Beetroot juice lowers blood pressure and improves endothelial function in pregnant eNOS+/− mice: importance of nitrate-independent effects

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Running title

Vascular effects of beetroot juice in pregnant mice

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Key Points

- Maternal hypertension is associated with increased rates of pregnancy pathologies, including fetal growth restriction, due at least in part to reductions in nitric oxide (NO) bioavailability and associated vascular dysfunction.

- Dietary nitrate supplementation, from beetroot juice (BRJ), has been shown to increase NO bioavailability and improve cardiovascular function in both preclinical and clinical studies.

- This study is the first to investigate effects of dietary nitrate supplementation in a pregnant animal model. Importantly, effects of nitrate-containing BRJ were compared with both ‘placebo’ (nitrate-depleted) BRJ as well as water to control for potential nitrate-independent effects.

- Our data show novel, nitrate-independent effects of BRJ to lower blood pressure and improve vascular function in endothelial nitric oxide synthase knockout (eNOS⁻/⁻) mice.

- These findings suggest potential beneficial effects of BRJ supplementation in pregnancy, and emphasise the importance of accounting for nitrate-independent effects of BRJ in study design and interpretation.
Abstract

Maternal hypertension associates with adverse pregnancy outcomes, including fetal growth restriction (FGR), due in part to reductions in nitric oxide (NO) bioavailability. We hypothesized that maternal dietary nitrate administration would increase NO bioavailability to reduce systolic blood pressure (SBP), improve vascular function and increase fetal growth in pregnant endothelial NO synthase knockout (eNOS⁻/⁻) mice, which exhibit hypertension, endothelial dysfunction and FGR.

Pregnant wildtype (WT) and eNOS⁻/⁻ mice were supplemented with nitrate-containing beetroot juice (BRJ+) from gestational day (GD) 12.5. Control mice received an equivalent dose of nitrate-depleted BRJ (BRJ⁻) or normal drinking water. At GD17.5, maternal SBP was measured; at GD18.5, maternal nitrate/nitrite concentrations, uterine artery (UtA) blood flow and endothelial function were assessed, and pregnancy outcomes determined.

Plasma nitrate concentrations were increased in both WT and eNOS⁻/⁻ mice supplemented with BRJ+ (P<0.001), whereas nitrite concentrations were increased only in eNOS⁻/⁻ mice (P<0.001). BRJ⁻ did not alter nitrate/nitrite concentrations. SBP was lowered and UtA endothelial function enhanced in eNOS⁻/⁻ mice supplemented with either BRJ+ or BRJ⁻, indicating nitrate-independent effects of BRJ. Improvements in endothelial function in eNOS⁻/⁻ mice were abrogated in the presence of 25mM KCl, implicating enhanced EDH signalling in BRJ treated animals. At GD18.5, eNOS⁻/⁻ fetuses were significantly smaller than WT animals (P<0.001), however BRJ supplementation did not affect fetal weight.

BRJ may be a beneficial intervention in pregnancies associated with hypertension, endothelial dysfunction and reduced NO bioavailability. Our data showing biological effects of non-nitrate components of BRJ have implications for both interpretation of previous findings and in the design of future clinical trials.

Key words: Nitrate, nitric oxide, pregnancy, blood pressure, beetroot juice
Teresa Tropea is a vascular physiologist at the Maternal and Fetal Health Research Centre in Manchester, UK, where she moved after receiving her PhD from the University of Calabria, Italy. Since her doctoral studies, her main research interests have focused on maternal vascular signalling throughout gestation and on the role of materno-placental interactions in determining fetal outcomes, in both human and animal models. Her work on dietary, and more recently, pharmacological interventions in high-risk pregnancies aims to prevent life-threatening consequences for both maternal and fetal health.

Introduction

Hypertensive disorders affect up to 10% of pregnancies worldwide and confer significant risk of perinatal morbidity and mortality to both mother and baby (Hutcheon et al. 2011; ACOG 2013). These associations are largely driven by impaired maternal adaptation to pregnancy (Morton et al. 2017), resulting in increased vascular resistance and reduced utero-placental blood flow. Increased vascular resistance within the utero-placental circulation, clinically indicated by the presence of
abnormal high pulsatility index in Doppler flow-velocity waveforms (Groenenberg et al. 1989), can in turn lead to fetal growth restriction (FGR) as a result of reduced placental oxygen and nutrient delivery to the fetus. Treatment options for hypertensive pregnant women are limited, due to known or suspected teratogenic effects of anti-hypertensive medications on the developing fetus (Sibai, 2001). Therefore, the development of new approaches to manage or treat maternal hypertension remains a research priority.

The endogenous vasodilator nitric oxide (NO) is critically involved in mediating maternal vascular adaptation to pregnancy (Kublickiene et al. 1997; Poston et al. 1995) and maintaining the low-resistance/high-flow uteroplacental vascular system (Osol & Mandala, 2009) needed to sustain sufficient nutrient supply to the fetus. Endogenously, NO is derived from oxidation of the amino acid L-arginine by the NO synthase (NOS) enzymes, of which there are three isoforms (neuronal, inducible and endothelial; nNOS, iNOS and eNOS, respectively). eNOS-derived NO plays a crucial role throughout pregnancy (Krause et al. 2011) and reduced NO production and/or bioavailability is associated with pregnancy complications, including FGR (Schiessl et al. 2006; Krause et al. 2013).

In addition to NOS-derived NO, there is now abundant evidence that NO can be generated by an alternative NOS-independent pathway: the reduction of exogenous or dietary nitrate to nitrite, and subsequently NO (Lundberg & Weitzberg, 2010; Lundberg & Govoni, 2004; Bryan et al. 2008). Numerous studies have demonstrated significant therapeutic potential of dietary nitrate to lower blood pressure and to improve blood flow in non-pregnant humans (Kapil et al. 2010; Kapil et al. 2015; Lidder & Webb, 2013) and animals (Ferguson et al. 2013; Ferguson et al. 2014; Ghosh et al. 2013; Guimaraes et al. 2019). Herein, we tested the hypothesis that maternal nitrate supplementation, delivered via beetroot juice (BRJ), would reduce blood pressure, improve vascular function and increase fetal growth in the endothelial NO synthase knockout (eNOS−/−) mouse, an established model of maternal hypertension associated with vascular dysfunction and FGR (Kusinski et al. 2012).
Methods

Ethical approval

This study was conducted in accordance with the UK Animals (Scientific Procedures) Act of 1986, under Home Office Project Licences 40/3385 and P9755892D. All protocols were approved by the Local Ethical Review Process of the University of Manchester. Authors confirm the present study complies with the policies and regulation of The Journal of Physiology for animal experimentation and ethical principles.

Experimental animals

Endothelial NO synthase knockout mice (eNOS\(^{-/-}\); stock number 002684; N=134), were purchased from Jackson Laboratories (Bar Harbor, ME, United States). C57BL/6J mice (Envigo, Huntington, UK; N=115), as the background strain for eNOS\(^{-/-}\) mice, were used as wildtype control mice (WT). All animals were housed in individually ventilated cages maintained under a constant 12 h light/dark cycle at 21–23°C; food (BK001 diet, Special Dietary Services, United Kingdom) and water were provided ad libitum. Female mice (10-18 weeks old) were mated overnight with genotype-matched male mice (8-26 weeks old) and checked the following morning. The presence of a vaginal plug was defined as gestational day (GD)0.5 (estimated term GD19.5). At GD12.5, mice were weighed and then randomly assigned using a block randomisation strategy, to one of three treatment groups: 1) nitrate supplemented, given beetroot juice containing nitrate (BRJ+) added to the normal drinking water at a dose delivering ~1mmol/kg/day nitrate (Beet it™, James White Drinks, Ipswich, UK); 2) nitrate-depleted beetroot juice (BRJ-), with mice receiving an equivalent volume of BRJ in drinking water (‘placebo’ juice, Beet it™, James White Drinks, Ipswich, UK), and 3) regular drinking water only. Bottles were changed daily until GD18.5, when mice were killed by cervical dislocation; maternal blood samples were collected and maternal and fetal tissues harvested.
Measurement of nitrate and nitrite concentrations in BRJ and plasma

To determine plasma concentrations of nitrate and nitrite, maternal blood samples (N=65; 9-13 dams in each experimental group) were collected into capillary blood collection tubes (Microvette® CB 300, Sarstedt). Blood was centrifuged immediately (5 mins at 5000 rpm) and plasma removed and stored at −80°C until analysis. Plasma samples (10 μl) were injected into a dedicated HPLC system (ENO-20; EiCom, Japan) using a Hamilton syringe, as previously described (Montenegro et al. 2016). Subsequently, nitrite and nitrate were separated by reverse phase/ion exchange chromatography followed by nitrate reduction to nitrite by cadmium and reduced copper. Nitrite was derivatized using Griess reagent to form diazo compounds and detected at 540 nm. To determine plasma concentrations of nitrate and nitrite, data were collected and analyzed using the PowerChrom software (V 2.7.9, eDAQ).

Nitrate and nitrite concentrations of BRJ, and of the drinking water used in the animal facility, were determined by chemiluminescence after reductive cleavage and subsequent release of NO into the gas phase, as previously described (Lundberg & Govoni, 2004). Briefly, samples were directly introduced into the reduction solution of a microreaction purge vessel coupled with a condenser and heating jacket unit (Sievers, Boulder, CO, USA). A rapid-response chemiluminescence NO system (Aerocrine AB, Stockholm, Sweden) was used to detect the NO signals. The data obtained were further analyzed with the Windows Azur platform and the levels of nitrate were calculated and reported in mM (mmol/L) by comparing the area under the curve to the known concentration of nitrate standards.
Maternal blood pressure

In a subset of animals (N=69; 8-14 dams in each experimental group), maternal systolic blood pressure (SBP) was determined prior to mating and at GD17.5, using a validated non-invasive tail-cuff method (LE5001; Pan Lab, Spain; Whitesall et al. 2004). Maternal heart rate was also recorded.

Ex vivo uterine artery vascular function

At GD18.5, uterine arteries (UtA) were used for ex vivo assessment of vascular reactivity, using wire myography (N=101; 14-21 dams in each experimental group). Main branch UtA were collected and cleaned of surrounding adipose tissue in ice-cold physiologic salt solution (PSS; in mM, 117 NaCl, 25 NaHCO₃, 4.69 KCl, 2.4 MgSO₄, 1.6 CaCl₂, 1.18 KH₂PO₄, 6.05 glucose, 0.034 EDTA; pH 7.4). Arterial segments (approximately 2mm in length) were mounted on two 40 μm steel wires in a myograph chamber (Model 620 M, Danish MyoTechnologies, Denmark) and immersed in 6 ml of 5%CO₂/20% oxygen/75% nitrogen gassed PSS, maintained at 37°C. Arteries were then normalized to 0.9 of luminal pressure (L)₁₃.₃ kPa, through a series of stepwise increases in luminal diameter to determine their optimal resting tension, in accordance with Mulvany’s normalization procedure (Mulvany & Halpern, 1977).

Following 20 minutes of equilibration, arteries underwent two separate exposures to a depolarizing solution (KPSS; 120 mM KCl in PSS, equimolar substitution of KCl for NaCl). After washing with PSS, a concentration-response curve to the thromboxane mimetic U46619 (10⁻¹⁰ - 2x10⁻⁶ M, Cayman Chemicals) was obtained and used to calculate the EC₈₀ concentration of U46619. Endothelium-dependent relaxation to acetylcholine (ACh, 10⁻¹⁰-10⁻⁵ M) was assessed in arteries pre-constricted with an EC₈₀ dose of U46619.

For studies using inhibitors of NOS-dependent and COX-dependent endothelial relaxation, separate vessels were pre-incubated with either L-NAME+L-NNA (each at 10⁻⁴M, Cayman Chemicals) or indomethacin (10⁻⁵M), respectively. To determine the contribution of endothelium-dependent hyperpolarization (EDH), ACh relaxation was assessed in the presence of modified PSS containing 25mM KCl (substitution of KCl for NaCl in PSS to maintain osmolality; Gerber et al. 1998). For all
inhibitor experiments, vessels were pre-incubated for 30 minutes prior to U46619 pre-constriction and ACh concentration-response curve; the inhibitor solution remained in the bath throughout this entire procedure. Dose-response relaxation in each experimental condition was expressed as a percentage change in tension from the level of pre-constriction achieved with an EC$_{80}$ dose of U46619. Maximum relaxation ($V_{\text{max}}$) to ACh was expressed as a percentage of the maximum effect of the dose-response. Effects of each inhibitor on the sensitivity of ACh-induced relaxation are expressed as the molar concentration of ACh causing 50% of the maximal relaxation in the presence of inhibitors, and expressed as logarithm ($\log \text{EC}_{50}$).

Uterine artery doppler ultrasound

In order to investigate changes in UtA vascular function in vivo, a separate group of mice to those used for myography studies (N=64; 7-16 dams in each experimental group) was used at GD18.5 to image the UtA transcutaneously, using an ultrasound biomicroscope (Vevo 2100, VisualSonics). Mice were anesthetized with inhaled isoflurane (1 L/min; 4% induction, ~1.5-2% maintenance) in oxygen and placed on a heated table. Body temperature was maintained between 35 and 36°C using an infrared lamp and monitored using a rectal probe. Respiratory and heart rates were also monitored. Fur was removed from the abdomen using depilatory cream and pre-warmed ultrasound gel applied. Scans were performed using an MS 550D probe (Visualsonics; 22–55 MHz, 15 mm maximum depth of penetration, 14.08 mm maximum width). The maternal bladder was identified and UtA Doppler waveforms were obtained from both left and right UtA proximal to the internal iliac artery. This was a terminal procedure, after which mice were killed by cervical dislocation. The highest point of the systolic waveform defined the peak systolic velocity (PSV), the end point of the diastolic waveform defined the end diastolic velocity (EDV). Measurements of both PSV and EDV obtained from three consecutive cardiac cycles were averaged and resulting values used to calculate UtA Resistance Index ($RI=\frac{\text{PSV-EDV}}{\text{PSV}}$) and Pulsatility Index ($PI=\frac{\text{PSV-EDV}}{\text{Mean Velocity}}$).
Drugs and chemicals

Chemicals and pharmacological agents used in this study were purchased from Sigma Aldrich, unless otherwise stated.

Data analysis

Data were assessed for normality and are expressed as mean ± SD (for parametric data) or median and range (for non-parametric data). The experimental N = number of dams/litters; for fetal and placental weight data, litter averages are presented. Non-parametric data were transformed prior to analysis using 2-way ANOVA (treatment, genotype and interaction as main factors), with post-hoc testing and adjustment for multiple comparisons, as appropriate, performed using Prism 7 software (GraphPad Software Inc, La Jolla, CA). Statistical significance was defined as P < 0.05.

Results

There were no differences in maternal body weight between groups at GD0.5. WT animals had a significantly greater body weight gain across gestation compared with eNOS−/− mice (effect of genotype, P = 0.0220), but there was no effect of treatment on maternal body weight gain in either WT or eNOS−/− mice (WT H2O, 14.8 ± 2.8 g; WT BRJ+, 15.1 ± 2.9 g; WT BRJ−, 14.6 ± 2.6 g; eNOS−/− H2O, 13.9 ± 1.7 g; eNOS−/− BRJ+, 14.5 ± 1.8 g; eNOS−/− BRJ−, 13.6 ± 2.1 g). Maternal fluid intake was slightly but significantly increased in WT compared with eNOS−/− mice (effect of genotype, P = 0.0455), and was significantly greater in BRJ supplemented animals compared with water control animals within each genotype group (effect of treatment, P<0.0001; WT H2O, 9.5 ± 1.8 mL/day; WT BRJ+, 11.2 ± 2.2 mL/day, P = 0.0115 vs WT H2O; WT BRJ−, 12.3 ± 2.4 mL/day, P = 0.0001 vs WT H2O; eNOS−/− H2O, 9.05 ± 2.1 mL/day; eNOS−/− BRJ+, 10.9 ± 2.2 mL/day, P = 0.0009 vs eNOS−/− H2O; eNOS−/− BRJ−, 11.0 ± 1.9 mL/day, P = 0.0008 vs eNOS−/− H2O).
Effects of maternal BRJ supplementation on plasma nitrate and nitrite concentrations

Concentrations of nitrate/nitrite in the batches of BRJ shots used across these studies were measured, with BRJ+ having 92.4 mmol/L nitrate/nitrite (range: 78.8-112.8 mmol/L) and BRJ- having 0.1 mmol/L nitrate/nitrite (range: 0.04-0.16 mmol/L). Concentrations of nitrate/nitrite in the drinking water ranged between 0.009 – 0.011 mmol/L. There was no significant difference in maternal plasma nitrate or nitrite concentrations in control WT compared with eNOS\(^{-/-}\) mice (i.e. drinking water only groups; Figure 1). Maternal plasma nitrate concentrations were significantly increased by BRJ+ supplementation in both WT and eNOS\(^{-/-}\) mice (effect of treatment, \(P<0.0001\); Figure 1A). In contrast, plasma nitrite concentrations were significantly elevated in BRJ+ supplemented eNOS\(^{-/-}\) dams only (effect of treatment, \(P<0.0001\), Figure 1B), suggesting enhanced conversion of nitrate to nitrite in these animals. No increase in nitrate or nitrite was seen in animals supplemented with BRJ-.

Maternal BRJ supplementation lowers systolic blood pressure in pregnant eNOS\(^{-/-}\) mice

eNOS\(^{-/-}\) mice displayed significantly higher pre-pregnancy SBP compared with WT controls (WT: 112.6 ± 7.1 mmHg; eNOS\(^{-/-}\): 136.2 ± 6.4 mmHg; \(P<0.0001\), unpaired t-test). There was a significant effect of maternal BRJ supplementation to lower SBP at GD17.5 (effect of treatment, \(P=0.0067\); Figure 2). Surprisingly, this blood pressure-lowering effect was not related to the nitrate content of the juice, as there was a reduction in SBP of ~8 mmHg in eNOS\(^{-/-}\) dams supplemented with either BRJ+ or BRJ-. There was no significant effect of BRJ supplementation on SBP in WT dams.
Maternal BRJ supplementation enhances uterine artery endothelial-dependent relaxation

Responses to the endothelial-dependent vasodilator ACh were significantly attenuated in UtA of eNOS<sup>−/−</sup> mice compared with WT animals (P<0.0001; Figure 3). BRJ supplementation did not alter responses to ACh in UtA from WT mice (Figure 3A), but both BRJ+ and BRJ− groups significantly enhanced endothelial-dependent relaxation in eNOS<sup>−/−</sup> mice (Figure 3B), again suggesting nitrate-independent effects of BRJ on vascular function.

To interrogate which component(s) of endothelial-dependent relaxation might be affected by BRJ treatment, we performed additional experiments in the presence of inhibitors of cyclooxygenase (COX) enzymes (indomethacin, 10<sup>−5</sup>M), NOS enzymes (L-NAME + L-NNA; 10<sup>−4</sup>M) or EDH (using PSS containing 25mM KCl to block relaxation dependent upon hyperpolarization). In WT animals drinking water only, endothelial responses to ACh were significantly attenuated by indomethacin, L-NAME + L-NNA and 25mM KCl (Figure 4A, C, E). Interestingly, ACh responses in eNOS<sup>−/−</sup> mice were unaffected by either indomethacin or L-NAME + L-NNA (Figure 4B, D), whereas 25mM KCl nearly abolished endothelial relaxation in eNOS<sup>−/−</sup> mice (Figure 4F).

Despite the absence of a treatment effect under control conditions in WT mice, significant effects of BRJ supplementation were revealed on UtA vascular function after inhibition of COX (P<0.001; Figure 5A) and NOS (P<0.05; Figure 5C) enzymes. Pre-incubation with indomethacin significantly increased the sensitivity to ACh in WT mice supplemented with BRJ when compared to same-genotype water control (effect of treatment, P = 0.0105; Log EC<sub>50</sub>: WT H<sub>2</sub>O, -6.83 ± 0.8; WT BRJ+, -8.10 ± 0.6, P = 0.0011 vs WT H<sub>2</sub>O; WT BRJ−, -7.63 ± 0.7, P = 0.0202 vs WT H<sub>2</sub>O, respectively). In the presence of 25mM KCl, ACh responses were attenuated in WT mice, but a significant effect of treatment remained (P<0.05; Figure 5E).

In eNOS<sup>−/−</sup> mice, pre-incubation with indomethacin further enhanced ACh responses in UtA of dams supplemented with either BRJ+ or BRJ− (Figure 5B); maximum relaxation to ACh was significantly increased in both BRJ+ or BRJ− animals (effect of genotype P < 0.0001; effect of treatment, P = 0.0030. Vmax: eNOS<sup>−/−</sup> H<sub>2</sub>O, 59.42 ± 23.6 %; eNOS<sup>−/−</sup> BRJ+, 85.49 ± 10.6 %, P = 0.0001 vs eNOS<sup>−/−</sup> H<sub>2</sub>O;
eNOS\textsuperscript{-/-} BRJ-, 85.92 ± 13.7 %, P = 0.0002 vs eNOS\textsuperscript{-/-} H\textsubscript{2}O). Following pre-incubation with NOS inhibitors, there was no longer a significant effect of BRJ supplementation to enhance UtA relaxation (effect of treatment, P=0.066; Figure 5D). In the presence of 25mM KCl, ACh responses were markedly attenuated in eNOS\textsuperscript{-/-} UtA; furthermore, this condition abolished the effects of BRJ to enhance endothelial function (Figure 5F).

**In vivo assessment of UtA haemodynamic parameters**

To determine whether the observed effects of BRJ supplementation on UtA vascular reactivity translated to changes in hemodynamic parameters in vivo, UtA blood flow-velocity measurements were obtained using micro-ultrasound. Under general anaesthesia, heart rate was not different between genotypes, whereas respiration rate (in breaths/min) was significantly higher in eNOS\textsuperscript{-/-} mice compared with WT animals, at GD18.5 (effect of genotype, P<0.001; WT H\textsubscript{2}O, 58.1 ± 6.8; WT BRJ+, 69.5 ± 9.0; WT BRJ, 62.85 ± 16.1; eNOS\textsuperscript{-/-} H\textsubscript{2}O, 81.7 ± 9.5; eNOS\textsuperscript{-/-} BRJ+, 72.17 ± 10.9; eNOS\textsuperscript{-/-} BRJ, 76.4 ± 12.9 breaths/min). Peak systolic velocity (PSV) was significantly lower (P = 0.0238) whereas end diastolic velocity (EDV) was not (P=0.07) in eNOS\textsuperscript{-/-} mice compared with WT animals (Table 1). Maternal BRJ supplementation did not affect PSV or EDV in either genotype; resistance index (RI) and pulsatility index (PI) were also unchanged in mice supplemented with BRJ (Table 1).

**Pregnancy outcomes**

At GD18.5, there was no significant difference in litter size between eNOS\textsuperscript{-/-} and WT mice (Table 2). Fetal weights were significantly reduced in eNOS\textsuperscript{-/-} mice compared with WT mice (P<0.0001, Table 2). Supplementation with BRJ had no effect on either fetal or placental weights, in either genotype group (Table 2).
Discussion

The present study is the first investigation to determine whether maternal BRJ supplementation may be of therapeutic benefit in an established pregnant animal model exhibiting maternal hypertension, endothelial dysfunction and FGR.

As previously reported, eNOS−/− mice display significantly higher SBP compared with WT controls (Shesely et al. 1996). In the present study, we found significant effects of maternal BRJ supplementation to lower blood pressure and improve endothelial function in eNOS−/− mice. A growing body of evidence from both preclinical and clinical studies has shown beneficial effects of BRJ supplementation, attributed to its high nitrate content, to lower blood pressure (Kapil et al. 2010; Kapil et al. 2015; Ferguson et al. 2013; Ghosh et al. 2013) and improve blood flow (Ferguson et al. 2013; Walker et al. 2019). In contrast to previous studies in which the effects of BRJ were compared with either a nitrate-depleted placebo juice (as used in this study; BRJ−) or water alone, we have here included both control arms side-by-side and have for the first time shown that there are highly significant and biologically important vascular effects of BRJ that are not related to its nitrate content. Nitrate-depleted BRJ exerted similar effects on maternal SBP and UtA vascular function compared with nitrate-containing BRJ, strongly suggesting that other bioactive compounds of the juice are involved.

In alignment with findings of the present study, a recent systematic review and meta-analysis of trials using BRJ as an intervention to lower blood pressure in humans suggested the potential importance for nitrate-independent effects of BRJ (Bahadoran et al. 2017). The authors identified that the effect of BRJ to lower blood pressure was attenuated if BRJ− was used as the control compared with another control such as water, highlighting potential nitrate-independent effects of BRJ (Bahadoran et al. 2017). In addition, a very recent study (Mills et al. 2020) comparing effects of nitrate-containing and nitrate-depleted ‘placebo’ BRJ in non-pregnant adults reported no difference in SBP between these arms across the 24-week intervention period. However, in both of these groups, peripheral BP did decrease by ~6.5 mmHg compared with baseline SBP. The similarity of these changes to those reported in the current study are notable, and again suggestive of nitrate-independent effects of BRJ. Our current findings now confirm the importance of nitrate-independent effects of BRJ on cardiovascular function. These data have implications for the interpretation of previous studies, but more importantly should inform the design of future intervention trials.
There are numerous bioactive phytochemicals contained within BRJ, including ascorbic acid, carotenoids, polyphenols and flavonoids (Kujala et al. 2002; Ninfali & Angelino, 2013) that have the potential to mediate effects on SBP and vascular function. To start to explore the mechanism(s) by which BRJ enhances endothelial function, we investigated the potential contribution of the three main endothelial mediators that regulate vascular tone; prostacyclin (PGI₂), NO and EDH. In the presence of the COX inhibitor, indomethacin, BRJ significantly increased ACh responses in both WT and eNOS⁻/⁻ mice; sensitivity to ACh and Vmax were significantly higher in UtA of WT and eNOS⁻/⁻ mice supplemented with BRJ. Previous evidence has indicated that COX-derived vasoconstrictive factors, such as prostaglandin H2 (PGH₂) and thromboxane A2 (TXA₂), can impair endothelium-dependent relaxation by inactivating NO formation (Taddei et al. 1997). We speculate that incubation with indomethacin may have removed vasoconstrictive effects of PGH₂ and TXA₂, thereby potentiating ACh responses. Our data are consistent with previous studies showing that indomethacin increases ACh-induced relaxation in isolated aortic rings of spontaneously hypertensive and Wistar Kyoto rats (De Angelis et al. 2004). Inhibition of NOS enzymes similarly led to an enhancement of ACh responsiveness, but in WT mice only. In eNOS⁻/⁻ mice, NOS inhibition appeared to abolish the effects of BRJ+ on ACh responses, yet a borderline effect of enhanced relaxation remained in BRJ⁻ animals. These data implicate potential nitrate-dependent effects of BRJ on ACh responses in eNOS⁻/⁻ animals, an effect that requires further investigation.

In an attempt to isolate the non-NO, non-PGI₂ effects of BRJ implicated by the above experiments, we used 25mM KCl to inhibit relaxation dependent upon hyperpolarization. As previously shown (Brandes et al. 2000), this approach highlights a marked difference between WT and eNOS⁻/⁻ mice in EDH-like pathways; in the presence of 25mM KCl, ACh responses were modestly attenuated in UtA from WT mice, whereas in eNOS⁻/⁻ mice, incubation with 25mM KCl almost completely abolished ACh-induced relaxation. This upregulation of an EDH-like K⁺ efflux pathway in eNOS⁻/⁻ mice has been suggested to be a compensatory mechanism to offset the NO deficiency and normalize vascular tone (Scotland et al. 2001; Waldron et al. 1999). It is therefore interesting that, in the absence of any inhibitors, the effects of BRJ to enhance endothelial function were seen only in eNOS⁻/⁻ mice, whereas treatment effects were absent in the presence of 25mM KCl in this strain. Our data have led us to hypothesize that the effects of BRJ act to increase an EDH-like component of endothelial
function; further detailed studies are needed to confirm this. In terms of identifying active component(s) of BRJ, it is interesting to note that previous studies investigating the effects of red wine polyphenols on vascular function have reported similar findings in vascular tissues exposed to polyphenolic compounds, namely increased NO and EDH formation (Duarte et al. 2004; Ndiaye et al. 2005). Future studies will focus on determining whether the effects we report here of BRJ supplementation on blood pressure and endothelial function are specific to pregnancy or to eNOS−/− mice. However, the fact that in WT animals BRJ supplementation clearly causes significant alterations in endothelial-dependent vasodilatory pathways suggests that the effects we have shown are likely to be of more widespread relevance.

Reduced utero-placental blood flow and elevated vascular resistance are predictive of FGR.

Our in vivo results demonstrate that PSV is significantly reduced in UtA of eNOS−/− compared with WT mice and EDV showed a trend towards reduction, in agreement with previous studies (Kulandavelu et al. 2012; Poudel et al. 2013); however, there were no significant effects of BRJ supplementation on UtA flow-velocity measurements or resistance indices. At present these findings suggest that the improved UtA endothelial responses demonstrated ex vivo may not translate to an increase in blood flow/reduction in vascular resistance in vivo, or alternatively that the effects of anaesthesia might reduce or mask any differences. Of note, isoflurane has been shown to vasodilate the vasculature, in part through endothelial-dependent effects on smooth muscle cells, with some studies showing such effects are mediated by specific K+ channels (Cason et al. 1994; Zhou et al. 1998). Thus, it is possible that the effects of BRJ to enhance EDH, as shown by our ex vivo data, is abrogated in our in vivo experiments by the effects of isoflurane on K+ channel function.

In agreement with recently published data (Peleli et al. 2016), we found no difference in circulating concentrations of nitrate or nitrite between WT and eNOS−/− animals in the water-control groups. The significant increase in maternal plasma nitrate concentrations in both WT and eNOS−/− mice supplemented with BRJ+ replicates previous findings using a similar dose of dietary nitrate (Ferguson et al. 2013; Peleli et al. 2016), showing an ~4-fold increase in circulating nitrate levels compared with either water-treated or BRJ- control animals. In agreement with the work of Carlstrom and colleagues (Peleli et al. 2016), we found that plasma nitrite responses to the same dose of dietary
nitrate were markedly enhanced in eNOS−/− animals when compared with WT controls. The previous study identified xanthine oxidoreductase (XOR) as the likely mediator of this enhanced nitrate-nitrite conversion, as concurrent administration of the selective XOR inhibitor, febuxostat, abolished this effect (Peleli et al. 2016). It is also interesting to note that in the present study, the increase in plasma nitrate and nitrite in response to BRJ+ supplementation varied markedly between animals. One significant limitation of studies administering substances in the drinking water is that researchers cannot control for exact volumes consumed by each mouse, nor the timing of ingestion relative to sampling. It is possible that differences in both the biochemical and functional responses to treatment in our studies are related to the timeframe between dosing and tissue harvest. In terms of the potential for differences in fluid intake to affect blood pressure, the small increase in fluid intake in BRJ-treated animals might be expected to increase SBP, rather than decrease it (Jordan et al. 2000), lending greater support to our findings that BRJ intake per se is responsible for the lowering of BP.

In agreement with our own data and those of others (Kusinski et al. 2012; Renshall et al. 2018; Kulandavelu et al. 2012; Poudel et al. 2013; Kulandavelu et al. 2013), fetal weights were significantly reduced in eNOS−/− mice compared with WT mice. Contrary to our hypothesis, and despite significantly increasing plasma nitrate and nitrite concentrations and improving UtA vascular function, BRJ+ supplementation did not increase fetal weight at GD18.5 in eNOS−/− mice. The lack of effect on both UtA blood flow (albeit under anaesthesia) and fetal weight, after administration of BRJ+, potentially reflects a limitation of using this genetically-modified model, in which the homozygous knockout fetus may have significantly limited genetic growth potential. It is possible that the eNOS−/− FGR phenotype is refractory to therapeutic intervention, as the majority of studies to date have reported minimal or no effect of interventions on fetal weight in this strain of mice. Studies aimed at improving FGR via administration of bioactive compounds such as resveratrol (Poudel et al. 2013), pomegranate juice (Finn-Sell et al. 2018), melatonin (Renshall et al. 2018) and tanshinone IIA (Morton et al. 2015), failed to increase fetal weight in eNOS−/− mice. To our knowledge, only administration of the antioxidant Tempol was able to improve fetal weight in this mouse model of FGR, and this only to a modest degree (Stanley et al. 2012). Alternatively, it could be that the reduction in maternal blood pressure seen at GD17.5 would lead to a reduction in placental perfusion, as has been previously speculated, albeit not supported by evidence in clinical studies (von Dadelszen & Magee, 2002; Magee et al. 2016). Arguing against this latter point,
However, are results of a recent study aiming to determine whether maternal nitrite supplementation, in the form of sodium nitrite, was able to lower maternal blood pressure and rescue FGR in hypertensive, L-NAME-treated rats (Goncalves-Rizzi et al. 2016). In this study, maternal nitrite treatment was reported to significantly lower maternal blood pressure and to lead to an increase in fetal weight, albeit in a small number of animals.

In terms of translation to pregnant women, two recent trials (Ormesher et al. 2018; Volino-Souza et al. 2018), including one by our own group (Ormesher et al. 2018), have investigated whether maternal BRJ supplementation could be beneficial in pregnant women. Notably, in both of these studies, a double-blind placebo-controlled design was used, and hence only BRJ+ and BRJ- arms were included. In our trial (Ormesher et al. 2018), which included 40 women consuming a dose of beetroot juice daily for a total of 8 days, there was no effect of BRJ treatment on blood pressure across the 8 days, compared with baseline. However, we did find a significant correlation between the change in plasma nitrite concentration and the decrease in diastolic blood pressure following acute BRJ+ supplementation, suggesting potential efficacy of BRJ+ supplementation to lower blood pressure in those hypertensive pregnant women able to effectively convert ingested nitrate to nitrite and NO (Ormesher et al. 2018). In the other trial, a crossover design was used to examine the effects of a single dose of BRJ on endothelial function, in a total of 12 pregnant women (Volino-Souza et al. 2018). In this study, the authors demonstrated that BRJ+ significantly increased flow-mediated vasodilation, with no change reported in the BRJ- group (Volino-Souza et al. 2018). Taken together, these data indicate that there may still be benefits of nitrate supplementation from BRJ in pregnant women to improve cardiovascular function. Our present results suggest that nitrate-independent effects of BRJ need to be taken into account when assessing the efficacy of BRJ supplementation.

In summary, we have shown that maternal BRJ supplementation can reduce SBP in pregnant eNOS−/− mice and improve vascular function, likely through effects on an EDH-like component of endothelial-dependent signaling. The nitrate-independent effects of BRJ warrant further investigation, and are of importance for informing the design of future preclinical and clinical studies using this dietary intervention.
Additional information

Competing interests

J.O.L. and E.W. are inventors on patents related to the therapeutic use of inorganic nitrate and nitrite. All other authors declare no conflict of interests.

Author contributions

T.T., S.L.G., C.P.S., M.W. and E.C.C. conceived and designed the experiments. T.T., L.J.R, C.N., E.W., J.O.L., A.L.D, V.T., D.S., M.W., S.L.G., C.P.S. and E.C.C acquired, analysed or interpreted experimental data. T.T., S.L.G., C.P.S. and E.C.C. drafted and/or critically revised the manuscript for important intellectual content. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Authors’ Translational Perspective

Maternal hypertensive disorders are common, affecting around 10% of pregnancies, and associate with increased risk of morbidity and mortality for both mother and baby. Treatment options are
limited due to effects of anti-hypertensive medications on the fetus, and the development of new approaches to manage maternal hypertension remains a research priority.

There is significant interest in the therapeutic potential of inorganic nitrate supplementation in cardiovascular medicine. Supplementation with beetroot juice (BRJ), containing high levels of inorganic nitrate, remains one of the primary interventions used in both preclinical and clinical studies. Here, we tested the hypothesis that maternal nitrate supplementation, from BRJ, will reduce blood pressure, improve vascular function and increase fetal growth in the endothelial nitric oxide synthase knockout (eNOS−/−) mouse, a model of maternal hypertension associated with vascular dysfunction and fetal growth restriction.

We have shown that BRJ supplementation lowers blood pressure and improves endothelial function in eNOS−/− mice. In contrast to previous studies, in which effects of nitrate-rich BRJ were compared with either nitrate-depleted placebo juice or water, we have included both control arms and have shown for the first time significant and biologically important effects of BRJ, independent of its nitrate content. Nitrate-depleted BRJ exerted similar effects on maternal blood pressure and vascular function compared with nitrate-containing BRJ, strongly suggesting that other bioactive compounds are important.

These data have significant implications for the interpretation of previous studies, in which effects of BRJ supplementation have been attributed solely to its nitrate content. Furthermore, these data should inform the design of future trials using this dietary approach.
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Cason BA, Shubayev I & Hickey RF (1994). Blockade of adenosine triphosphate-sensitive potassium channels eliminates isoflurane-induced coronary artery vasodilation. Anesthesiology 81, 1245-28A.


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Figure 1. Maternal plasma nitrate and nitrite concentrations in BRJ supplemented dams at GD 18.5

Plasma nitrate concentrations (A) were significantly elevated in BRJ+ supplemented dams. Nitrite concentrations (B) were significantly elevated only in eNOS⁻/⁻ BRJ+ supplemented dams. ***P<0.001 BRJ+ vs H₂O (same-genotype); §§§P<0.001 eNOS⁻/⁻ vs WT. n=9-13 dams per group.
Figure 2. BRJ supplementation reduces SBP in eNOS⁻/⁻ mice

Maternal BRJ supplementation lowered SBP at GD17.5. This effect was significant in eNOS⁻/⁻ mice.

*P<0.05 BRJ+ vs H₂O; #P<0.05 BRJ- vs H₂O; §§§P<0.001 eNOS⁻/⁻ vs WT. N=8-14 dams per group.
Maternal BRJ supplementation did not affect UtA endothelial responses to ACh in WT mice (A) but significantly enhanced relaxation in eNOS−/− mice (B). *P<0.05, **P<0.01 BRJ+ vs H2O; #P<0.05 BRJ- vs H2O. N=14-21 dams per group.
Figure 4. Endothelial responses to ACh in drinking water control animals are affected by inhibitors of COX, NOS and EDHF. Incubation with Indomethacin (A, B), L-NAME+L-NNA (C, D) and 25mM KCl (E, F) attenuated ACh responses, with differential effects in WT compared with eNOS−/− mice. Ø P<0.05, Ø Ø P<0.01, Ø Ø Ø P<0.001. N=6-15 dams per group.
Figure 5. BRJ supplementation alters UtA endothelium-dependent relaxation components. In UtA of WT mice, inhibition of COX- (A) and NOS- (C) dependent pathways revealed effects of BRJ supplementation on non-prostaglandin/non-NO induced relaxation; 25 mM KCl (E) attenuated but did not completely prevent these effects of BRJ in WT mice. In eNOS−/− mice supplemented with BRJ, inhibition of COX (B) enhanced endothelial responses to ACh compared with H2O controls. NOS inhibition blunted (D) and 25 mM KCl abolished (F) effects of BRJ in this strain. *P<0.05, **P<0.01, ###P<0.001 BRJ+ vs H2O; #P<0.05, ##P<0.01, ####P<0.001 BRJ vs H2O. N= 6-15 dams per group.
Table 1. BRJ supplementation has no significant effect on uterine artery haemodynamic parameters measured under general anaesthesia using micro-ultrasound at GD18.5.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>eNOS⁻/⁻</th>
<th>Treat</th>
<th>Gen</th>
<th>Int</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>H₂O</td>
<td>BRJ+</td>
<td>BRJ⁻</td>
<td>H₂O</td>
<td>BRJ+</td>
</tr>
<tr>
<td>PSV (mm/s)</td>
<td>427.2 ± 98.8</td>
<td>401.5 ± 126.3</td>
<td>463.9 ± 76.2</td>
<td>366.1 ± 99.4</td>
<td>383.7 ± 143.5</td>
</tr>
<tr>
<td>EDV (mm/s)</td>
<td>228.8 ± 60.5</td>
<td>216.1 ± 75.5</td>
<td>254.5 ± 43.7</td>
<td>191.4 ± 57.6</td>
<td>223.1 ± 91.8</td>
</tr>
<tr>
<td>RI</td>
<td>0.468 ± 0.05</td>
<td>0.464 ± 0.03</td>
<td>0.451 ± 0.05</td>
<td>0.480 ± 0.06</td>
<td>0.424 ± 0.06</td>
</tr>
<tr>
<td>PI</td>
<td>0.658 ± 0.11</td>
<td>0.643 ± 0.08</td>
<td>0.625 ± 0.10</td>
<td>0.687 ± 0.15</td>
<td>0.567 ± 0.11</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. Treat, effect of treatment; Gen, effect of genotype; Int, interaction between genotype and treatment. § P < 0.05 WT vs eNOS⁻/⁻; NS, not significant. PSV, peak systolic velocity; EDV, end diastolic velocity; RI, resistance index; PI, pulsatility index. N=7-16 dams per group.
Table 2. Litter size, fetal and placental weights at GD18.5

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>eNOS^{−/−}</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>H_{2}O</td>
<td>BRJ+</td>
</tr>
<tr>
<td>Litter size (pups/dam)</td>
<td>7.0±1.9</td>
<td>7.3±1.8</td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td>1.135±0.059</td>
<td>1.139±0.054</td>
</tr>
<tr>
<td>Placental weight (mg)</td>
<td>79.1±6.4</td>
<td>76.9±5.7</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. Treat, effect of treatment; Gen, effect of genotype; Int, interaction between genotype and treatment. §§§ P < 0.001 WT vs eNOS^{−/−}; NS, not significant. N=27-36 litters per group.