**Low dose dietary nitrate improves endothelial dysfunction and plaque stability
in the ApoE^{-/-} mouse fed a high fat diet.**

JR Bakker^{*1}, NP Bondonno^{*1}, TA Gaspari², BK Kemp-Harper², AJ McCashney², JM
Hodgson^{1,3}, KD Croft¹, NC Ward^{1,4}

¹*School of Medicine & Pharmacology, University of Western Australia, Perth
Australia*

²*Cardiovascular Disease Program, Biomedicine Discovery Institute, Department of
Pharmacology, Monash University, Melbourne Australia*

³*School of Medical and Health Sciences, Edith Cowan University, Perth Australia*

⁴*School of Biomedical Sciences & Curtin Health Innovation Research Institute,
Curtin University, Perth Australia*

**These authors contributed equally to this work.*

Address for correspondence:

Dr NC Ward*

School of Medicine & Pharmacology

University of Western Australia

GPO Box X2213

Perth WA 6847

Tel: +61 8 92240392

Fax: +61 8 92240246

Email: Natalie.ward@uwa.edu.au

*Present Address:

School of Biomedical Sciences

Curtin University

GPO Box U1987

Perth WA 6845

Tel: +61 8 92667520

Fax: +61 8 92667424

Email: Natalie.ward@curtin.edu.au

Abstract

Background: Nitric oxide (NO) is an important vascular signalling molecule. NO is synthesised endogenously by endothelial nitric oxide synthase (eNOS). An alternate pathway is exogenous dietary nitrate, which can be converted to nitrite and then stored or further converted to NO and used immediately. Atherosclerosis is associated with endothelial dysfunction and subsequent lesion formation. This is thought to arise due to a reduction in the bioavailability and/or bioactivity of endogenous NO.

Aim: To determine if dietary nitrate can protect against endothelial dysfunction and lesion formation in the ApoE^{-/-} mouse fed a high fat diet (HFD).

Methods & Results: ApoE^{-/-} fed a HFD were randomized to receive (i) high nitrate (10mmol/kg/day, n=12), (ii) moderate nitrate (1mmol/kg/day, n=8), (iii) low nitrate (0.1mmol/kg/day, n=8), or (iv) sodium chloride supplemented drinking water (control, n=10) for 10 weeks. A group of C57BL6 mice (n=6) received regular water and served as a healthy reference group. At 10 weeks, ACh-induced vessel relaxation was significantly impaired in ApoE^{-/-} mice versus C57BL6. Mice supplemented with low or moderate nitrate showed significant improvements in ACh-induced vessel relaxation compared to ApoE^{-/-} mice given the high nitrate or sodium chloride. Plaque collagen expression was increased and lipid deposition reduced following supplementation with low or moderate nitrate compared to sodium chloride, reflecting increased plaque stability with nitrate supplementation. Plasma nitrate and nitrite levels were significantly increased in all three groups fed the nitrate-supplemented water.

Conclusion: Low and moderate dose nitrate significantly improved endothelial function and atherosclerotic plaque composition in ApoE^{-/-} mice fed a HFD.

Keywords: dietary nitrate, endothelial function, atherosclerosis, eNOS.

Introduction

Nitric oxide (NO) is an important vascular signalling molecule that plays a major role in the control of vascular function and tone, as well as the prevention of atherosclerosis and cardiovascular disease (CVD).¹ There is substantial epidemiological evidence to suggest that a diet rich in fruits and vegetables has beneficial effects on CVD.^{2,3} Vegetables are a major source of dietary nitrate and there are several clinical trials demonstrating beneficial effects of dietary nitrate on both blood pressure and vascular function.⁴⁻⁶

The majority of the body's NO is synthesized endogenously, while dietary nitrate provides an alternate source.⁷ Within the body, NO is produced by endothelial cells via conversion of L-arginine to citrulline by endothelial nitric oxide synthase (eNOS). Once released, NO diffuses to the underlying smooth muscle layer, stimulates production of cyclic guanosine monophosphate (cGMP), which results in changes to intracellular calcium concentration and subsequent relaxation of the vessel wall.⁸ Dietary nitrate, found predominantly in green leafy vegetables, represents an alternate source of NO. It is well absorbed, and approximately 25% is then secreted into the saliva and 20% of this (~5% of ingested nitrate) is converted to nitrite in the mouth. The nitrite is then swallowed and absorbed where it can have direct vasodilatory effects or be stored to act as a pool of NO.^{7,9} This nitrate-nitrite derived NO pool represents a NOS-independent pathway that can be used to supplement endogenous NO supplies or replace them when they are compromised.

Atherosclerosis is characterised by a build up of lipid in the vessel wall, which coupled with macrophage accumulation, inflammation and oxidative stress, results in lesion development and plaque formation.¹⁰ Endothelial dysfunction is an early risk factor for CVD and is thought to be one of the initial steps in the development of atherosclerosis.¹¹ Damaged endothelial cells have been shown to express selective adhesion molecules and the subsequent adhesion of monocytes can further suppress the release of NO. This continual build up of monocytes and macrophages, coupled with lipid accumulation in the vessel wall not only contributes to plaque formation, but also reduces vessel diameter, further exacerbating endothelial dysfunction.^{10, 12}

There is very little data available on the effects of dietary nitrate on the development of atherosclerosis. A previous study in cholesterol fed rabbits observed that supplementation with L-arginine, the precursor to eNOS derived NO, improved endothelial function and blocked the development of atherosclerotic plaques.¹³ This L-arginine supplementation was also shown to increase macrophage apoptosis in intimal lesions¹⁴, while another study suggested that NO signalling in monocytes plays a role in plaque stability.¹⁵ More recently, a study in the LDL receptor knockout mouse found no improvements in blood pressure or atherosclerosis following high dose nitrate supplementation (1g/L).¹⁶

It is clear that further investigation into the beneficial effects of dietary nitrate on both endothelial dysfunction and the subsequent development of atherosclerosis is needed. When considering these studies however, it is essential to take into account the dose of nitrate given. This is particularly important given the suggestion that a negative cross-talk exists between the endogenous NO synthase and exogenous nitrate-nitrite-NO pathways.¹⁷ With this in mind, the aim of this study was to investigate the effect of low, moderate and high dose dietary nitrate supplementation on endothelial dysfunction and atherosclerotic lesion formation in the ApoE^{-/-} mouse fed a high fat diet (HFD).

Methods

Animal study

The aims of this study were achieved via two separate animal studies, using male ApoE^{-/-} mice on a C57BL6 background. The ApoE^{-/-} mouse is a well established model of atherosclerosis that has been shown to develop spontaneous hypercholesterolaemia and atherosclerotic lesions when fed a HFD.¹⁸

Study 1 investigated supplementation with high dose nitrate (HDN) and included 6 male wild-type (WT) C57BL6 mice and 24 male ApoE^{-/-} mice on a C57BL6 background. The WT mice were given specialised animal house drinking water (ddH₂O + HCl) and acted as a healthy reference group. The ApoE^{-/-} mice were randomly allocated to one of two treatment groups: (i) ddH₂O + NaNO₃ (10 mmol/kg/day, high dose nitrate, n=12), or (ii) ddH₂O + NaCl (1% w/v, control, n=12).

Study 2 investigated the effect of low and moderate dose nitrate (LDN and MDN respectively). In this study 60 male ApoE^{-/-} mice on a C57BL6 background were randomised into one of three treatment groups: (i) ddH₂O + NaNO₃ (0.1 mmol/kg/day, low dose nitrate, n=20), (ii) ddH₂O + NaNO₃ (1 mmol/kg/day, moderate dose nitrate, n=20), or (iii) ddH₂O + NaCl (1 mmol/kg/day, control, n=20). Half the mice in each group were analysed at 2 weeks (short-term study), while the remaining half were analysed at 10 weeks (long-term study).

In both studies, NaCl was added to the water of the ApoE^{-/-} mice not receiving nitrate and the mice on the low nitrate to maintain a comparable osmotic pressure and to account for the presence of sodium. In study 1, 10 g/L of NaCl was added to the control water and no NaCl was added to the high dose nitrate water. In study 2, 293 mg/L of NaCl was added to the control water, while 263 mg/L was added to the low dose nitrate water. No NaCl was added to the moderate dose nitrate water. All animals were between 6-8 weeks of age at the beginning of the study and were kept at 23±2°C under a 12 hr light-dark cycle. All mice were given a week to acclimatise, after which they were fed a HFD (SF10-110, Glen Forrest Stockfeed, Australia) for 10 weeks, along with the allocated drinking water. The HFD contained 35% energy from carbohydrate, 42% energy from fat and 23% energy from protein. All mice were allowed ad libitum access to both food and water and body weight was checked weekly. The Royal Perth Hospital Animal Ethics Committee (R519/13-14 and R527/14-15) approved the use of animals and all experiments were conducted in accordance with the NHMRC guidelines for the care and use of laboratory animals.

Vascular function studies

After treatment periods, animals were anaesthetised using methoxyflurane gas (Biopharm, Australia). Blood samples were taken via cardiac puncture with continued inhalation of methoxyflurane and placed into tubes containing EDTA. Urine samples were removed via bladder puncture. Central incision exposed the abdominal and thoracic cavities and the heart was then perfused with saline (0.9%) until the liver

appeared pale. The abdominal aorta was stripped of connective and adipose tissue and transferred to Krebs-Henseleit buffer (Sigma-Aldrich), which had been oxygenated with 95% O₂/5% CO₂.

The aortas were incubated in 95% O₂/5% CO₂ at 37°C for 1 hr before being cut into ~2mm segments, which were mounted on parallel wires in isolated tissue baths using the small vessel myography system 620M (DMT, Denmark). Vessels were primed with KPSS buffer (containing 123.7 mM KCl, 1.17 mM MgSO₄, 1.18 mM KH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, 0.03 mM EDTA and 5.5 mM glucose) and mechanically stretched in a step-wise procedure to 9mN. Following pre-constriction with increasing doses of phenylephrine (Phe; 10⁻⁹ to 10⁻⁵ M) assessment of endothelium dependent relaxation was determined via relaxation to increasing doses of acetylcholine (ACh; 10⁻¹⁰ to 10⁻⁵ M). Pre-constriction with phenylephrine was then repeated and endothelium independent relaxation was determined by measuring relaxation response to increasing doses of sodium nitroprusside (SNP; 10⁻⁹ to 10⁻⁵ M) were performed as previously described.¹⁹

Atherosclerotic lesion size and composition

Atherosclerotic plaque area was determined in the brachiocephalic artery (BCA) of ApoE^{-/-} mice from Study 2 as previously described.²⁰ Collagen content and lipid deposition was determined in the atherosclerotic plaques as previously described.²¹ Plaque stability was calculated as the ratio of collagen:lipid in each plaque.^{22, 23}

Plasma nitrate, nitrite and NOx concentrations

Blood taken for analysis of nitrate, nitrite and NOx concentration was stored on ice immediately after collection and the plasma separated from red cells within 10 mins. Plasma nitrate and nitrite status was determined using a gas chromatography mass spectrometry (GCMS) method as previously described.²⁴ Plasma nitrosothiols were determined using a gas phase ozone based chemiluminescence assay as previously described.²⁵ Urinary cGMP was analysed as an indirect measure of NO production using a commercially available kit (Cayman Chemicals).

Protein expression

Expression of eNOS (BD Biosciences), p-eNOS (ser1177, Cell Signalling), iNOS (Cell Signalling), p-AMPK (thr172, Cell Signalling), AMPK (Cell Signalling), p-Akt (ser473, Cell Signalling), Akt (Cell Signalling), HO-1 (Enzo Lifesciences), gp96phox (Cell Signalling) and p47phox (Cell Signalling) proteins were analysed via western blot on isolated aortic tissue as previously described.¹⁹

Plasma and urinary F₂-isoprostane analysis

Plasma and urinary F₂-isoprostane concentrations were determined using an isotope dilution GCMS method, as previously described.²⁶ Urinary concentrations were corrected for creatinine to account for variations in volume.

Plasma cholesterol levels

Plasma cholesterol levels were determined using a Cholesterol Assay Kit (Roche, Australia).

Statistical analysis

All data is presented as mean $\pm$ SEM. Vascular function data was analysed using mixed models with post hoc adjustment for multiple comparisons (SAS, version 9.3). All other analysis was performed using t-tests or one-way ANOVA with post-hoc comparisons, as appropriate (SPSS, version 21).

Results

Nitrate diet and animal characteristics

The concentration of nitrate found in the HFD is described in Table 1. Based on previous studies²⁷ food consumption was assumed to be 5g chow per day and water consumption approximately 6 mL per day. In study 1, there was no significant difference in weight per se between the control mice and ApoE^{-/-} mice, and no effect of high nitrate treatment on weight gain in the ApoE^{-/-} mice. In study 2, there was no significant difference in weight gain between ApoE^{-/-} mice fed the HFD alone or those supplemented with low or moderate dose nitrate (Table 2). As expected, the ApoE^{-/-} mice had significantly elevated plasma cholesterol levels compared to the WT mice (Table 2).

The effect of dietary nitrate supplementation on vascular function

In study 1, investigating the effect of high dose nitrate on ACh-mediated vessel relaxation in WT and ApoE^{-/-} mice fed a HFD, we found that the ApoE^{-/-} mice had significantly impaired vascular function compared to the WT mice (Figure 1a). The addition of high dose nitrate to the HFD diet for 10 weeks had no beneficial effect on the vascular dysfunction (Figure 1a).

In study 2, we investigated the effect of low and moderate dose nitrate on ACh-mediated vessel relaxation in ApoE^{-/-} mice fed a HFD. In the short-term (2 week) study, analysis revealed a non-significant trend towards improved vascular function in mice receiving either low or moderate dose nitrate compared to control (Figure 1b). Long-term (10 week) supplementation with dietary nitrate, at both the low and moderate doses, resulted in significant improvements in ACh-mediated vessel relaxation (Figure 1c) compared to mice fed the HFD supplemented with NaCl.

There were no significant differences in vessel relaxation response to the NO donor SNP in either study, between any of the treatment groups (included as supplemental data).

The effect of dietary nitrate supplementation on atherosclerotic lesion size and composition

In study 1, atherosclerotic lesions were observed in the aortic arch of the ApoE^{-/-} mice, but not the WT control mice (Figure 2a) and the mean atherosclerotic lesion area was significantly increased in ApoE^{-/-} mice compared to WT mice. There was no effect of high dose nitrate supplementation on cross-sectional lesion area in the ApoE^{-/-} mice (Figure 2a).

In study 2, mice from all three treatment groups showed atherosclerotic lesion formation in the aortic arch. Supplementation with low or moderate dose nitrate increased plaque collagen expression (Figure 2b) and reduced plaque lipid deposition (Figure 2c). This resulted in an improvement in overall plaque stability, calculated as the collagen:lipid ratio (Figure 2d). Supplementation with low or moderate dose nitrate was also associated with a small, non-significant reduction in plaque area (Figure 2e).

The effect of dietary nitrate supplementation on NO production

In study 1, plasma levels of both nitrate and nitrite were significantly increased in the ApoE^{-/-} mice fed a HFD supplemented with high dose nitrate (Figure 3a). The levels of nitrate were approximately 20 fold higher than the plasma levels of nitrite.

In study 2, short-term nitrate supplementation resulted in significant increases in plasma nitrate and nitrite only in ApoE^{-/-} mice supplemented with moderate dose nitrate (Figure 3b). Long-term (10 week) nitrate supplementation resulted in a significant difference in overall concentrations of nitrite and other nitroso compounds (S-nitrosothiols, iron-nitrosyls, N-nitrosamines) in the ApoE^{-/-} mice supplemented with moderate dose nitrate compared to both control and low dose nitrate (Figure 3c). Urinary cGMP production was assessed in mice receiving the low and moderate dose nitrate, however this was not significantly different compared to the control ApoE^{-/-} mice (Figure 3d)

The effect of dietary nitrate supplementation on eNOS signalling pathways

Analysis of eNOS protein expression and eNOS phosphorylation was assessed in the thoracic aorta of mice in study 2. There was no significant difference in total eNOS protein expression or eNOS phosphorylation at the serine 1177 residue between control mice and mice supplemented with low or moderate dose nitrate (Figure 4a). There was a significant reduction in p47phox expression following treatment with the moderate dose nitrate (figure 4b). However there was no significant effect of low or moderate dose nitrate supplementation on iNOS, pAMPK/AMPK, pAkt/Akt, gp91phox, or HO-1 expression in isolated thoracic aortas (Figure 4b).

The effect of dietary nitrate supplementation on oxidative stress

In study 1, plasma F₂-isoprostane levels were not significantly different between WT and ApoE^{-/-} mice. Supplementation with high dose nitrate had no effect on plasma F₂-isoprostanes (Figure 5a). In study 2, there was no significant difference in urinary F₂-

isopropanes between the control, low nitrate and moderate nitrate dose groups (Figure 5b).

Discussion

We have previously shown that ApoE^{-/-} mice fed a HFD develop hypercholesterolemia, which leads to both endothelial dysfunction and the development of atherosclerosis.²⁸ In the present study, we confirm our finding, with study 1 indicating the presence of both endothelial dysfunction and atherosclerotic lesions in the ApoE^{-/-} mice, but not the WT mice, fed a HFD. Although supplementation with high dose nitrate had no effect on either vessel relaxation or lesion formation, study 2 revealed that low or moderate dose nitrate supplementation significantly improved the endothelial dysfunction associated with this mouse model. This improvement in endothelial function was also associated with an increase in plaque collagen expression and a reduction in plaque lipid deposition, which represents an increase in overall plaque stability. There were small reductions in plaque size following low and moderate dose nitrate supplementation. To our knowledge, this is the first study to demonstrate a beneficial effect of low to moderate dose nitrate supplementation on both atherosclerotic plaque composition and endothelial dysfunction in the ApoE^{-/-} mouse.

Endothelial dysfunction is one of the first steps in the pathogenesis of atherosclerosis, occurring at the earliest stages of disease development and preceding any evidence of disease.^{29,30} The dysfunction is primarily thought to arise due to a reduction in the bioavailability and/or bioactivity of the important vasodilator NO and approaches that restore NO levels represent an attractive therapeutic option. Beetroot and green leafy vegetables are particularly rich in nitrate, which when absorbed, can be converted to nitrite and stored to act as a reserve pool or converted into NO for direct action.³¹ Several clinical trials have demonstrated beneficial improvements following nitrate supplementation in both vascular function, as measured by flow mediated dilatation and cGMP concentrations,³²⁻³⁴ and blood pressure.^{4,5} In the present study we have demonstrated for the first time that chronic low to moderate dose nitrate supplementation not only prevents the development of endothelial dysfunction in the ApoE^{-/-} mice fed a HFD, but is associated with improvements in plaque composition and stability. This improvement in endothelial dysfunction following the low and moderate dose nitrate was observed as early as two weeks after treatment began, and was highly significant at ten weeks. These results suggest an immediate and direct effect of nitrate supplementation in restoring NO supplies, with beneficial effects extending beyond simply restoring normal vascular function.

Our study is in contrast to a recent study carried out in the LDL receptor knock-out mouse (LDLr^{-/-}), where no improvements in blood pressure or atherosclerosis were seen following 14 weeks of nitrate supplementation.¹⁶ However, in this study, the dose of nitrate given was 1g/L,¹⁶ which is comparable to the high dose nitrate treatment group in our first study where we saw no beneficial effects on either

vascular function or atherosclerosis. The dose given in our low and moderate nitrate treatment groups, where we did see beneficial effects on endothelial function and atherosclerosis, corresponds to < 0.01g/L and 0.1 g/L respectively. There was also a trend towards a small increase in aortic eNOS expression in the mice fed the low and moderate dose nitrate, suggesting that the mechanisms behind the beneficial effects of low and moderate dose nitrate on endothelial function may still be through direct effects on eNOS, including via increased expression, phosphorylation or nitrosylation. However, while beneficial, these effects may be small and/or transient, making them difficult to detect using western blot methods. In addition, there was also a trend towards increases in both AMPK and Akt phosphorylation following low and moderate dose nitrate. These are important kinases in the eNOS pathway and support the suggestion that dietary nitrate may mediate its beneficial effects via the eNOS pathway. Furthermore, there may be other steps in the eNOS signalling pathway not studied herein, including Hsp90 association, eNOS dimer formation and eNOS uncoupling that play a role in the observed beneficial effects. The role of eNOS uncoupling and subsequent superoxide production may be important to consider in future studies, as previous studies have shown that this plays a role in altered NO production in the ApoE^{-/-} mouse.^{35, 36}

The lack of effect seen at the higher dose may be due to a negative cross-talk between the endogenous eNOS and exogenous nitrate-nitrite-NO pathways. Indeed, nitrate/nitrite derived NO and related nitrogen species may affect the activity of kinases and phosphatases which modulate eNOS activity.³⁷ In a recent study, Carlstrom et al showed that at moderate doses, nitrate suppressed aortic eNOS phosphorylation at ser1177 (an activation site), while inducing eNOS phosphorylation at thr495 (an inactivation site).¹⁷ Although we didn't observe any indications of cross-talk in the aortas of our mice fed the moderate dose, it should be noted that only healthy rats and mice were used in Carlstrom's study, in contrast to the atherosclerotic model used in our study. It may be that cross-talk between the endogenous and exogenous pathways is altered under different physiological conditions or diseased states. Furthermore, the nature of the cross-talk may be dose-dependent such that higher doses of nitrate exert a negative feedback on eNOS function. Such interplay may account for the lack of improvement in endothelial function in the ApoE^{-/-} mice fed the high nitrate diet in study 1.

In addition to endothelial dysfunction, atherosclerosis is also characterised by the build up of lipid in the vessel wall. This is accompanied by monocyte adhesion, macrophage accumulation, inflammation and oxidative stress, all of which leads to lesion development and plaque formation.¹⁰ A key component of plaque development is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes (NOXs). Expressed in the artery wall, they are a source of reactive oxygen species (ROS) that may contribute to oxidative stress and inflammation as well as promote lesion formation.²¹ Within the ApoE^{-/-} mouse, NOX2 is the predominant source of (ROS), with the isoform expressed in endothelial cells, adventitial fibroblasts and

monocytes and macrophages.³⁸ Studies in ApoE^{-/-} mice with p47phox deletion (a NOX organiser protein) have shown significantly reduced lesion formation compared to ApoE^{-/-} mice alone.³⁸ Although we saw no effect on aortic expression of gp91phox (NOX2), we did observe a significant reduction in aortic p47phox expression, a key regulatory subunit of NOX2, in the mice receiving the moderate dose nitrate. This was accompanied by increased plaque collagen content and reduced lipid deposition in both the low and moderate dose nitrate treated mice. This increase in the collagen:lipid ratio indicates an overall increase in plaque stability in the mice treated with both low and moderate dose nitrate. Although plaque area did not differ significantly different between the groups, such an observation is not unexpected as the innominate artery is one of the first areas to develop plaque and therefore one of the hardest to cause regression. However, the change in plaque composition is an important finding as this highlights the role dietary nitrate plays in reducing lipid content and increasing collagen expression, leading to a plaque that overall has a lower risk of rupturing compared to the ApoE^{-/-} mouse that didn't receive nitrate. The reduction in p47phox expression, coupled with the beneficial effects on atherosclerotic plaque composition, provides further evidence for the beneficial effects of nitrate supplementation and suggests that the protective effects of dietary nitrate extend beyond just preventing endothelial dysfunction.

As a secondary explanation, we also investigated the aortic expression of heme oxygenase-1 (HO-1). HO-1 is an inducible enzyme involved in the metabolism of heme, which has been shown to play a role in both vascular function and atherosclerosis.³⁹ We have previously shown that supplementation with quercetin, an important dietary flavonoid, protects the endothelium from oxidant-induced damage as well as reduces atherosclerotic lesion size in ApoE^{-/-} mice, in part via a HO-1 dependent pathway.²⁸ We did observe a trend towards increased aortic expression of HO-1 in mice fed low or moderate dose nitrate compared to control, suggesting that the beneficial effects of dietary nitrate may act in part via a HO-1 dependent pathway, however further investigation is warranted.

The increase in both cholesterol levels and lesion formation we observed in the ApoE^{-/-} mice compared to the WT mice following a HFD, confirms the use of this animal as a model of atherosclerosis. Interestingly, we saw no difference in the level of oxidative stress in these animals (compared to the WT mice), as measured by plasma and urinary F₂-isoprostanes, a biomarker of lipid peroxidation.²⁶ Oxidative stress is thought to contribute to endothelial dysfunction via scavenging of NO by reactive oxygen species, as well as promoting the formation of lesions.⁴⁰ The lack of effect on markers of oxidative stress following nitrate supplementation in either study however, suggests that the improvements to vascular function and atherosclerotic plaque formation following the low and moderate nitrate doses occurred independently of oxidative stress. It is worth considering however, that both plasma and urinary measurement of lipid peroxidation may reflect overall systemic oxidative stress. A more relevant measure might be to investigate aortic levels of F₂-isoprostanes or

ROS. However, limitations of tissue availability in the present study prevented us from doing this.

We saw significant increases in plasma nitrate and nitrite in mice supplemented with high dose nitrate. In the mice receiving low or moderate dose nitrate however, we only observed significant increases in the moderate dose group. Previous human studies have seen increases in plasma nitrate or nitroso species at similar times to improvements in clinical measures such as blood pressure and endothelial function.^{25, 41} However, in agreement with our study, other animal studies have failed to see increases in plasma nitroso species, despite improvements in vascular function.^{17, 42} In the most recent study, Marsch et al initially observed elevated plasma nitrate and nitrite levels, which subsequently decreased over time, with plasma nitrite levels reaching baseline by 14 weeks.¹⁶ This discrepancy may be due to a number of reasons, including; the use of a single bolus dose in acute human studies, methodological differences in measuring NO status and the likely small increases in plasma NOx seen with the low dose nitrate supplementation. It may be that these plasma increases, while significant enough to have a beneficial effect, particularly over a chronic feeding period, are too small to detect with available methods. It should also be considered that a negative feedback system exists, even at low dose nitrate supplementation levels. Such a system could mean that endogenous supplies are reduced or there's enhanced clearance from the body once the NO status has reached some sort of equilibrium through dietary supplementation.

Our own unpublished data demonstrates that there is ~2mg of nitrate per gram of green leafy vegetables, with 62mg of nitrate corresponding to 1mmol. The low dose nitrate used in the present study is therefore comparable to a single large serve (100g) of high nitrate green leafy vegetables in a human diet. The moderate dose nitrate would require a concentrated blend (1kg) of high nitrate green leafy vegetables. These amounts could be reduced with consumption of beetroot. The high dose nitrate, for which we saw no beneficial effects, could only be achieved via the use of nitrate supplements, which would also contain high levels of sodium. Although a typical mouse diet is not expected to include significant amounts of nitrate, the improvements we've observed following low and moderate dose nitrate provide strong evidence for a beneficial effect following a small dietary change. While we are limited to a certain extent by the use of an animal model of atherosclerosis, our findings provide evidence that a relatively small and easily achievable dietary change can provide significant beneficial effects. Future, longer-term studies in humans, particularly those at high risk of CVD or consuming very low dietary nitrate, are warranted.

In conclusion, the present study has demonstrated for the first time, that supplementation with low and moderate dose nitrate can improve endothelial function and atherosclerotic plaque composition in the ApoE^{-/-} mouse fed a HFD. Although the exact mechanism remains to be elucidated, these findings provide further evidence for the potential beneficial effects of a diet rich in vegetables on CVD.

Acknowledgements

NCW acknowledges the previous support of a MRF/UWA Postdoctoral Fellowship. JMH acknowledges the support of a NHMRC Senior Research Fellow Fellowship. JR Bakker acknowledges the support of a Faculty of Medicine, Dentistry & Health Sciences Bachelor of Medical Science Scholarship.

Disclosures

None.

Highlights:

- Dietary nitrate represents an alternative source of nitric oxide (NO), via conversion to NO through the nitrate-nitrite-NO pathway.
- Chronic supplementation with dietary nitrate can protect against the development of endothelial dysfunction in ApoE^{-/-} mice fed a high fat diet.
- Chronic supplementation with dietary nitrate, increased atherosclerotic plaque collagen content and reduced lipid deposition, thereby increasing plaque stability in the ApoE^{-/-} mouse fed a high fat diet.
- Dietary nitrate represents an alternative form of NO when endogenous systems are compromised.

References

1. Forstermann U and Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation*. 2006;113:1708-14.
2. Bazzano LA, He J, Ogden LG, Loria CM, Vupputuri S, Myers L and Whelton PK. Fruit and vegetable intake and risk of cardiovascular disease in US adults: the first National Health and Nutrition Examination Survey Epidemiologic Follow-up Study. *Am J Clin Nutr*. 2002;76:93-9.
3. Liu S, Manson JE, Lee IM, Cole SR, Hennekens CH, Willett WC and Buring JE. Fruit and vegetable intake and risk of cardiovascular disease: the Women's Health Study. *Am J Clin Nutr*. 2000;72:922-8.
4. Kapil V, Khambata RS, Robertson A, Caulfield MJ and Ahluwalia A. Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2, double-blind, placebo-controlled study. *Hypertension*. 2015;65:320-7.
5. Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, Macallister R, Hobbs AJ, Webb AJ and Ahluwalia A. Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertension*. 2010;56:274-81.
6. Bondonno CP, Yang X, Croft KD, Considine MJ, Ward NC, Rich L, Puddey IB, Swinney E, Mubarak A and Hodgson JM. Flavonoid-rich apples and nitrate-rich spinach augment nitric oxide status and improve endothelial function in healthy men and women: a randomized controlled trial. *Free Radic Biol Med*. 2012;52:95-102.
7. Lundberg JO and Weitzberg E. NO generation from nitrite and its role in vascular control. *Arterioscler Thromb Vasc Biol*. 2005;25:915-22.
8. Sessa WC. eNOS at a glance. *J Cell Sci*. 2004;117:2427-9.
9. Lundberg JO and Govoni M. Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic Biol Med*. 2004;37:395-400.
10. Stocker R and Keaney JF, Jr. Role of oxidative modifications in atherosclerosis. *Physiol Rev*. 2004;84:1381-478.
11. Celermajer DS, Sorensen KE, Bull C, Robinson J and Deanfield JE. Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. *J Am Coll Cardiol*. 1994;24:1468-74.
12. Glass CK and Witztum JL. Atherosclerosis. the road ahead. *Cell*. 2001;104:503-16.
13. Boger RH, Bode-Boger SM, Brandes RP, Phivthong-ngam L, Bohme M, Nafe R, Mugge A and Frolich JC. Dietary L-arginine reduces the progression of atherosclerosis in cholesterol-fed rabbits: comparison with lovastatin. *Circulation*. 1997;96:1282-90.
14. Wang BY, Ho HK, Lin PS, Schwarzscher SP, Pollman MJ, Gibbons GH, Tsao PS and Cooke JP. Regression of atherosclerosis: role of nitric oxide and apoptosis. *Circulation*. 1999;99:1236-41.
15. Seehaus S, Shahzad K, Kashif M, Vinnikov IA, Schiller M, Wang H, Madhusudhan T, Eckstein V, Bierhaus A, Bea F, Blessing E, Weiler H, Frommhold D, Nawroth PP and Isermann B. Hypercoagulability inhibits monocyte transendothelial migration through protease-activated receptor-1-, phospholipase-Cbeta-, phosphoinositide 3-kinase-, and nitric oxide-dependent signaling in monocytes and promotes plaque stability. *Circulation*. 2009;120:774-84.

16. Marsch E, Theelen TL, Janssen BJ, Briede JJ, Haenen GR, Senden JM, van Loon LJ, Poeze M, Bierau J, Gijbels MJ, Daemen MJ and Sluimer JC. The effect of prolonged dietary nitrate supplementation on atherosclerosis development. *Atherosclerosis*. 2016;245:212-21.
17. Carlstrom M, Liu M, Yang T, Zollbrecht C, Huang L, Peleli M, Borniquel S, Kishikawa H, Hezel M, Persson AE, Weitzberg E and Lundberg JO. Cross-talk Between Nitrate-Nitrite-NO and NO Synthase Pathways in Control of Vascular NO Homeostasis. *Antioxid Redox Signal*. 2014.
18. Zhang SH, Reddick RL, Piedrahita JA and Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science*. 1992;258:468-71.
19. Shen Y, Croft KD, Hodgson JM, Kyle R, Lee IL, Wang Y, Stocker R and Ward NC. Quercetin and its metabolites improve vessel function by inducing eNOS activity via phosphorylation of AMPK. *Biochem Pharmacol*. 2012;84:1036-44.
20. Bullen ML, Miller AA, Dharmarajah J, Drummond GR, Sobey CG and Kemp-Harper BK. Vasorelaxant and antiaggregatory actions of the nitroxyl donor isopropylamine NONOate are maintained in hypercholesterolemia. *Am J Physiol Heart Circ Physiol*. 2011;301:H1405-14.
21. Sobey CG, Judkins CP, Rivera J, Lewis CV, Diep H, Lee HW, Kemp-Harper BK, Broughton BR, Selemidis S, Gaspari TA, Samuel CS and Drummond GR. NOX1 deficiency in apolipoprotein E-knockout mice is associated with elevated plasma lipids and enhanced atherosclerosis. *Free Radic Res*. 2015;49:186-98.
22. Blessing E, Preusch M, Kranzhofer R, Kinscherf R, Marx N, Rosenfeld ME, Isermann B, Weber CM, Kreuzer J, Grafe J, Katus HA and Bea F. Anti-atherosclerotic properties of telmisartan in advanced atherosclerotic lesions in apolipoprotein E deficient mice. *Atherosclerosis*. 2008;199:295-303.
23. Nakamura M, Lee DP and Yeung AC. Identification and treatment of vulnerable plaque. *Rev Cardiovasc Med*. 2004;5 Suppl 2:S22-33.
24. Yang X, Bondonno CP, Indrawan A, Hodgson JM and Croft KD. An improved mass spectrometry-based measurement of NO metabolites in biological fluids. *Free Radic Biol Med*. 2013;56:1-8.
25. Bondonno CP, Croft KD, Puddey IB, Considine MJ, Yang X, Ward NC and Hodgson JM. Nitrate causes a dose-dependent augmentation of nitric oxide status in healthy women. *Food Funct*. 2012;3:522-7.
26. Mori TA, Croft KD, Puddey IB and Beilin LJ. An improved method for the measurement of urinary and plasma F2-isoprostanes using gas chromatography-mass spectrometry. *Anal Biochem*. 1999;268:117-25.
27. Bachmanov AA, Reed DR, Beauchamp GK and Tordoff MG. Food intake, water intake, and drinking spout side preference of 28 mouse strains. *Behav Genet*. 2002;32:435-43.
28. Shen Y, Ward NC, Hodgson JM, Puddey IB, Wang Y, Zhang D, Maghzal GJ, Stocker R and Croft KD. Dietary quercetin attenuates oxidant-induced endothelial dysfunction and atherosclerosis in apolipoprotein E knockout mice fed a high-fat diet: a critical role for heme oxygenase-1. *Free Radic Biol Med*. 2013;65:908-15.
29. Rathod KS, Velmurugan S and Ahluwalia A. A 'green' diet-based approach to cardiovascular health? Is inorganic nitrate the answer? *Mol Nutr Food Res*. 2015.
30. Bonetti PO, Lerman LO and Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol*. 2003;23:168-75.
31. Bondonno CP, Croft KD and Hodgson JM. Dietary nitrate, nitric oxide and cardiovascular health. *Crit Rev Food Sci Nutr*. 2015:0.

32. Bondonno CP, Liu AH, Croft KD, Ward NC, Puddey IB, Woodman RJ and Hodgson JM. Short-term effects of a high nitrate diet on nitrate metabolism in healthy individuals. *Nutrients*. 2015;7:1906-15.
33. Velmurugan S, Kapil V, Ghosh SM, Davies S, McKnight A, Aboud Z, Khambata RS, Webb AJ, Poole A and Ahluwalia A. Antiplatelet effects of dietary nitrate in healthy volunteers: involvement of cGMP and influence of sex. *Free Radic Biol Med*. 2013;65:1521-32.
34. Velmurugan S, Gan JM, Rathod KS, Khambata RS, Ghosh SM, Hartley A, Van Eijl S, Sagi-Kiss V, Chowdhury TA, Curtis M, Kuhnle GG, Wade WG and Ahluwalia A. Dietary nitrate improves vascular function in patients with hypercholesterolemia: a randomized, double-blind, placebo-controlled study. *The American journal of clinical nutrition*. 2016;103:25-38.
35. Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, Tarpey M, Fukai T and Harrison DG. Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation*. 2001;103:1282-8.
36. Alp NJ, McAteer MA, Khoo J, Choudhury RP and Channon KM. Increased endothelial tetrahydrobiopterin synthesis by targeted transgenic GTP-cyclohydrolase I overexpression reduces endothelial dysfunction and atherosclerosis in ApoE-knockout mice. *Arterioscler Thromb Vasc Biol*. 2004;24:445-50.
37. Kolluru GK, Siamwala JH and Chatterjee S. eNOS phosphorylation in health and disease. *Biochimie*. 2010;92:1186-98.
38. Judkins CP, Diep H, Broughton BR, Mast AE, Hooker EU, Miller AA, Selemidis S, Dusting GJ, Sobey CG and Drummond GR. Direct evidence of a role for Nox2 in superoxide production, reduced nitric oxide bioavailability, and early atherosclerotic plaque formation in ApoE^{-/-} mice. *Am J Physiol Heart Circ Physiol*. 2010;298:H24-32.
39. Stocker R and Perrella MA. Heme oxygenase-1: a novel drug target for atherosclerotic diseases? *Circulation*. 2006;114:2178-89.
40. Munzel T, Gori T, Bruno RM and Taddei S. Is oxidative stress a therapeutic target in cardiovascular disease? *European heart journal*. 2010;31:2741-8.
41. Hobbs DA, Goulding MG, Nguyen A, Malaver T, Walker CF, George TW, Methven L and Lovegrove JA. Acute ingestion of beetroot bread increases endothelium-independent vasodilation and lowers diastolic blood pressure in healthy men: a randomized controlled trial. *The Journal of nutrition*. 2013;143:1399-405.
42. Carlstrom M, Larsen FJ, Nystrom T, Hezel M, Borniquel S, Weitzberg E and Lundberg JO. Dietary inorganic nitrate reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice. *Proc Natl Acad Sci U S A*. 2010;107:17716-20.

Table 1: Analysis of nitrate and nitrite content of HFD and drinking water

Sample	Nitrate	Nitrite
HFD food pellet	9.9 µg/g	0.8 µg/g
ddH₂O*	0.5 µg/mL	0.023 µg/mL
ddH₂O + NaCl	0 µg/mL	0 µg/mL
ddH₂O + NaNO₃		
10 mmol (high dose)	1301 µg/mL	26.6 µg/mL
1 mmol (moderate dose)	130 µg/mL	3 µg/mL
0.1 mmol (low dose)	13 µg/mL	0.3 µg/mL

*specialised animal house drinking water.

Table 2: Body weight and plasma cholesterol levels at 10 weeks.

Treatment	Body weight (g)	Cholesterol (mM)
C57BL6 WT (n=6)	42.8±1.8 g	3.4±0.38
ApoE^{-/-}		
ddH₂O + NaCl (n=12)	32.6±0.9 g	13.6±0.43
ddH₂O + NaNO₃		
10 mmol (high dose, n=12)	37.3±1.2 g	14.8±0.72
1 mmol (moderate dose, n=10)	33.9±1.4 g	ND
0.1 mmol (low dose, n=10)	33.1±1.1 g	ND

Results presented as mean±SEM. ND: not determined.

Figure 1: Endothelial-dependent relaxation in response to increasing doses of acetylcholine (ACh) in (a) C57BL6 mice (n=6, closed circles), ApoE^{-/-} mice fed a HFD (n=12, closed squares) and ApoE^{-/-} mice fed a HFD supplemented with high dose nitrate for 10 weeks (n=12, closed triangles); (b) ApoE^{-/-} mice fed a HFD (n=10, closed squares), ApoE^{-/-} mice fed a HFD supplemented with low dose nitrate (n=10, open squares) and ApoE^{-/-} mice fed a HFD supplemented with moderate dose nitrate (n=10, open triangles) at 2 weeks; (c) ApoE^{-/-} mice fed a HFD (n=10, closed squares), ApoE^{-/-} mice fed a HFD supplemented with low dose nitrate (n=10, open squares) and ApoE^{-/-} mice fed a HFD supplemented with moderate dose nitrate (n=10, open triangles) at 10 weeks. *p<0.05.

Figure 2: (a) Photomicrographs of sections of brachiocephalic artery (BCA) and atherosclerotic lesion size in C57BL6 WT (n=6), ApoE^{-/-} fed a HFD (n=12) and ApoE^{-/-} fed a HFD supplemented with high dose nitrate (HDN) for 10 weeks (n=12), *p<0.05 versus WT mice. (b) Bright field microscopy (x20 mag), polarised microscopy (x20 mag) and collagen expression in picrosirius red stained BCA sections from ApoE^{-/-} mice fed HFD (n=10), HFD + low dose nitrate (LDN, n=10) and HFD + moderate dose nitrate (MDN, n=10) for 10 weeks, *p=0.01 versus HFD and **p=0.006 versus HFD. (c) Bright field microscopy (x20 mag) and lipid deposition in oil red O stained BCA sections from ApoE^{-/-} mice fed HFD (n=10), HFD + LDN (n=10) and HFD + MDN (n=10) for 10 weeks, *p<0.05 versus HFD. (d) Plaque stability score (collagen:lipid) in BCA from ApoE^{-/-} mice fed HFD (n=10), HFD + LDN (n=10) and HFD + MDN (n=10) for 10 weeks, *p=0.001 versus HFD. (e) Bright field microscopy (x20 mag) and total plaque area in BCA from ApoE^{-/-} mice fed a HFD, HFD + LDN and HFD + MDN for 10 weeks.

Figure 3: GCMS analysis of plasma nitrate (closed bar) and nitrite (hatched bar) following (a) high dose nitrate (HDN, n=12) for 10 weeks (*p<0.005 versus WT and ApoE^{-/-}) and (b) low dose nitrate (LDN, n=10) and moderate dose nitrate (MDN, n=10) for 2 weeks (**p<0.05 versus ApoE^{-/-}). (c) Total NOx measured by chemiluminescence following LDN (n=10) and MDN (n=10) for 10 weeks. (d) Urinary cGMP concentration in ApoE^{-/-} fed a HFD (n=10), HFD + LDN (n=10) and HFD + MDN (n=10) (**p<0.05 for trend and v LDN).

Figure 4: Aortic (a) eNOS and p-eNOS (ser1177) and (b) iNOS, p-AMPK (thr172)/AMPK, p-Akt (ser473)/Akt, gp91phox, p47phox and HO-1 expression in ApoE^{-/-} fed a HFD (n=10), a HFD supplemented with LDN (n=9) and a HFD supplemented with MDN (n=10) for 10 weeks. *p<0.005 versus ApoE^{-/-} + HFD.

Figure 5: (a) plasma and (b) urinary F₂-isoprostane concentrations in ApoE^{-/-} fed a HFD supplemented with HDN (n=12), LDN (n=10) or MDN (n=10) for 10 weeks.

Supplementary Figure 1: Endothelial-independent relaxation in response to increasing doses of SNP in (a) C57BL6 mice (n=6, closed circles), ApoE^{-/-} mice fed a HFD (n=12, closed squares) and ApoE^{-/-} mice fed a HFD supplemented with high dose nitrate for 10 weeks (n=12, closed triangles); and (b) ApoE^{-/-} mice fed a HFD (n=10, closed squares), ApoE^{-/-} mice fed a HFD supplemented with low dose nitrate (n=10, open squares) and ApoE^{-/-} mice fed a HFD supplemented with moderate dose nitrate (n=10, open triangles) at 10 weeks. *p<0.05.