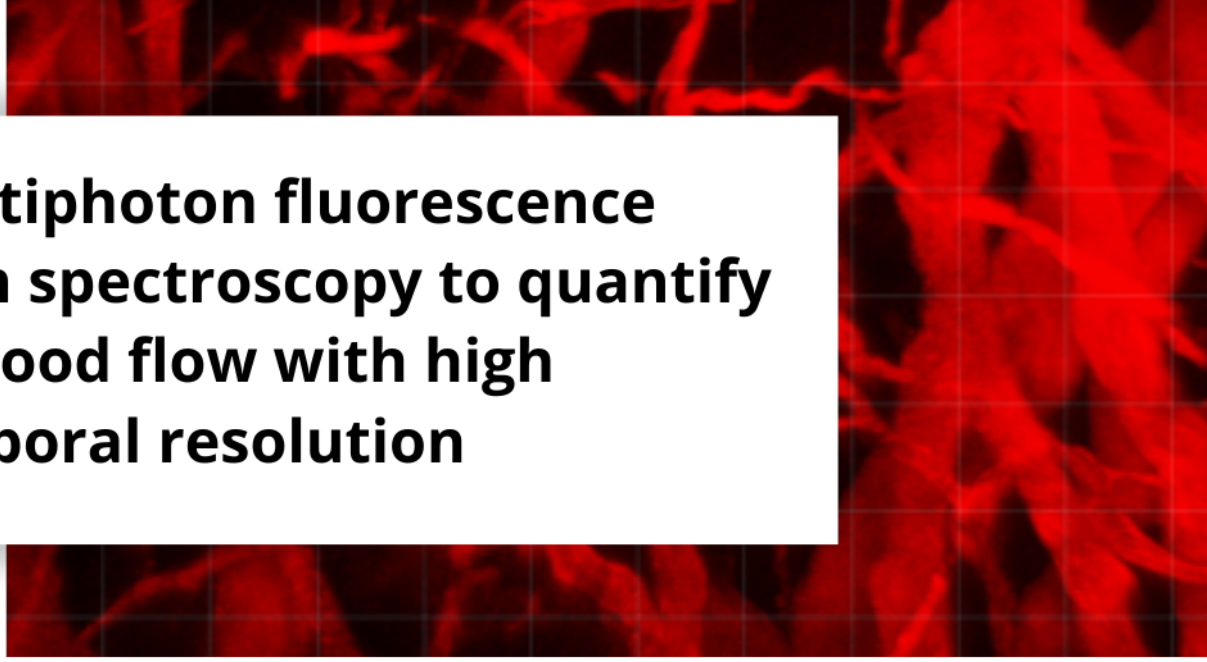

In vivo multiphoton fluorescence correlation spectroscopy to quantify cerebral blood flow with high spatiotemporal resolution

Cerebral blood flow (CBF) measurements provide critical information about physiological and pathological processes within the central nervous system (CNS). The complex microvascular network plays a fundamental role within the CNS, where neuronal activity regulates the flow of nutrients. Understanding blood flow dynamics with high spatial and temporal resolution is essential to understanding the role of vascular dysfunction in a variety of pathological processes. Using multiphoton in vivo fluorescence correlation spectroscopy (FCS), blood flow rates can be determined at individual pixels with sub-micron resolution.

Free Webinar
November 12, 2020
11 am EST

Sponsored by:

ZEISS

Provided by:

CURRENT PROTOCOLS
A Wiley Brand

WILEY

J Physiol 598.18 (2020) pp 4079–4092

The Journal of Physiology

Beetroot juice lowers blood pressure and improves endothelial function in pregnant eNOS$^{-/-}$ mice: importance of nitrate-independent effects

Teresa Tropea[1,2], Lewis J. Renshall[1,2], Carina Nihlen[3], Eddie Weitzberg[3], Jon O. Lundberg[3], Anna L. David[4], Vassilis Tsatsaris[5], Daniel J. Stuckey[6], Mark Wareing[1,2], Susan L. Greenwood[1,2], Colin P. Sibley[1,2] and Elizabeth C. Cottrell[1,2]

[1]*Maternal and Fetal Health Research Centre, Division of Developmental Biology and Medicine, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK*
[2]*Manchester Academic Health Science Centre, Manchester University NHS Foundation Trust, St. Mary's Hospital, Manchester, UK*
[3]*Department of Physiology and Pharmacology, Karolinska Institute, Stockholm, SE-171 77, Sweden*
[4]*Elizabeth Garrett Anderson Institute for Women's Health, University College London, London, UK*
[5]*Obstetrics and Gynecology Unit, Maternité Port-Royal, APHP, Paris V, Paris, France*
[6]*Centre for Advanced Biomedical Imaging, University College London, London, UK*

Edited by: Laura Bennet & Suzanne Miller

Linked articles: This article is highlighted in a Perspectives article by Torrens & Feelisch. To read this article, visit https://doi.org/10.1113/JP280091.

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, the 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 emphasize the importance of accounting for nitrate-independent effects of BRJ in study design and interpretation.

Abstract Maternal hypertension is associated with adverse pregnancy outcomes, including fetal growth restriction (FGR), due in part to reductions in nitric oxide (NO) bioavailability. We

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.

DOI: 10.1113/JP279655

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 were 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 was 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 25 mM KCl, implicating enhanced EDH signalling in BRJ− treated animals. At GD18.5, $eNOS^{-/-}$ fetuses were significantly smaller than WT animals ($P < 0.001$), but 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.

(Received 6 February 2020; accepted after revision 27 April 2020; first published online 5 May 2020)
Corresponding author Dr T. Tropea: Maternal & Fetal Health Research Centre, Division of Developmental Biology & Medicine, School of Medical Sciences, Faculty of Biology, Medicine and Health, University of Manchester, St Mary's Hospital, Manchester M13 9WL, UK. Email: teresa.tropea@manchester.ac.uk

Introduction

Hypertensive disorders affect up to 10% of pregnancies worldwide and confer significant risks 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 (Poston *et al.* 1995; Kublickiene *et al.* 1997) 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 & Govoni, 2004; Bryan *et al.* 2008; Lundberg & Weitzberg, 2010). 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, 2015; Lidder & Webb, 2013) and animals (Ferguson *et al.* 2013, 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. The authors confirm that the present study complies with the policies and regulation of *The Journal of Physiology* for animal experimentation and ethical principles.

Experimental animals

eNOS$^{-/-}$ mice (stock number 002684; $n = 134$) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). 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, UK) 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 randomization 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 ∼1 mmol kg^{-1} day^{-1} 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 were 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, Nümbrecht, Germany). Blood was centrifuged immediately (5 min 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, Tokyo, 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 analysed using the PowerChrom software (V 2.7.9, eDAQ, Denistone East, NSW, Australia).

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 analysed with the Windows Azur platform and the levels of nitrate were calculated and reported in mM (mmol l^{-1}) 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 physiological salt solution (PSS; in mM, 117 NaCl, 25 $NaHCO_3$, 4.69 KCl, 2.4 $MgSO_4$, 1.6 $CaCl_2$, 1.18 KH_2PO_4, 6.05 glucose, 0.034 EDTA; pH 7.4). Arterial segments (∼2 mm in length) were mounted on two 40 µm steel wires in a myograph chamber (Model 620M, Danish MyoTechnologies, Aarhus, Denmark) and immersed in 6 ml of 5% CO_2/20% 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 min 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}–2×10^{-6}M, Cayman Chemicals, Ann Arbor, MI, USA) was obtained and used to calculate the EC_{80} concentration of U46619. Endothelium-dependent relaxation to acetylcholine (ACh, 10^{-10}–10^{-5} M) was

assessed in arteries pre-constricted with an EC_{80} dose of U46619.

For studies using inhibitors of NOS-dependent and cyclooxygenase (COX)-dependent endothelial relaxation, separate vessels were pre-incubated with either L-NAME + L-NNA (each at 10^{-4} M, Cayman Chemicals) or indomethacin (10^{-5} M), respectively. To determine the contribution of endothelium-dependent hyperpolarization (EDH), ACh relaxation was assessed in the presence of modified PSS containing 25 mM 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 min prior to U46619 pre-constriction and the 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_{max}) to ACh was expressed as a percentage of the maximum effect of the dose-response. The 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 are expressed as a logarithm (Log EC_{50}).

Uterine artery doppler ultrasound

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, Toronto, Canada). Mice were anaesthetized with inhaled isoflurane (1 l min^{-1}; 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 was 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), and 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 were used to calculate the UtA Resistance Index [RI = (PSV − EDV)/PSV] and Pulsatility Index [PI = (PSV − EDV)/mean velocity].

Drugs and chemicals

Chemicals and pharmacological agents used in this study were purchased from Sigma Aldrich (St Louis, MO, USA), 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 two-way ANOVA (treatment, genotype and their 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, USA). 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 H_2O, 14.8 ± 2.8 g; WT BRJ+, 15.1 ± 2.9 g; WT BRJ−, 14.6 ± 2.6 g; $eNOS^{-/-}$ H_2O, 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 H_2O, 9.5 ± 1.8 ml day^{-1}; WT BRJ+, 11.2 ± 2.2 ml day^{-1}, $P = 0.0115$ *vs.* WT H_2O; WT BRJ−, 12.3 ± 2.4 ml day^{-1}, $P = 0.0001$ *vs.* WT H_2O; $eNOS^{-/-}$ H_2O, 9.05 ± 2.1 ml day^{-1}; $eNOS^{-/-}$ BRJ+, 10.9 ± 2.2 ml day^{-1}, $P = 0.0009$ *vs.* $eNOS^{-/-}$ H_2O; $eNOS^{-/-}$ BRJ−, 11.0 ± 1.9 ml day−, $P = 0.0008$ *vs.* $eNOS^{-/-}$ H_2O).

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^{-1} nitrate/nitrite (range: 78.8–112.8 mmol l^{-1}) and BRJ- having 0.1 mmol l^{-1} nitrate/nitrite (range: 0.04–0.16 mmol l^{-1}). Concentrations of nitrate/nitrite in the drinking water

ranged between 0.009 and 0.011 mmol l^{-1}. 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; Fig. 1). Maternal plasma nitrate concentrations were significantly increased by BRJ+ supplementation in both WT and eNOS$^{-/-}$ mice (effect of treatment, $P < 0.0001$; Fig. 1*A*). In contrast, plasma nitrite concentrations were significantly elevated in BRJ+-supplemented eNOS$^{-/-}$ dams only (effect of treatment, $P < 0.0001$, Fig. 1*B*), 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 SBP 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, $P = 0.0067$; Fig. 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 UtA endothelial-dependent relaxation

Responses to the endothelial-dependent vasodilator ACh were significantly attenuated in UtA of eNOS$^{-/-}$ mice compared with WT animals ($P < 0.0001$; Fig. 3). BRJ supplementation did not alter responses to ACh in UtA from WT mice (Fig. 3*A*), but both BRJ+ and BRJ− significantly enhanced endothelial-dependent relaxation in eNOS$^{-/-}$ mice (Fig. 3*B*), 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 COX enzymes (indomethacin, 10^{-5} M), NOS enzymes (L-NAME + L-NNA; 10^{-4} M) or EDH (using PSS containing 25 mM 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 25 mM KCl (Fig. 4*A*, *C* and *E*). Interestingly, ACh responses in eNOS$^{-/-}$ mice were unaffected by either indomethacin or L-NAME + L-NNA (Fig. 4*B* and *D*), whereas 25 mM KCl nearly abolished endothelial relaxation in eNOS$^{-/-}$ mice (Fig. 4*F*).

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 ($P < 0.001$; Fig. 5*A*) and NOS ($P < 0.05$; Fig. 5*C*) enzymes. Pre-incubation with indomethacin significantly increased the sensitivity to ACh in WT mice supplemented with BRJ when compared to same-genotype water controls (effect of treatment, $P = 0.0105$; Log EC_{50}: WT H_2O, −6.83 ± 0.8; WT BRJ+, −8.10 ± 0.6, $P = 0.0011$ *vs.* WT H_2O; WT BRJ−, −7.63 ± 0.7, $P = 0.0202$ *vs.* WT H_2O, respectively). In the presence of 25 mM KCl, ACh responses were attenuated in WT mice, but a significant effect of treatment remained ($P < 0.05$; Fig. 5*E*).

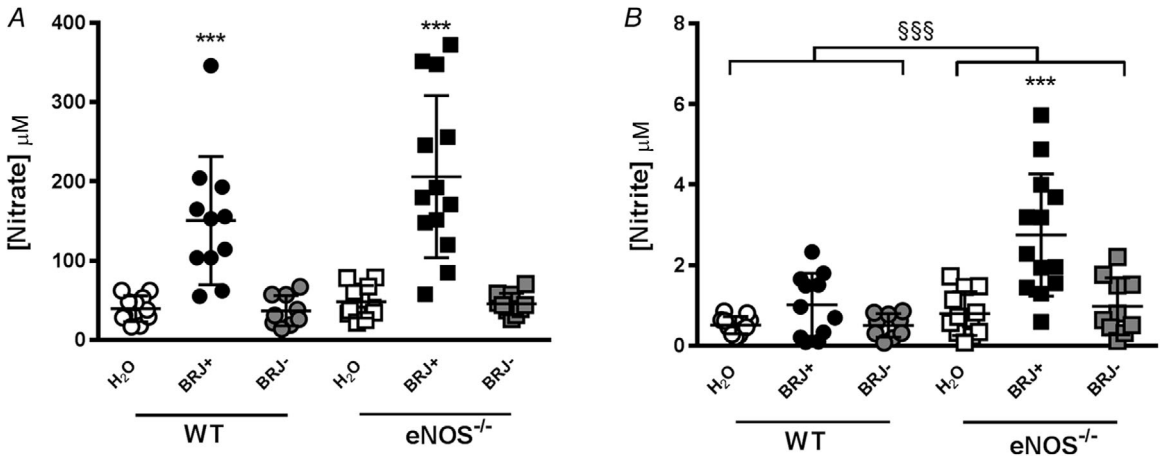

Figure 1. Maternal plasma nitrate and nitrite concentrations in BRJ− supplemented dams at GD18.5
A, plasma nitrate concentrations were significantly elevated in BRJ+-supplemented dams. *B*, nitrite concentrations were significantly elevated only in eNOS$^{-/-}$ BRJ+-supplemented dams. ***$P < 0.001$ BRJ+ *vs.* H_2O (same-genotype); §§§$P < 0.001$ eNOS$^{-/-}$ *vs.* WT. $n = 9$–13 dams per group.

In eNOS$^{-/-}$ mice, pre-incubation with indomethacin further enhanced ACh responses in UtA of dams supplemented with either BRJ+ or BRJ− (Fig. 5*B*); maximum relaxation to ACh was significantly increased in both BRJ+ or BRJ− animals (effect of genotype, $P < 0.0001$; effect of treatment, $P = 0.0030$. V_{max}: eNOS$^{-/-}$ H_2O, 59.42 ± 23.6 %; eNOS$^{-/-}$ BRJ+, 85.49 ± 10.6 %, $P = 0.0001$ *vs.* eNOS$^{-/-}$ H_2O; eNOS$^{-/-}$ BRJ−, 85.92 ± 13.7%, $P = 0.0002$ *vs.* eNOS$^{-/-}$ H_2O). Following pre-incubation with NOS inhibitors, there was no longer a significant effect of BRJ supplementation to enhance UtA relaxation (effect of treatment, $P = 0.066$; Fig. 5*D*). In the presence of 25 mM KCl, ACh responses were markedly attenuated in eNOS$^{-/-}$ UtA; furthermore, this condition abolished the effects of BRJ to enhance endothelial function (Fig. 5*F*).

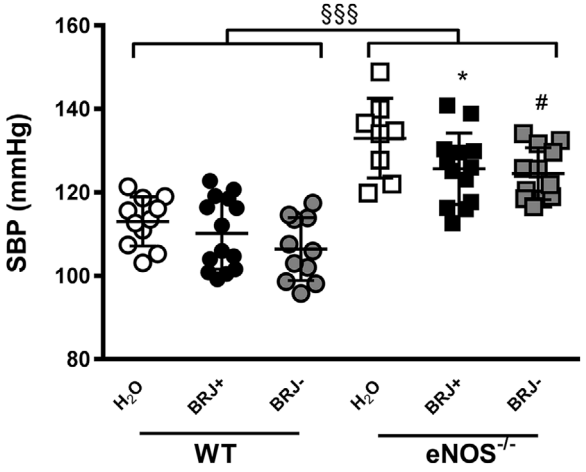

Figure 2. BRJ supplementation reduces SBP in eNOS$^{-/-}$mice
Maternal BRJ supplementation lowered SBP at GD17.5. This effect was significant in eNOS$^{-/-}$ mice. *$P < 0.05$ BRJ+ *vs.* H_2O; #$P < 0.05$ BRJ− *vs.* H_2O; §§§$P < 0.001$ eNOS$^{-/-}$ *vs.* WT. $n = 8$–14 dams per group.

In vivo assessment of UtA haemodynamic parameters

To determine whether the observed effects of BRJ supplementation on UtA vascular reactivity translated to changes in haemodynamic 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^{-1}) was significantly higher in eNOS$^{-/-}$ mice compared with WT animals, at GD18.5 (effect of genotype, $P < 0.001$; WT H_2O, 58.1 ± 6.8; WT BRJ+, 69.5 ± 9.0; WT BRJ, 62.85 ± 16.1; eNOS$^{-/-}$ H_2O, 81.7 ± 9.5; eNOS$^{-/-}$ BRJ+, 72.17 ± 10.9; eNOS$^{-/-}$ BRJ, 76.4 ± 12.9 breaths min^{-1}). PSV was significantly lower ($P = 0.0238$) whereas EDV was not ($P = 0.07$) in eNOS$^{-/-}$ mice compared with WT animals (Table 1). Maternal BRJ supplementation did not affect PSV or EDV in either genotype; RI and 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$^{-/-}$ and WT mice (Table 2). Fetal weights were significantly reduced in eNOS$^{-/-}$ 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

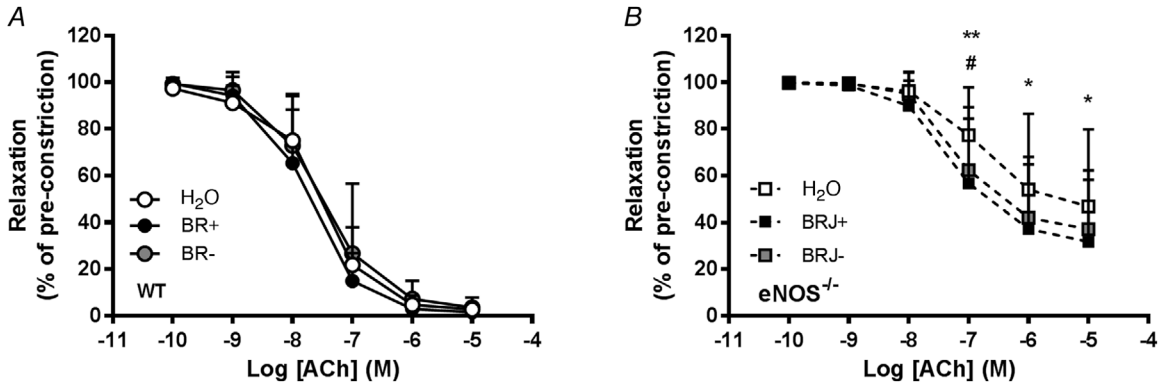

Figure 3. BRJ supplementation enhances UtA endothelial function
Maternal BRJ supplementation did not affect UtA endothelial responses to ACh in WT mice (*A*) but significantly enhanced relaxation in eNOS$^{-/-}$ mice (*B*). *$P < 0.05$, **$P < 0.01$ BRJ+ *vs.* H_2O; #$P < 0.05$ BRJ− *vs.* H_2O. $n = 14$–21 dams per group.

(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, 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, here we have 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 the findings of the present study, a recent systematic review and meta-analysis of trials using

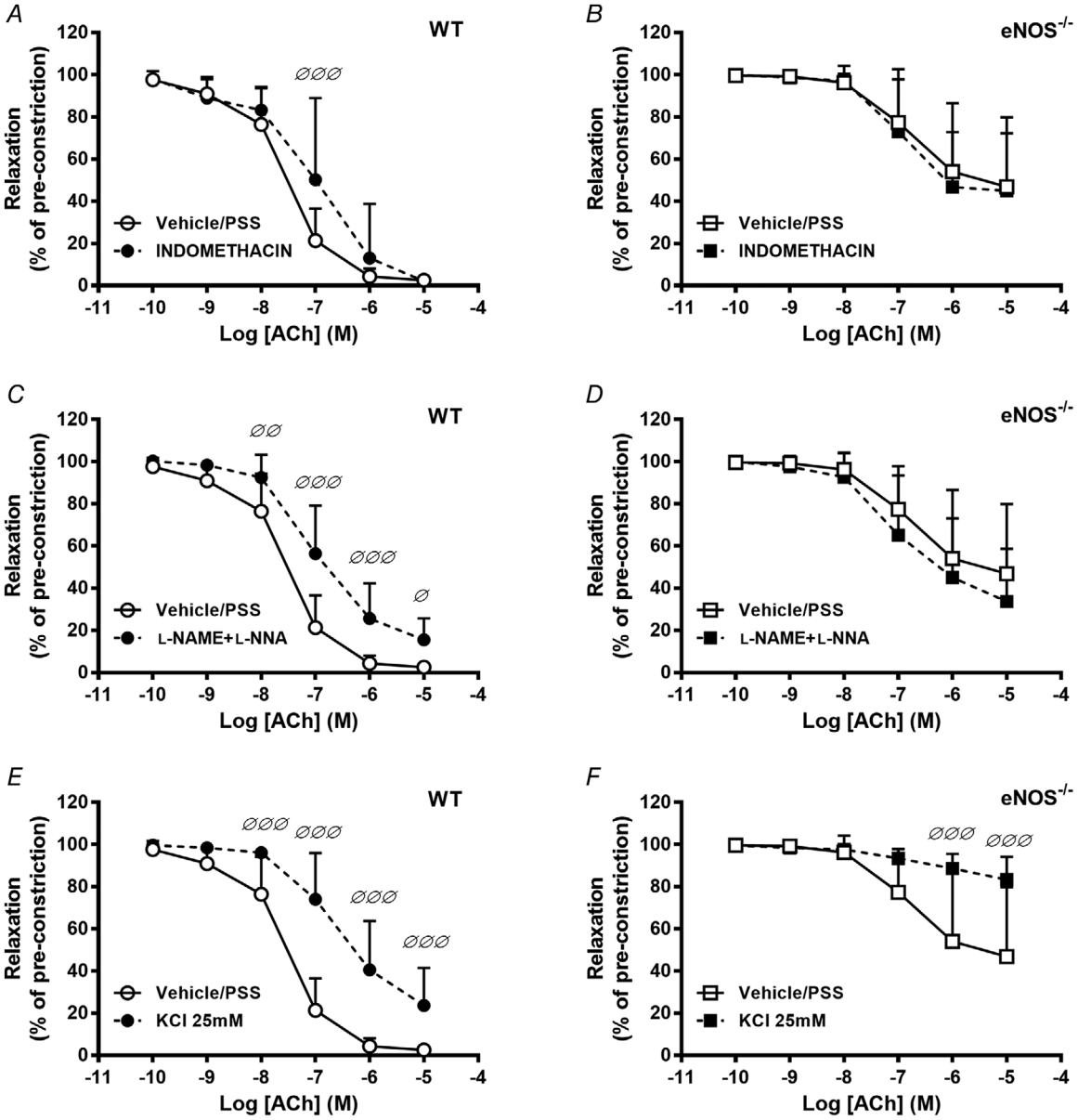

Figure 4. Endothelial responses to ACh in drinking water control animals are affected by inhibitors of COX, NOS and EDHF
Incubation with indomethacin (*A*, *B*), L-NAME + L-NNA (*C*, *D*) and 25 mM KCl (*E*, *F*) attenuated ACh responses, with differential effects in WT compared with eNOS$^{-/-}$ mice. Ø$P < 0.05$, ØØ$P < 0.01$, ØØØ$P < 0.001$. n = 6–15 dams per group.

BRJ as an intervention to lower blood pressure in humans suggested the potential importance of 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 the 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 is notable, and again suggests nitrate-independent effects of BRJ. Our current findings now confirm the importance of nitrate-independent effects of BRJ on cardiovascular

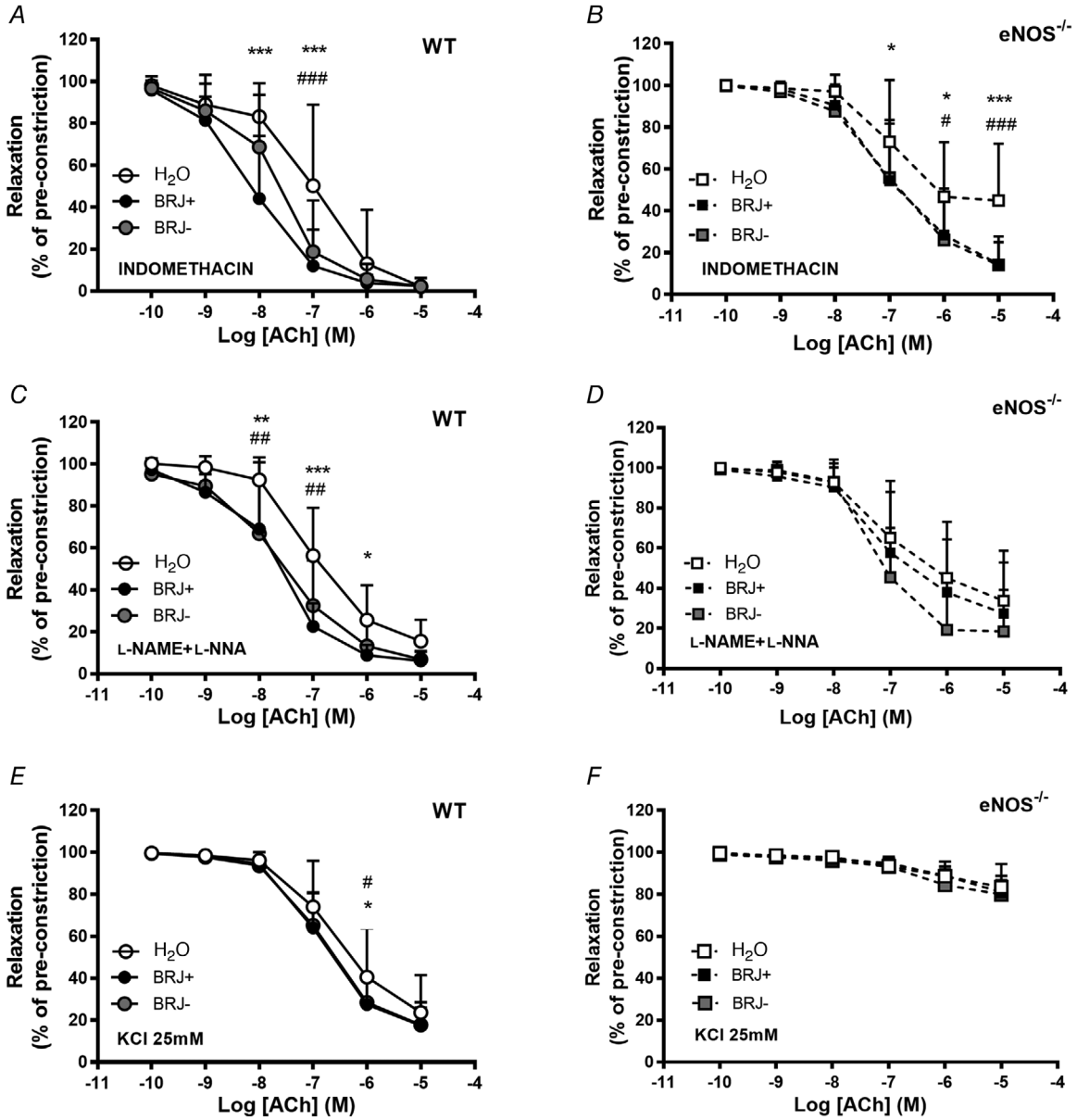

Figure 5. BRJ supplementation alters UtA endothelium-dependent relaxation components
In UtA of WT mice, inhibition of COX- (*A*) and NOS- (*C*) dependent pathways revealed effects of BRJ supplementation on non-prostacyclin/non-NO-induced relaxation; *E*, 25 mM KCl attenuated but did not completely prevent these effects of BRJ in WT mice. *B*, in eNOS$^{-/-}$ mice supplemented with BRJ, inhibition of COX enhanced endothelial responses to ACh compared with H_2O 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.* H_2O; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ BR- *vs.* H_2O. $n = 6$–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

	WT			eNOS$^{-/-}$					
	H_2O	BRJ+	BRJ−	H_2O	BRJ+	BRJ−	Treat	Gen	Int
PSV (mm s^{-1})	427.2 ± 98.8	401.5 ± 126.3	463.9 ± 76.2	366.1 ± 99.4	383.7 ± 143.5	334.5 ± 100.9	n.s.	§	n.s.
EDV (mm s^{-1})	228.8 ± 60.5	216.1 ± 75.5	254.5 ± 43.7	191.4 ± 57.6	223.1 ± 91.8	182.7 ± 66.4	n.s.	$P = 0.07$	n.s.
RI	0.468 ± 0.05	0.464 ± 0.03	0.451 ± 0.05	0.480 ± 0.06	0.424 ± 0.06	0.466 ± 0.07	n.s.	n.s.	n.s.
PI	0.658 ± 0.11	0.643 ± 0.08	0.625 ± 0.10	0.687± 0.15	0.567 ± 0.11	0.658 ± 0.15	n.s.	n.s.	n.s.

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$^{-/-}$; n.s., 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, and fetal and placental weights at GD18.5

	WT			eNOS$^{-/-}$					
	H_2O	BRJ+	BRJ−	H_2O	BRJ+	BRJ−	Treat	Gen	Int
Litter size (pups per dam)	7.0 ± 1.9	7.3 ± 1.8	6.8 ± 2.0	7.1 ± 1.4	7.4 ± 1.2	6.6 ± 1.9	n.s.	n.s.	n.s.
Fetal weight (g)	1.135 ± 0.059	1.139 ± 0.054	1.143 ± 0.069	0.992 ± 0.065	1.005 ± 0.044	0.999 ± 0.055	n.s.	§§§	n.s.
Placental weight (mg)	79.1 ± 6.4	76.9 ± 5.7	78.8 ± 6.4	79.7 ± 5.6	77.7 ± 3.9	80.5 ± 7.2	n.s.	n.s.	n.s.

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$^{-/-}$; n.s., not significant. $n = 27$–36 litters per group.

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 (PGI_2), 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 V_{max} 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 (PGH_2) and thromboxane A2 (TXA_2), 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 PGH_2 and TXA_2, 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-PGI_2 effects of BRJ implicated by the above experiments, we used 25 mM 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 25 mM KCl, ACh responses were modestly attenuated in UtA from WT mice, whereas in eNOS$^{-/-}$ mice, incubation with 25 mM 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 (Waldron *et al.* 1999; Scotland *et al.* 2001). 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 25 mM 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, are 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 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 probable 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 the 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 (Kulandavelu *et al.* 2012, 2013; Kusinski *et al.* 2012; Poudel *et al.* 2013; Renshall *et al.* 2018), 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 their 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 BRJ 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 vasodilatation, 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, probably through effects on an EDH-like component of endothelial-dependent signalling. 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.

References

ACOG (2013). Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' task force on hypertension in pregnancy. *Obstet Gynecol* **122**, 1122–1131.

Bahadoran Z, Mirmiran P, Kabir A, Azizi F & Ghasemi A (2017). The nitrate-independent blood pressure-lowering effect of beetroot juice: a systematic review and meta-analysis. *Adv Nutr* **8**, 830–838.

Brandes RP, Schmitz-Winnenthal FH, Félétou M, Gödecke A, Huang PL, Vanhoutte PM, Fleming I & Busse R (2000). An endothelium-derived hyperpolarizing factor distinct from NO and prostacyclin is a major endothelium-dependent vasodilator in resistance vessels of wild-type and endothelial NO synthase knockout mice. *Proc Natl Acad Sci U S A* **97**, 9747–9752.

Bryan NS, Calvert JW, Gundewar S & Lefer DJ (2008). Dietary nitrite restores NO homeostasis and is cardioprotective in endothelial nitric oxide synthase-deficient mice. *Free Radic Biol Med* **45**, 468–474.

Cason BA, Shubayev I & Hickey RF (1994). Blockade of adenosine triphosphate-sensitive potassium channels eliminates isoflurane-induced coronary artery vasodilation. *Anesthesiology* **81**, 1245-28A.

De Angelis A, Rinaldi B, Capuano A, Rossi F & Filippelli A (2004). Indomethacin potentiates acetylcholine-induced vasodilation by increasing free radical production. *Br J Pharmacol* **142**, 1233–1240.

Duarte J, Andriambeloson E, Diebolt M & Andriantsitohaina R (2004). Wine polyphenols stimulate superoxide anion production to promote calcium signaling and endothelial-dependent vasodilatation. *Physiol Res* **53**, 595–602.

Ferguson SK, Hirai DM, Copp SW, Holdsworth CT, Allen JD, Jones AM, Musch TI & Poole DC (2013). Impact of dietary nitrate supplementation via beetroot juice on exercising muscle vascular control in rats. *J Physiol* **591**, 547–557.

Ferguson SK, Hirai DM, Copp SW, Holdsworth CT, Allen JD, Jones AM, Musch TI & Poole DC (2014). Dose dependent effects of nitrate supplementation on cardiovascular control and microvascular oxygenation dynamics in healthy rats. *Nitric Oxide* **39**, 51–58.

Finn-Sell SL, Cottrell EC, Greenwood SL, Dilworth MR, Cowley EJ, Sibley CP & Wareing M (2018). Pomegranate juice supplementation alters utero-placental vascular function and fetal growth in the $eNOS^{-/-}$ mouse model of fetal growth restriction. *Front Physiol* **9**, 1145.

Gerber RT, Anwar MA & Poston L (1998). Enhanced acetylcholine induced relaxation in small mesenteric arteries from pregnant rats: an important role for endothelium-derived hyperpolarizing factor (EDHF). *Br J Pharmacol* **125**, 455–460.

Ghosh SM, Kapil V, Fuentes-Calvo I, Bubb KJ, Pearl V, Milsom AB, Khambata R, Maleki-Toyserkani S, Yousuf M, Benjamin N, Webb AJ, Caulfield MJ, Hobbs AJ & Ahluwalia A (2013). Enhanced vasodilator activity of nitrite in hypertension: critical role for erythrocytic xanthine oxidoreductase and translational potential. *Hypertension* **61**, 1091–1102.

Goncalves-Rizzi VH, Possomato-Vieira JS, Sales Graca TU, Nascimento RA & Dias-Junior CA (2016). Sodium nitrite attenuates hypertension-in-pregnancy and blunts increases in soluble fms-like tyrosine kinase-1 and in vascular endothelial growth factor. *Nitric Oxide* **57**, 71–78.

Groenenberg IA, Wladimiroff JW & Hop WC (1989). Fetal cardiac and peripheral arterial flow velocity waveforms in intrauterine growth-retardation. *Circulation* **80**, 1711–1717.

Guimaraes DD, Cruz JC, Carvalho-Galvao A, Zhuge Z, Marques SM, Naves LM, Persson AEG, Weitzberg E, Lundberg JO, Balarini CM, Pedrino GR, Braga VA & Carlstrom M (2019). Dietary nitrate reduces blood pressure in rats with angiotensin II-induced hypertension via mechanisms that involve reduction of sympathetic hyperactivity. *Hypertension* **73**, 839–848.

Hutcheon JA, Lisonkova S & Joseph KS (2011). Epidemiology of pre-eclampsia and the other hypertensive disorders of pregnancy. *Best Pract Res Clin Obstet Gynaecol* **25**, 391–403.

Jordan J, Shannon JR, Black BK, Ali Y, Farley M, Costa F, Diedrich A, Robertson RM, Biaggioni I & Robertson D (2000). The pressor response to water drinking in humans: a sympathetic reflex? *Circulation* **101**, 504–509.

Kapil V, Khambata RS, Robertson A, Caulfield MJ & Ahluwalia A (2015). Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2, double-blind, placebo-controlled study. *Hypertension* **65**, 320–327.

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 & Ahluwalia A (2010). Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertension* **56**, 274–281.

Krause BJ, Carrasco-Wong I, Caniuguir A, Carvajal J, Farias M & Casanello P (2013). Endothelial eNOS/arginase imbalance contributes to vascular dysfunction in IUGR umbilical and placental vessels. *Placenta* **34**, 20–28.

Krause BJ, Hanson MA & Casanello P (2011). Role of nitric oxide in placental vascular development and function. *Placenta* **32**, 797–805.

Kublickiene KR, Cockell AP, Nisell H & Poston L (1997). Role of nitric oxide in the regulation of vascular tone in pressurized and perfused resistance myometrial arteries from term pregnant women. *Am J Obstet Gynecol* **177**, 1263–1269.

Kujala TS, Vienola MS, Klika KD, Loponen JM & Pihlaja K (2002). Betalain and phenolic compositions of four beetroot (*Beta vulgaris*) cultivars. *Eur Food Res Technol* **214**, 505–510.

Kulandavelu S, Whiteley KJ, Bainbridge SA, Qu D & Adamson SL (2013). Endothelial NO synthase augments fetoplacental blood flow, placental vascularization, and fetal growth in mice. *Hypertension* **61**, 259–266.

Kulandavelu S, Whiteley KJ, Qu D, Mu J, Bainbridge SA & Adamson SL (2012). Endothelial nitric oxide synthase deficiency reduces uterine blood flow, spiral artery elongation, and placental oxygenation in pregnant mice. *Hypertension* **60**, 231–238.

Kusinski LC, Stanley JL, Dilworth MR, Hirt CJ, Andersson IJ, Renshall LJ, Baker BC, Baker PN, Sibley CP, Wareing M & Glazier JD (2012). eNOS knockout mouse as a model of fetal growth restriction with an impaired uterine artery function and placental transport phenotype. *Am J Physiol Regul Integr Comp Physiol* **303**, R86–93.

Lidder S & Webb AJ (2013). Vascular effects of dietary nitrate (as found in green leafy vegetables and beetroot) via the nitrate-nitrite-nitric oxide pathway. *Br J Clin Pharmacol* **75**, 677–696.

Lundberg JO & Govoni M (2004). Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic Biol Med* **37**, 395–400.

Lundberg JO & Weitzberg E (2010). NO-synthase independent NO generation in mammals. *Biochem Biophys Res Commun* **396**, 39–45.

Magee LA, CHIPS Study Group, von Dadelszen P, Singer J, Lee T, Rey E, Ross S, Asztalos E, Murphy KE, Menzies J, Sanchez J, Gafni A, Gruslin A, Helewa M, Hutton E, Koren G, Lee SK, Logan AG, Ganzevoort JW, Welch R, Thornton JG & Moutquin JM (2016). Do labetalol and methyldopa have different effects on pregnancy outcome? Analysis of data from the Control of Hypertension In Pregnancy Study (CHIPS) trial. *BJOG* **123**, 1143–1151.

Mills CE, Govoni V, Faconti L, Casagrande ML, Morant SV, Crickmore H, Iqbal F, Maskell P, Masani A, Nanino E, Webb AJ & Cruickshank JK (2020). A randomised, factorial trial to reduce arterial stiffness independently of blood pressure: Proof of concept? The VaSera trial testing dietary nitrate and spironolactone. *Br J Clin Pharmacol* **86**: 891–902.

Montenegro MF, Sundqvist ML, Nihlén C, Hezel M, Carlström M, Weitzberg E & Lundberg JO (2016). Profound differences between humans and rodents in the ability to concentrate salivary nitrate: Implications for translational research. *Redox Biol* **10**, 206–210.

Morton JS, Andersson IJ, Cheung PY, Baker P & Davidge ST (2015). The vascular effects of sodium tanshinone IIA sulphonate in rodent and human pregnancy. *PLoS One* **10**, e0121897.

Morton JS, Care AS & Davidge ST (2017). Mechanisms of uterine artery dysfunction in pregnancy complications. *J Cardiovasc Pharmacol* **69**, 343–359.

Mulvany M & Halpern W (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res* **41**, 19–26.

Ndiaye M, Chataigneau M, Lobysheva I, Chataigneau T & Schini-Kerth VB (2005). Red wine polyphenol-induced, endothelium-dependent NO-mediated relaxation is due to the redox-sensitive PI3-kinase/Akt-dependent phosphorylation of endothelial NO-synthase in the isolated porcine coronary artery. *FASEB J* **19**, 455–457.

Ninfali P & Angelino D (2013). Nutritional and functional potential of *Beta vulgaris cicla* and *rubra*. *Fitoterapia* **89**, 188–199.

Ormesher L, Myers JE, Chmiel C, Wareing M, Greenwood SL, Tropea T, Lundberg JO, Weitzberg E, Nihlen C, Sibley CP, Johnstone ED & Cottrell EC (2018). Effects of dietary nitrate supplementation, from beetroot juice, on blood pressure in hypertensive pregnant women: A randomised, double-blind, placebo-controlled feasibility trial. *Nitric Oxide* **80**, 37–44.

Osol G & Mandala M (2009). Maternal uterine vascular remodeling during pregnancy. *Physiology (Bethesda)* **24**, 58–71.

Peleli M, Zollbrecht C, Montenegro MF, Hezel M, Zhong J, Persson EG, Holmdahl R, Weitzberg E, Lundberg JO & Carlstrom M (2016). Enhanced XOR activity in eNOS-deficient mice: Effects on the nitrate-nitrite-NO pathway and ROS homeostasis. *Free Radic Biol Med* **99**, 472–484.

Poston L, McCarthy AL & Ritter JM (1995). Control of vascular resistance in the maternal and feto-placental arterial beds. *Pharmacol Ther* **65**, 215–239.

Poudel R, Stanley JL, Rueda-Clausen CF, Andersson IJ, Sibley CP, Davidge ST & Baker PN (2013). Effects of resveratrol in pregnancy using murine models with reduced blood supply to the uterus. *PLoS One* **8**, e64401.

Renshall LJ, Morgan HL, Moens H, Cansfield D, Finn-Sell SL, Tropea T, Cottrell EC, Greenwood S, Sibley CP, Wareing M & Dilworth MR (2018). Melatonin increases fetal weight in wild-type mice but not in mouse models of fetal growth restriction. *Front Physiol* **9**, 1141.

Schiessl B, Strasburger C, Bidlingmaier M, Mylonas I, Jeschke U, Kainer F & Friese K (2006). Plasma- and urine concentrations of nitrite/nitrate and cyclic Guanosinemonophosphate in intrauterine growth restricted and preeclamptic pregnancies. *Arch Gynecol Obstet* **274**, 150–154.

Scotland RS, Chauhan S, Vallance PJ & Ahluwalia A (2001). An endothelium-derived hyperpolarizing factor-like factor moderates myogenic constriction of mesenteric resistance arteries in the absence of endothelial nitric oxide synthase-derived nitric oxide. *Hypertension* **38**, 833–839.

Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, Sherman PA, Sessa WC & Smithies O (1996). Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* **93**, 13176–13181.

Sibai BM (2001). Antihypertensive drugs during pregnancy. *Semin Perinatol* **25**, 159–164.

Stanley JL, Andersson IJ, Hirt CJ, Moore L, Dilworth MR, Chade AR, Sibley CP, Davidge ST & Baker PN (2012). Effect of the anti-oxidant tempol on fetal growth in a mouse model of fetal growth restriction. *Biol Reprod* **87**, 1–8.

Taddei S, Virdis A, Ghiadoni L, Magagna A & Salvetti A (1997). Cyclooxygenase inhibition restores nitric oxide activity in essential hypertension. *Hypertension* **29**, 274–279.

von Dadelszen P & Magee LA (2002). Fall in mean arterial pressure and fetal growth restriction in pregnancy hypertension: An updated metaregression analysis. *J Obstet Gynaecol Can.* **24**, 941–945.

Volino-Souza M, de Oliveira GV & Alvares TS (2018). A single dose of beetroot juice improves endothelial function but not tissue oxygenation in pregnant women: a randomised clinical trial. *Br J Nutr* **120**, 1006–1013.

Waldron GJ, Ding H, Lovren F, Kubes P & Triggle CR (1999). Acetylcholine-induced relaxation of peripheral arteries isolated from mice lacking endothelial nitric oxide synthase. *Br J Pharmacol* **128**, 653–658.

Walker MA, Bailey TG, McIlvenna L, Allen JD, Green DJ & Askew CD (2019). Acute dietary nitrate supplementation improves flow mediated dilatation of the superficial femoral artery in healthy older males. *Nutrients* **11**, 954.

Whitesall SE, Hoff JB, Vollmer AP & D'Alecy LG (2004). Comparison of simultaneous measurement of mouse systolic arterial blood pressure by radiotelemetry and tail-cuff methods. *Am J Physiol Heart Circ Physiol* **286**, H2408–2415.

Zhou X, Abboud W, Manabat NC, Salem MR & Crystal GJ (1998). Isoflurane-induced dilation of porcine coronary arterioles is mediated by ATP-sensitive potassium channels. *Anesthesiology* **89**, 182–189.

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 read and approved the final version of the 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.

Funding

This work was supported by a Fellowship from the British Heart Foundation to E.C.C. (FS/15/31/31 418). A.L.D. is supported by the National Institute for Health Research University College London Hospitals Biomedical Research Centre. D.J.S. is supported by a Fellowship from the British Heart Foundation (FS/15/33/31 608).

Acknowledgements

We thank Dr Nicholas Ashton for his technical help with systolic blood pressure measurements, and the technical staff at the University of Manchester's Biological Services Facility, particularly Renata Orzechowska, for excellent care of the experimental animals used in these studies.

Keywords

beetroot juice, blood pressure, nitrate, nitric oxide, pregnancy

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Statistical Summary Document

Translational Perspective

Maternal hypertensive disorders are common, affecting around 10% of pregnancies, and are associated with an 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.