

The effects of garlic extract upon endothelial function, vascular inflammation, oxidative stress and insulin resistance in adults with type 2 diabetes at high cardiovascular risk. A pilot double blind randomized placebo controlled trial

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Abstract

Background and aims

Endothelial dysfunction, vascular inflammation and oxidative stress have been integrally linked to the pathogenesis of both type 2 diabetes and cardiovascular disease. Aged Garlic Extract (AGE), a potent antioxidant, has been shown in previous studies to attenuate these novel risk factors in a non-diabetic population.

Aims

This study tested the hypothesis that AGE may improve endothelial function, oxidative stress, vascular inflammation and insulin resistance in high risk cardiovascular subjects with type 2 diabetes.

Methods

A double blind, placebo controlled crossover pilot study was performed in 26 subjects with type 2 diabetes who received 1200 mg of AGE or placebo daily for 4 weeks with a 4 week washout period. Plasma HsCRP was measured as a marker of inflammation. Plasma TAOS, blood GSH/GSSG and plasma LHP were measured as markers of oxidative stress/anti-oxidant defense. Insulin resistance was measured using the HOMA-IR method. Endothelial function was measured using change in the reflective index (RI) post-salbutamol using digital photoplethysmography and urinary albumin/creatinine ratio was measured as a biochemical surrogate. Measurements were taken at baseline and after intervention with AGE or placebo.

Results

Of the 26 patients studied (male 17, female 9), age was 61 ± 8 years (mean \pm 1 SD), HbA1c $7.2 \pm 1.1\%$, BP $130/75 \pm 15.9/9.8$ mmHg, total cholesterol 4.2 ± 0.81 mmol/l, triglyceride 2.11 ± 1.51 mmol/l, and HDL cholesterol 1.04 ± 0.29 mmol/l. The majority of patients were being treated with metformin (59%), aspirin (50%) and statin (96%) therapy. 36% were treated with an ACEI. There were no changes in these therapies throughout the study.

Treatment with AGE had no significant effect upon the above metabolic parameters including insulin resistance. Treatment with AGE also had no significant effect on markers of endothelial function (plethysmography), oxidative stress (TAOS, GSH/GSSG, LHP) or inflammation (HsCRP).

Conclusion

In this group of type 2 diabetic patients at high cardiovascular risk, 4 weeks treatment with AGE did not significantly improve endothelial function, vascular inflammation, oxidative stress or insulin resistance.

1. Introduction

Adequate treatment of the traditional risk factors for vascular disease is given equal priority to blood glucose control in patients with diabetes. This has given significant improvements in expected lifespan in type 2 diabetes. However, cardiovascular and cerebrovascular diseases remain responsible for 80% of diabetes related mortality (Campbell, Newton, Patel, Jacobs, & Gapstur, 2012).

Many of the traditional risk factors share similar underlying biochemical processes such as oxidative stress, vascular inflammation and endothelial dysfunction which could explain their contribution to the complications of diabetes. Further consideration shows these processes to be present even before the development of diabetes (Lüa et al., 2009, Perticone et al., 2008, Su et al., 2008a and Su et al., 2008b) and they also seem to have a fundamental role in the pathogenesis of diabetic complications (see Fig. 1). This study investigates whether treatment with an antioxidant can affect these novel risk factors in a typical diabetes outpatient population.

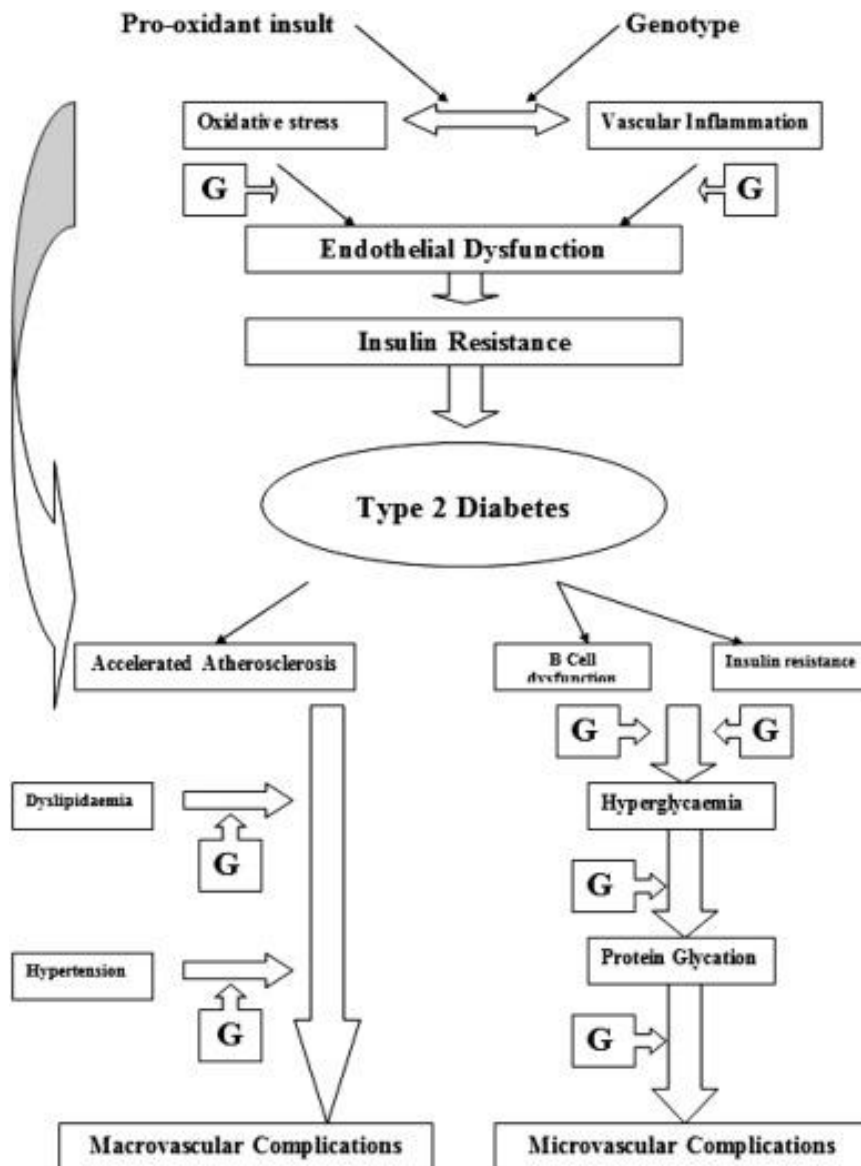


Fig. 1. Diagram to show the common soil hypothesis of the inter-relationship between oxidative stress, vascular inflammation and endothelial dysfunction in type 2 diabetes. The points at which garlic may be effective are marked with a G (Ahmad et al., 2006, Liu et al., 2007, Ried et al., 2013, Vazquez-Prieto et al., 2010, Wang et al., 2015 and Williams et al., 2005).

Aged Garlic Extract (AGE) is prepared by storing sliced raw garlic in 15–20% ethanol solution for 20 months at room temperature. It is administered either in liquid form or capsules. It has been shown to be a potent antioxidant (Imai et al., 1994) and has beneficial effects on markers of oxidative stress (Dillon et al., 2002), inflammation (Budoff et al., 2009) and endothelial function (Weiss et al., 2006) in vitro or animal models. AGE has been used in previous human studies and has been shown to be safe (Nakagawa et al., 1984).

2. Methods

2.1. Subjects

26 subjects with type 2 diabetes who were deemed to be at high cardiovascular risk (deemed to be at 30% risk of a cardiovascular event within the next 10 years using a prediction algorithm (Wilson et al., 1998)) were recruited between 2007 and 2009. All subjects gave written informed consent. The Hampshire and Isle of Wight Research Ethics Committee gave their approval for this study.

The inclusion criteria included type 2 diabetes patients aged between 18 and 70 years, who were not treated with insulin. Exclusion criteria included established cardiovascular or cerebrovascular disease and treatment with insulin or warfarin (Table 1).

Table 1

Baseline subject characteristics

Mean age	Mean duration of diabetes	% of smokers	Male	Mean BMI	Mean baseline HbA1c
49.8years	4.9years	25	72%	32	7.2%

2.2. Protocol

Physical examination was undertaken and baseline measurements taken (BMI and BP). An ECG was also performed to exclude occult ischaemic heart disease. Baseline fasting investigations included measurements of plasma lipids (total cholesterol, high density lipoprotein cholesterol and triglyceride) serum urea and serum electrolytes, liver function tests and INR. Glycemic control was measured using HbA1c and fructosamine. Measurements were also made of insulin resistance, endothelial function, vascular inflammation and oxidative stress as described below.

Once baseline assessments had been concluded, the subjects were given either Aged Garlic Extract (kyolic) or a placebo. Double blind, randomized allocation of the placebo or garlic treatment was undertaken. Subjects took 4 capsules per day (1200 mg) for 4 weeks. There was then a 4 week washout period and then the subjects entered the crossover arm (see Fig. 2). Compliance levels were monitored with a tablet count at the end of each 4 week treatment period.

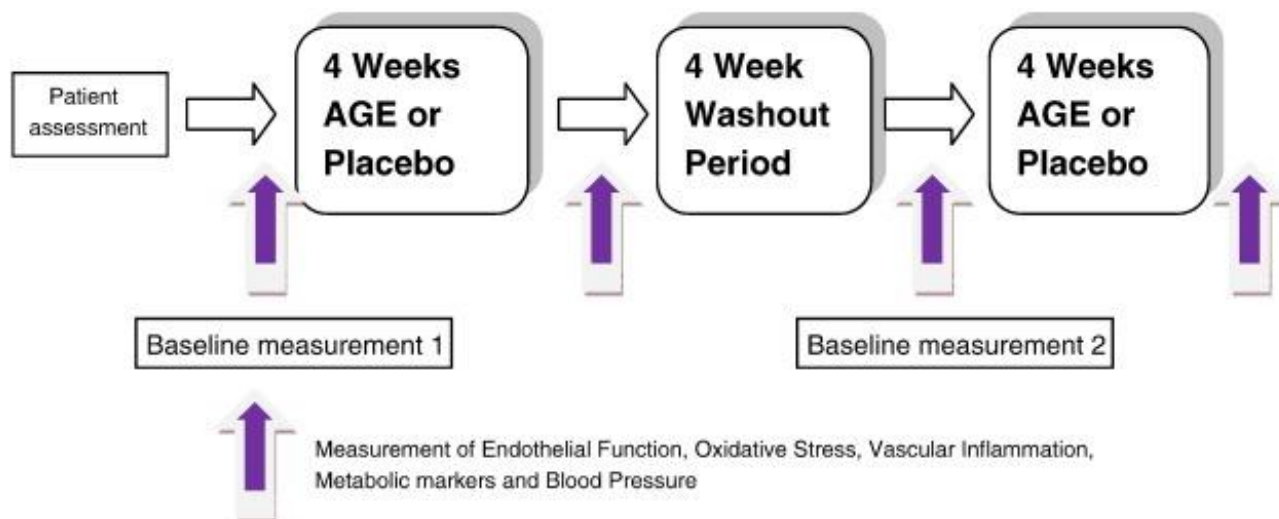


Fig. 2. Diagram of clinical protocol.

2.3. Photoplethysmography

Digital photoplethysmography is a non-invasive measurement of vasoactive endothelial function.

Measurements were made to determine the digital volume waveform [DVW] using the photoplethysmography apparatus (Micro Medical Pulse Trace, Rochester, Kent, UK). This technique was previously described and validated in diabetic and non-diabetic populations by Chowienczyk et al. (1999). This technique has also been validated against macrovascular brachial FMD (Rambaran et al., 2008).

Each subject had the probe attached to an index finger for 20 minutes, resting supine, before measurements were taken. Digital pulse wave readings were taken at baseline and the software calculated reflective index (RI). This was then repeated following administration of a sublingual 500 mcg dose of GTN. GTN is an endothelium-independent vasodilator and thus acted as a control. These readings were then repeated following inhaled salbutamol (an endothelium dependent vasodilator). Three readings were taken at baseline and an average taken. Readings were taken at 3 and 5 minutes. A washout period of 20 minutes was allowed after which another reading was taken to confirm the return to baseline. Inhaled salbutamol (400 mcg) was administered using a standardized technique via a spacer device, and readings taken at 10, 12 and 15 minutes. An average of the readings was taken.

2.4. Assays

Metabolic markers including fasting glucose, insulin, HOMA-IR, fructosamine, lipid profile (total cholesterol, HDL cholesterol and triglycerides), liver function tests, urea and electrolytes were also measured at each visit.

HbA1c was measured by HPLC (Menarini Diagnostics, Wokingham, UK). Plasma total cholesterol concentration was measured by esterase and oxidase conversion (Advia 1650, Bayer Diagnostics, Newbury,

UK) and HDL cholesterol and plasma triglyceride concentration by enzymatic determination (Advia 1650, Bayer Diagnostics, Newbury, UK). The intra-assay coefficient of variation of these assays was < 2%.

Samples taken for HsCRP and oxidative stress markers were separated and frozen to – 80 °C at the date of collection. The assays were then run in single batches.

2.5. Insulin Resistance

Plasma insulin and fasting plasma glucose were collected at the beginning of each visit in each of the subjects. These were repeated at 5 minute intervals to give a total of 3 pairs of results and the average of these was used to calculate insulin resistance using the HOMA 2 model (Matthews et al., 1985).

2.6. Oxidative Stress Markers

Markers of oxidative stress and antioxidant defense included whole blood ratio of reduced and oxidized glutathione [GSH/GSSG] (enzymatic colorimetric assay (Tietze, 1969)), plasma total antioxidant status [TAOS] (enzymatic colorimetric assay (Laight et al., 1999)) and plasma lipid hydroperoxides [LHP] (enzymatic colorimetric assay (Ruiz et al., 1997)). This combination of assays gives a broad evaluation of total redox status and was carried out using previously described methods (see references). For all 3 assays the intra-assay % coefficient of variation (CV) is < 3, while the inter-assay CV is < 10.

2.7. Biochemical Markers of Inflammation

2.7.1. HsCRP

The mixture of HsCRP and the sample were measured on the DADA BEHRING BN ProSpec system analyzer. The result was then analyzed by comparison with a standard of known concentration. The assigned values of CRP were standardized against the international reference preparation BCR-CRM 470 (Whicher et al., 1994).

2.8. Statistics

Statistical software GraphPad InStat 3 and XLStat 2007 were used for statistical analysis.

The Kolmogorov–Smirnov test (KS test) was used to assess whether distributions were parametric or non-parametric.

Repeated measures of ANOVA (analysis of variance) were used to compare 4 sets of values obtained. These were: value at baseline; value after AGE intervention; value at baseline (after washout period) and value after placebo.

For parametric values, post-tests were undertaken using the Bonferroni method. Friedman's test with Dunn's post-test was used if the distribution was non-parametric.

Missing values were small in number (< 5%) and where possible were repeated. The remaining missing values occurred randomly and were excluded from the analysis (casewise deletion).

3. Results

The subjects were recruited from primary care diabetes lists and the diabetes clinic. 9 out of 26 subjects were taking an ACE inhibitor throughout the study, 25/26 were taking a statin, 16/26 were taking 75 mg aspirin and 19/26 were taking metformin. These medications remained unchanged during the study period. All parameters returned to baseline following washout period (Table 2).

Table 2
Baseline characteristics

Parameter	Mean baseline pre-placebo	Mean baseline pre-AGE
Plasma total cholesterol (mmol/l)	4.2±0.8	4.2±0.9
Plasma HDL cholesterol (mmol/l)	1.0±0.3	1.0±0.3
Plasma triglycerides (mmol/l)	1.6 IQR 1.2	1.4 IQR 0.7
Diastolic BP (mmHg)	74.8±9.8	74.7±7.5
Systolic BP (mmHg)	130.3±15.9	130.3±14.0
RI change post-salbutamol	8.0 IQR 4.7	6.5 IQR 9.7
Insulin resistance (HOMA-IR)	2.5±2.0	1.9±1.1
LHP (µM)	158.3±97.0	144.7±55.4
GSH/GSSG	17.0 IQR 15.1	18.8 IQR 21.2
Total glutathione (µM)	698.0±193.7	690.2±177.9
TAOS (µM)	62.9±3.6	63.1±3.2
HsCRP (mg/l)	1.8 IQR 2.1	2.0 IQR 1.8

There were no significant differences between these baseline measurements (paired t test or Wilcoxon signed rank test) suggesting that washout periods were effective.

Following treatment with AGE there were no statistically significant changes in weight, systolic blood pressure, diastolic blood pressure, total cholesterol, plasma HDL cholesterol, plasma triglycerides or fructosamine in comparison with placebo. Moreover, there were no statistically significant changes in endothelial function as measured by digital plethysmography. Finally, no difference was found in biochemical markers of oxidative stress and inflammation (Table 3).

Table 3

Summary of results.

Marker	Mean pre-placebo	Mean post-placebo	Mean pre-AGE	Mean post-AGE	P value (ANOVA)
Weight (kg)	98.7 ± 18.5	98.8 ± 18.4	98.2 ± 18.2	98.7 ± 18.2	0.24
Systolic BP (mmHg)	130.3 ± 15.9	131.6 ± 17.5	130.3 ± 14.0	130.8 ± 14.6	0.94
Diastolic BP (mmHg)	74.8 ± 9.8	75.1 ± 8.3	74.7 ± 7.5	73.9 ± 7.7	0.81
Total cholesterol (mmol/l)	4.2 ± 0.8	4.2 ± 0.9	4.2 ± 0.9	4.2 ± 0.8	0.96
Plasma HDL (mmol/l)	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.3	0.46
Plasma triglycerides (mmol/l)	1.6 IQR 1.2	1.5 IQR 1.1	1.4 IQR 0.7	1.4 IQR 0.8	0.04 [□]
Fructosamine (µmol/l)	284 ± 46	270 ± 33	274 ± 33	270 ± 33	0.88
RI post-GTN (%)	12.5 ± 8.2	11 ± 8.2	11 ± 5.7	12 ± 7.3	0.52
RI post-salbutamol (%)	8.0 IQR 4.7	9.0 IQR 9.5	6.5 IQR 7.7	6.5 IQR 9.7	0.95
Insulin resistance (HOMA-IR)	2.5 ± 2.0	2.0 ± 1.1	1.89 ± 1.1	1.7 ± 0.9	0.05 [□]
A/C ratio	0.8 IQR 1.6	0.6 IQR 1.6	0.5 IQR 1.55	0.9 IQR 1.5	0.43
HsCRP (mg/l)	1.8 IQR 2.1	2.0 IQR 1.6	2.0 IQR 1.8	1.9 IQR 1.9	0.90
TAOS (AEAC)	62.9 ± 3.6	63.6 ± 5.6	63.1 ± 3.2	64.0 ± 4.4	0.57
GSH/GSSG ratio	17 IQR 15.1	20.6 IQR 22.15	18.8 IQR 21.2	22.8 IQR 25.1	0.63
Total blood glutathione (µM)	698.9 ± 193.7	690.9 ± 169.1	690.2 ± 177.9	725.8 ± 224.5	0.67
Plasma LHP (µM)	158.3 ± 97	134.2 ± 29.7	144.7 ± 55.4	134.19 ± 41.1	0.41

* not significant on post hoc testing.

3.1. Adverse Events

Two subjects withdrew due to side effects from the garlic extract, namely indigestion. Two subjects withdrew due to concurrent, unconnected illness. None of the data was used in the analysis. Compliance was assessed by tablet count.

4. Discussion

The current pilot study found no significant effect of 4 weeks treatment with 1200 mg daily of Aged Garlic Extract on the metabolic parameters studied in our cohort of subjects.

Previous studies have shown that AGE had the potential to improve oxidative stress and it has the potential to have positive metabolic effects in diabetes.

4.1. AGE and Endothelial Function

Weiss et al. (2006) investigated the effects of AGE upon flow mediated dilatation in the brachial artery after induced acute homocysteinemia. This crossover study of 11 healthy individuals found subjects treated with AGE for 6 weeks had a 66% increase in FMD in the brachial artery, as measured by Doppler ultrasound, in comparison with the placebo-treated subjects. Acute homocysteinemia gives experimentally induced

endothelial dysfunction by reducing bioavailable nitric oxide at the endothelium. This may account for the differences seen in our study. Furthermore, this study recruited a very different cohort to ours (relatively young, healthy individuals with no cardiac risk factors or diabetes and not taking other medications) and in small numbers (n = 11).

Williams et al. (2005) investigated the effect of AGE upon endothelial function in men with established coronary artery disease in a crossover study of 15 subjects. In this study 2.4 g/day of AGE was used for a period of 2 weeks. This study employed ultrasound and Doppler measured brachial artery FMD as a measure of endothelial function and found a 44% increase in FMD from baseline following treatment with AGE over placebo. The limitations of this study include its small numbers and the relatively short duration of treatment. This study investigated non-diabetic subjects and all of the subjects were treated with statins and aspirin. However, despite good correlation of digital plethysmography with brachial FMD, it may be that brachial FMD may be a more sensitive tool for detecting relatively small degrees of change in vasomotor endothelial function.

In contrast, Gómez-Arbeláez et al. (2013) used brachial artery FMD in 46 individuals with metabolic syndrome. This group had been treated with 1200 mg AGE for 12 weeks but there was no significant change in FMD after this period.

Larijani et al. (2013) studied the effect of 1200 mg of AGE given for 1 year on the endothelial function of 65 healthy individuals. This study used digital thermal monitoring as a marker of endothelial function and did show a statistically significant improvement.

4.2. AGE and Markers of Inflammation

In vitro studies have shown reduction of oxidative inflammation in cell lines treated with garlic preparations (Hui et al., 2010 and Ide and Lau, 2001), however clinical studies have largely failed to replicate these results. Van Doorn (2006) studied the effect of garlic powder upon 90 overweight smokers at a dose of 2.1 g/day over a period of 3 months. This study showed garlic powder had no effect on plasma CRP or TNF- α levels in comparison to placebo. In the same study, Atorvastatin showed significant reductions in all plasma inflammatory markers. Williams et al. (2005) also showed no effect of AGE upon inflammatory markers in their study in individuals with coronary artery disease. Furthermore, Diego Gómez-Arbeláez et al. (Ridker et al., 2008) studied 46 patients with metabolic syndrome, though it is not clear how many of these subjects also had diabetes. This group showed no effect on CRP or IL-6 after 12 weeks of AGE. These results are supported by our study using AGE in a population with diabetes. Budoff et al. (2009) did show an improvement in plasma CRP levels however this was using a combination of antioxidants including AGE for 1 year.

4.3. AGE and Oxidative Stress

Budoff et al. (2009) investigated the effect of AGE treatment on microvascular endothelial function in subjects at moderate cardiovascular risk and showed significant changes following treatment with AGE. This randomized, placebo controlled study of 65 individuals used 1 year's treatment with AGE plus (AGE + Vitamin B6 + Vitamin B12 + folate + L-arginine) and measured endothelial function using digital thermal monitoring. Subjects were all treated with statins and 97% were treated with unspecified antihypertensive agents. Only 5% had diabetes. This study was larger and had a significantly longer duration of treatment than the present study, however it studied only a very small number of individuals with diabetes and used a combination of antioxidants. These findings have not been replicated consistently. Williams et al. (2005) showed no effect of AGE on oxidative stress markers in individuals with coronary artery disease. Studies using other garlic preparations have shown some improvement in oxidative markers but in very different cohorts (Duda et al., 2008, Durak et al., 2004a, Durak et al., 2004b and Koseoglu et al., 2010).

This pilot study was the largest study using Aged Garlic Extract in patients with diabetes to date, although the sample size remained small and the duration of treatment relatively short. Furthermore, the cohort studied had near optimal metabolic parameters (as per NICE guideline CG87) at baseline and therefore any clinically significant change in the parameters studied would have been difficult to achieve or detect. This cohort was receiving numerous vasoactive medications. These were metformin (59%), aspirin (50%) and statin therapy (96%). 36% were treated with an ACEI. These medications themselves have anti-inflammatory effects which could attenuate any benefit of anti-oxidant therapy.

The baseline plasma HsCRP in the present study was lower than similar studies (Koseoglu et al., 2010 and Van Doorn, 2006) (Jupiter study (Ridker et al., 2008) baseline 4.2 mg/l our baseline 2.07 mg/l and Korean study (Lee et al., 2009) 3.0 mg/l). The Jupiter study did also not include any subjects with diabetes and only 16% were treated with aspirin (Ridker et al., 2008). Furthermore, the group treated with Rosuvastatin in the Jupiter trial had a post-treatment plasma HsCRP of 2.2 mg/l; still higher than the pre-treatment baseline in the present study. This would suggest that despite the cardiovascular algorithm prediction of 30% risk of a cardiovascular event in the next 10 years, biochemically our cohort was not of high cardiovascular risk.

Endothelial function at baseline in our cohort was also largely normal. Baseline changes in reflective index following administration of salbutamol in our patients were in the range 6.5–8%. This is much closer to the control group (5.9%) cited by Chowienczyk et al. (1999) rather than the diabetes group (11.5%), suggesting that our cohort did not have significantly deranged endothelial function pre-treatment.

Our study used a small dose of AGE for a period of 4 weeks. This was based on previous studies (Dillon et al., 2002 and Budoff et al., 2009) using the same preparation of AGE. Comparison of dosage of garlic

products is difficult as studies use different preparations of AGE, the components of which are not standardized. Our results are consistent with the study of Williams et al. (2005) but the Budoff study (Budoff et al., 2009) suggests that a smaller dose (250 mg/day) of AGE alongside other antioxidants for a much longer period of administration (> 12 months) may be necessary to have an effect upon oxidative stress markers in a cohort treated with statins. Furthermore, a further study from the Budoff group (Budoff, 2006) showed that 1200 mg of AGE used for 1 year could have effects on markers of endothelial function and work by Gómez-Arbeláez et al. (2013) showed a positive effect of AGE 1200 mg/day for 12 weeks on adiponectin levels but no effect on markers of inflammation or endothelial function. Future studies could consider using AGE for longer periods in this study group.

The Dillon study (Dillon et al., 2002) suggests that other markers of oxidative stress may change more rapidly and it may be that measurement of F2-isoprostane 8-iso-prostaglandin is more sensitive than other markers of oxidative stress.

5. Conclusion

The results from the current study would suggest that there is no clinical benefit of adding AGE, in the short term, to usual medical therapy in this cohort with type 2 diabetes. However, further studies are required to assess the long term benefit of AGE and other garlic preparations in the diabetes population with different cardiovascular risk profiles.

In order to further investigate the properties of Aged Garlic Extract, future studies could examine a combination of antioxidants for a longer duration (Durak et al., 2004b and Ghanim et al., 2011) and examine a diabetic cohort with higher cardiovascular risk, such as those with established cardiac disease or those with preselected high levels of HsCRP (Ridker et al., 2008).

Acknowledgements

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