measured thiol levels at time zero after DDS-NHOH addition, showed a greater than 50% fall from control values within 5 min in both diabetic and non-diabetic samples. There was a significantly greater depletion in thiol levels at 5 min in diabetic compared with non-diabetic samples ($P < 0.05$; Fig. 3A) although there were no significant differences at 10, 15 and 20 min between the groups. At 3 weeks, thiol levels in the diabetic samples were greater than those of non-diabetic subjects at 10 and 20 min ($P < 0.001$; Fig. 3B), although there was no significant difference at 5 and 15 min. At 6 weeks, thiol levels in the diabetic samples were significantly greater compared with those of non-diabetics in three (10, 15 and 20) of the four time points (Fig. 4A; $P < 0.01$), although at 10 weeks, there were no significant differences between the diabetic and non-diabetic groups in thiol concentrations (Fig. 4B).

3.3. Other markers

At 3 weeks, there was no significant reduction in the diabetic subjects in their mean HbA_{1c} values (7.5 ± 1.7%) compared with their time zero values (9.5 ± 1.6%), however, at 6 weeks the diabetic HbA_{1c} values were significantly lower (6.6 ± 1.5%, $P < 0.05$) than those of time zero (9.5 ± 1.6%), but were not significantly different from the 3-week values. At 10 weeks, the mean HbA_{1c} values had risen to levels (10.1 ± 2.4%) which were significantly higher than the 6-week values (6.6 ± 1.5%, $P < 0.01$), but not significantly different from those of time zero (9.5 ± 1.6%). Non-diabetic HbA_{1c} values ranged from 2.5 to 3.5% over the time of the study. TAS values for the diabetics at time zero (1.2 ±

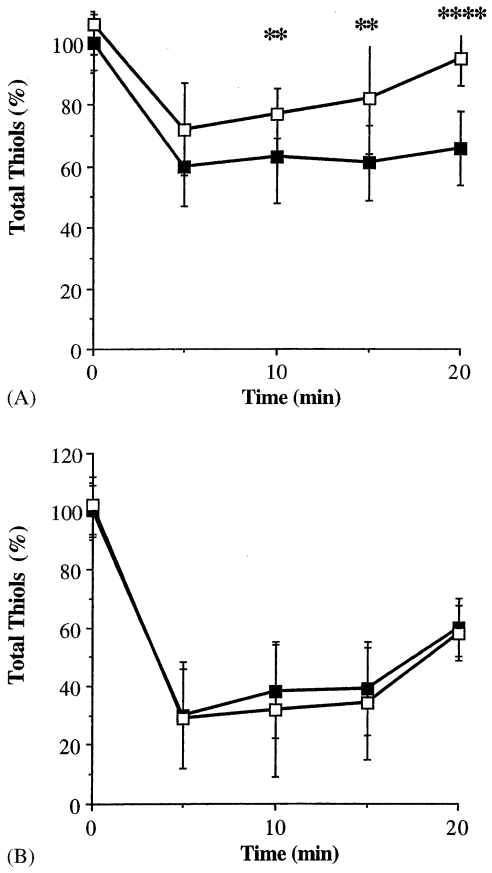

Fig. 4. Total thiol levels in vitro during dapsone hydroxylamine-mediated methaemoglobin formation in vitro in non-diabetic (■) and diabetic (□) erythrocytes isolated from volunteers. (A) After 6 weeks of daily supplementation of the diabetics with vitamin C (250 mg), vitamin E (200 IU) and α-lipoic acid (90 mg). (B) 4 weeks after the end of supplementation. Data expressed as a percentage of non-diabetic resting thiol levels. Values assayed in quadruplicate per subject, data represented as mean ± S.D., n = 4 per point; *; **; ***; denotes $P <$ 0.05; 0.01; 0.001; for significant differences between the observations from the non-diabetic and diabetic volunteer thiol levels.

0.16 mmol/l) were significantly lower than those of the non-diabetics (1.4 ± 0.2 mmol/l). After three (1.39 ± 0.24 vs. 1.42 ± 0.13 mmol/l) and 6 weeks (1.41 ± 0.13 vs. 1.44 ± 0.17 mmol/l), there were no significant differences between the two groups. At 10 weeks, the differential between the diabetic (1.24 ± 0.10 mmol/l) and non-diabetic subjects (1.45 ± 0.12 mmol/l) was again significant ($P < 0.05$). There were no significant differences between fructosamine values at time zero, for the diabetic subjects (1.78 ± 0.3 mmol/l) and at week 10, (1.7 ± 0.3 mmol/l). Non-diabetic values for fructosamine ranged from 0.9 to 1.8 mmol/l during the study.

4. Discussion

Many individual measured parameters have been employed successfully for the determination of the extent of oxidative stress in diabetic patients. These include enzyme activities, such as superoxide dismutase and catalase, as well as concentrations of other indices of oxidative stress such as thiobarbituric acid reactive substances (TBARS), TAS, LDL oxidation, GSH and ascorbate concentrations. The proposed use of the process of methaemoglobin formation as an index of resistance to oxidative stress is based on a number of factors. Erythrocytes are long-lived cells and it is established by the usage of HbA_{1c} that they reflect the adaptations and attritions which are consequences of hyperglycaemia. They can be easily sampled and contain high concentrations of glutathione which can be assayed rapidly and cheaply. The reaction of dapsone hydroxylamines with oxyhaemoglobin is thought to proceed as a co-oxidation, where the hydroxylamines are converted to nitrosoarenes and the oxyhaemoglobin to methaemoglobin; it is believed that GSH makes the major contribution to the reduction of the nitrosoarene to the hydroxylamine, which then reacts with another oxyhaemoglobin molecule in a cyclic manner (Kiese, 1966; Kramer et al., 1972). Eventually, the reaction of GSH with hydroxylamines results in the formation of the parent amine (Heilmair et al., 1991; Coleman and Jacobus, 1993). Normally, the rate-limiting step in the velocity of DDS-NHOH-mediated methaemoglobin formation is the erythrocyte's ability maintain intracellular GSH levels, which in turn sustain the oxidative process of haemoglobin oxidation (Coleman, 2000; Kiese, 1966; Scott and Rasbridge, 1973). During the intense stress of the methaemoglobin process it is likely that demand exceeds supply for GSH in diabetic erythrocytes, probably due to glycation at critical points in the GSH system; thus methaemoglobin formation is lower in diabetic erythrocytes compared with non-diabetics and erythrocytic thiol maintenance under the stress of the oxidative process is inferior to that of non-diabetics (Blakynty and Harding, 1992; Coleman and Baker, 2001; Coleman et al., 1994, 1996, 1998, 2001b; Coleman and Walker, 2000).

In this report, the reduction in methaemoglobin formation seen in the diabetic subjects at time zero was also reflected in an initial lower resistance to GSH depletion compared with non-diabetic erythrocytes. TAS levels were also lower in the diabetics compared with the non-diabetic volunteers, as has been shown previously (Seghrouchni et al., 2002). However, antioxidant supplementation appeared to prevent thiol levels in diabetics from falling in a progressive manner and also initially removed the differential in methaemoglobin formation, then paradoxically restored it at 6 weeks. The differential in TAS levels between the two subject groups was also removed by antioxidant supplementation. Interestingly, despite a considerable period of supplementation, measured resting total thiol levels in

the diabetic erythrocytes were not significantly different from those of non-diabetics.

Previous studies in vitro with diabetic erythrocytes, α-lipoic acid and DHLA showed a similar pattern, in that resting thiol levels were unchanged by these agents although intracellular thiol levels did not become depleted during methaemoglobin formation and measured methaemoglobin levels themselves were lower in the presence of these agents (Coleman et al., 2001a; Coleman and Baker, 2001; Coleman et al., 2001b). In this study, as in previous work, it is likely that α-lipoic acid and DHLA directly attenuated the hydroxylamine-mediated methaemoglobin formation process in a concentration-dependent manner and thus prevented the consumption of thiol which would normally occur in their absence. At 3 weeks, where the differential in methaemoglobin formation between the two experimental groups had been removed and non-diabetic haemoglobin oxidation had not decreased compared with time zero, it might be speculated that the concentrations of the DHLA and the other antioxidants present in the diabetic erythrocytes were sufficient to restore the deficiency in intracellular thiol maintenance without directly inhibiting methaemoglobin formation, so restoring methaemoglobin formation to parity with non-diabetics.

Although ascorbate also prevented thiol depletion in vitro, in contrast to DHLA and α-lipoate, ascorbate directly increased methaemoglobin formation in vitro at equivalent concentrations (Coleman et al., 2001b). As there was a net fall methaemoglobin formation in the presence of the antioxidants at 6 weeks in the diabetic erythrocytes in this study, it could be speculated that either by the end of the study, erythrocytic concentrations of DHLA and α-lipoic acid were greater than those of ascorbate, or that the thiols exerted a more potent intracellular effect at this dosage regimen. It is apparent from the progressive nature of methaemoglobin level reduction and thiol maintenance that stabilisation of the erythrocytic levels of the different supplements took some time to occur and may not have been completed in the short duration of this preliminary study.

Although HbA_{1c} is a measure of hyperglycaemia-mediated glycation, this report indicates that the antioxidant combination attenuates in vitro an oxidatively stressful process such as methaemoglobin formation as well as reducing haemoglobin glycation in vivo. Perhaps this combination of agents may not attenuate all damaging oxidative process, however, it is possible that antioxidant therapy may ameliorate hyperglycaemia-mediated glycation in vulnerable tissues such as the kidneys and vascular system (Borcea et al., 1999; Coleman et al., 2001a).

In previous studies, monoacetyl dapsone hydroxylamine (MADDS-NHOH) was used instead of DDS-NHOH to form methaemoglobin in vitro and diabetic erythrocytic thiol concentrations were depleted at early time points, while non-diabetic erythrocytes showed no depletion (Coleman and Baker, 2001; Coleman et al., 2001b). In the present study, DDS-NHOH caused significant depletion in non-diabetic erythrocytes also, which is in broad agreement with an earlier study with DDS-NHOH (Coleman and Jacobus, 1993). Over the initial 5–10 min of the methaemoglobin reaction process, it is possible that DDS-NHOH may be a more potent GSH depletor than MADDS-NHOH.

In summary, during the 6-week period, a daily supplement of a triple antioxidant combination was associated with a significant reduction in HbA_{1c} values and an improvement in plasma antioxidant capacity in a small group of diabetic subjects. The presence of α-lipoic acid in the combination negated the use of fructosamine values as an indicator of glycaemic control and led to a progressive fall in methaemoglobin formation and a consequent preservation of erythrocytic thiol levels. Future studies will concentrate on establishing the time to reach antioxidant 'steady state' and the relationship between in vivo HbA_{1c} and in vitro methaemoglobin and intracellular thiol values.

References

Anderson, M.E., 1985. Determination of GSH and GSH disulfide in biological samples. Methods Enzymol. 113, 548.

Baynes, J.W., 1991. Role of oxidative stress in development of complications in diabetes. Diabetes 40, 405.

Baynes, J.W., Thorpe, S.R., 1999. Role of oxidative stress in diabetic complications a new perspective on an old paradigm. Diabetes 48, 1.

Biewenga, G.P., Haenen, G.R.R.M., Bast, A., 1997. The pharmacology of the antioxidant lipoic acid. Gen. Pharmacol. 29, 315.

Blakynty, R., Harding, J.J., 1992. Glycation (non-enzymatic glycosylation) inactivates glutathione reductase. Biochem. J. 288, 303.

Blostein-Fujii, A., DiSilvestro, R.A., Frid, D., Katz, C., 1999. Short term citrus flavonoid supplimentation of type II diabetic women: no effect on lipoprotein oxidation tendencies. Free Radic. Res. 30, 315.

Borcea, V., Nourooz-Zadeh, J., Wolff, S.P., Klevesath, M., Hofmann, M., Urich, H., Wahl, P., Ziegler, R., Tritschler, H.J., Halliwell, B., Nawroth, P.P., 1999. Alpha-lipoic acid decreases oxidative stress even in diabetic patients with poor glycaemic control and albuminuria. Free Radic. Biol. Med. 26, 1495.

Bunn, H.F., Higgins, P.J., 1981. Reaction of monosaccharides with proteins-possible evolutionary significance. Science 213, 222.

Ceriello, A., Bortolotti, N., Motz, E., Lizzio, S., Catone, B., Assaloni, R., Tonutti, L., Taboga, C., 2001. Red wine protects diabetic patients from meal induced oxidative stress and thrombosis activation: a pleasant approach to the prevention of cardiovascular disease in diabetes. Eur. J. Clin. Invest. 31, 322.

Coleman, M.D., 2000. 'Commentary' the use of in vitro methaemoglobin generation to study antioxidant status in the diabetic erythrocyte. Biochem. Pharmacol. 60, 1409.

Coleman, M.D., 2001a. Editorial: 'antioxidants: unlocking their potential'. Environ. Toxicol. Pharmacol. 10, 139a.

Coleman, M.D., 2001b. Monitoring diabetic antioxidant status: a role of in vitro methaemoglobin formation. Environ. Toxicol. Pharmacol. 10, 207.

Coleman, M.D., Baker, C.D., 2001. Effects if the antioxidants dihydrolipoic acid and probucol on xenobiotic-mediated methaemoglobin formation in human diabetic and non-diabetic erythrocytes in vitro. Environ. Toxicol. Pharmacol. 9, 161.

Coleman, M.D., Walker, C.L., 2000. Effects of the antioxidants α-lipoic acid and α-tocopherol on xenobiotic-mediated methaemoglobin formation in human diabetic and non-diabetic erythrocytes in vitro. Environ. Toxicol. Pharmacol. 8, 127.

Coleman, M.D., Jacobus, D.P., 1993. Reduction of dapsone hydroxylamine to dapsone during methaemoglobin formation in human erythrocytes in vitro. Biochem. Pharmacol. 45, 1027.

Coleman, M.D., Simpson, J., Jacobus, D.P., 1994. Reduction of dapsone hydroxylamine to dapsone during methaemoglobin formation in human erythrocytes in vitro. III: effect of type I diabetes. Biochem. Pharmacol. 48, 1341.

Coleman, M.D., Ogg, M.S., Holmes, J.L., Gardiner, J.M., Jacobus, D.P., 1996. Studies on the differential sensitivity between diabetic and non-diabetic human erythrocytes to monoacetyl dapsone hydroxylamine-mediated methaemoglobin formation in vitro. Environ. Toxicol. Pharmacol. 1, 97.

Coleman, M.D., Hayes, P.J., Jacobus, D.P., 1998. Methaemoglobin formation due to nitrite, disulfiram, 4-aminophenol and monoacetyldapsone hydroxylamine in diabetic and non-diabetic human erythrocytes in vitro. Environ. Toxicol. Pharmacol. 5, 61.

Coleman, M.D., Eason, R.C., Bailey, C.J., 2001a. The therapeutic use of lipoic acid in diabetes: a current perspective. Environ. Toxicol. Pharmacol. 10, 167.

Coleman, M.D., Tolley, H.L., Desai, A.K., 2001b. Monitoring antioxidant effects using methaemoglobin formation in diabetic erythrocytes. Br. J. Diab. Vas. Dis. 1, 88.

Di Simplicio, P., De Georgio, L.A., Cardaioli, E., Lecis, K., Miceli, M., Rossi, R., Anichini, R., Mian, R., Seghieri, G., Franconi, F., 1995. Glutathione, glutathione utilizing enzymes and thioltransferase in platelets of insulin-dependent diabetic patients: relation with platelte aggregation and microangiopatic complications. Eur. J. Clin. Invest. 25, 665.

Diabetes Control and Complications Trial Research Group, 1993. The effect of intensive diabetes treatment on the development and progression of long-term complications in insulin-dependent diabetes mellitus: The Diabetes Control and Complications Trial. New Engl. J. Med. 329, 977.

Engelen, W., Keenoy, B.M.Y., Vertommen, J., De Leeuw, I., 2000. Effects of long-term supplementation with moderate pharmacological doses of vitamin E are saturable and reversible in patients with Type 1 diabetes. Am. J. Clin. Nutr. 72, 1142.

Heilmair, R., Karreth, S., Lenk, W., 1991. The metabolism of 4-aminobiphenyl in the rat II. Reaction of 4-hydroxy-4-aminobiphenyl with rat blood in vitro. Xenobiotica 21, 805.

Keenoy, B.M.Y., Vertommen, J., De Leeuw, I., 1999. The effect of flavonoid treatment on the glycation and antioxidant status in Type I diabetic patients. Diab. Nutr. Metab. 12, 256.

Kiese, M., 1966. The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines, and mechanisms of ferrihemoglobin formation. Pharmacol. Rev. 18, 1091.

Kramer, P.A., Glader, B.E., Li, T.K., 1972. Mechanism of methaemoglobin formation by diphenylsulphones. Biochem. Pharmacol. 21, 1265.

Miller, N.J., Rice-Evans, C., Davie, M.J., Gopinathan, V., Milner, A., 1993. A novel method for measuring antioxidant capacity and its application for monitoring the antioxidant status in premature neonates. Clin. Sci. 84, 407.

Nathan, D.M., Meigs, J., Singer, D.E., 1997. The epidermology of cardiovascular disease in type 2 diabetes mellitus: how sweet it is . . . or is it. Lancet 350, 4.

Scott, G.L., Rasbridge, M.R., 1973. The in vitro action of dapsone and its derivatives on normal and G6PD-deficient red cells. Br. J. Haematol. 24, 307.

Seghrouchni, I., Drai, J., Bannier, E., Riviere, J., Calmard, P., Garcia, I., Orgiazzi, J., Revol, A., 2002. Oxidative stress parameters in type I, type II and insulin-treated type-2 diabetes mellitus; insulin treatment efficiency. Clin. Chim. Acta 321, 89.

Skyler, J., 1996. Diabetic complications: the importance of glucose control. In: Brownlee, M.B., King, G.L. (Eds.), Endocrinology and Metabolism. 1996 Clinics of North America. WB Saunders.

Stratton, I.M., Adler, A., Neil, H.A.W., Matthews, D., Manely, S., Cull, A.C., Hadden, D., Turner, R.C., Holman, R.R., 2000. Association of glycaemia with microvascular and microvascular complications of type 2 diabetes (UKPDS 35): a prospective observational study. Br. Med. J. 321, 405.

Ting, H.H., Timimi, F.K., Boles, K.S., Creager, S.J., Ganz, P., Creager, M.A., 1996. Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. J. Clin. Invest. 97, 22.

Wautier, J.L., Wautier, M.P., Anderson, G.M., Hori, O., Zoukourian, C., Capron, L., Chappey, O., Yan, S.D., Brett, J., Guillausseau, P.J., Stern, D., 1994. Advanced glycation end products (AGEs) on the surface of diabetic erythrocytes bind to the vessel wall via a specific receptor inducing oxidant stress in the vasculature: a link between surface-associated AGEs and diabetic complications. Proc. Natl. Acad. Sci. USA 91, 9441.

Yoshida, K., Hirokawa, J., Tagami, S.S., Kawakami, Y., Urata, Y., Kondo, T., 1995. Weakened cellular scavenging activity against oxidative stress in diabetes mellitus: regulation of glutathione synthesis and efflux. Diabetologia 38, 201.