

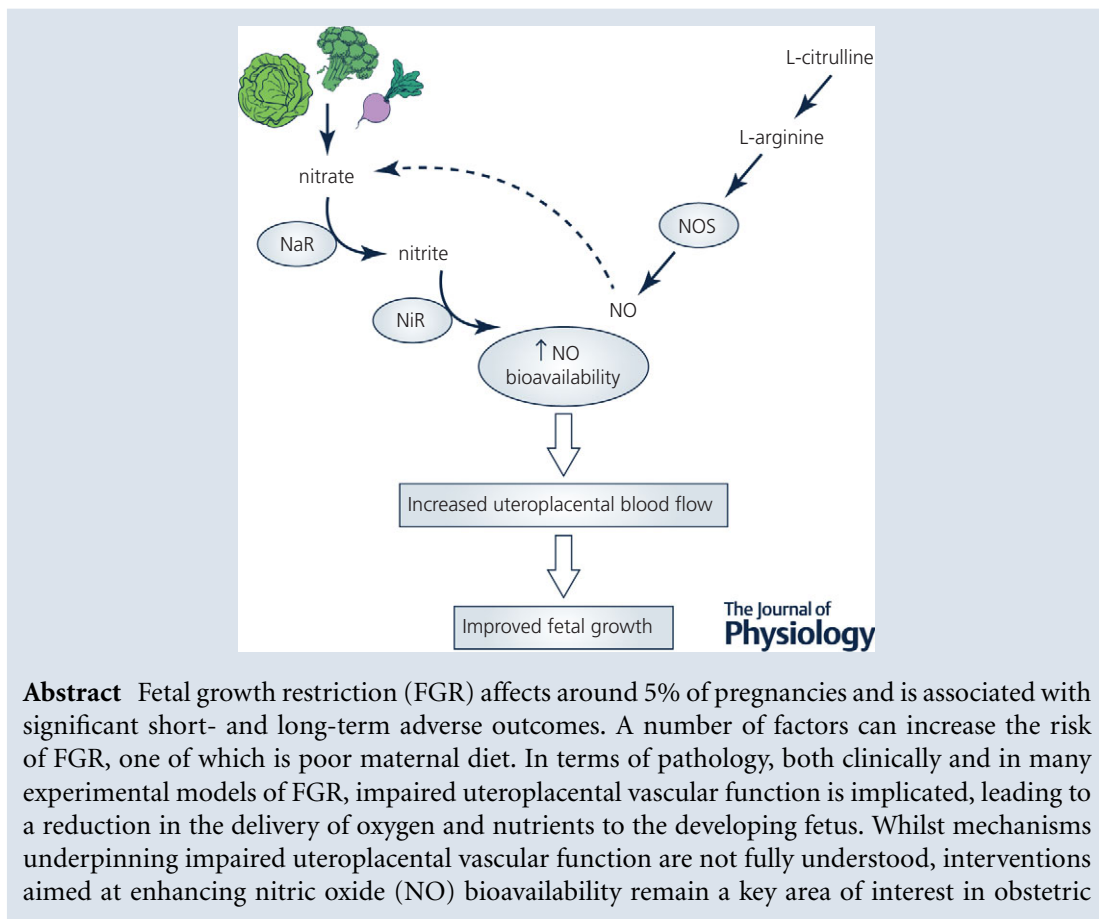


SYMPOSIUM REVIEW

Dietary interventions for fetal growth restriction – therapeutic potential of dietary nitrate supplementation in pregnancy

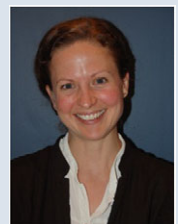
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Abstract Fetal growth restriction (FGR) affects around 5% of pregnancies and is associated with significant short- and long-term adverse outcomes. A number of factors can increase the risk of FGR, one of which is poor maternal diet. In terms of pathology, both clinically and in many experimental models of FGR, impaired uteroplacental vascular function is implicated, leading to a reduction in the delivery of oxygen and nutrients to the developing fetus. Whilst mechanisms underpinning impaired uteroplacental vascular function are not fully understood, interventions aimed at enhancing nitric oxide (NO) bioavailability remain a key area of interest in obstetric

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research. In addition to endogenous NO production from the amino acid L-arginine, via nitric oxide synthase (NOS) enzymes, research in recent years has established that significant NO can be derived from dietary nitrate, via the 'alternative NO pathway'. Dietary nitrate, abundant in green leafy vegetables and beetroot, can increase NO bioactivity, conferring beneficial effects on cardiovascular function and blood flow. Given the beneficial effects of dietary nitrate supplementation to date in non-pregnant humans and animals, current investigations aim to assess the therapeutic potential of this approach in pregnancy to enhance NO bioactivity, improve uteroplacental vascular function and increase fetal growth.

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Abstract figure legend Targeting the nitrate–nitrite–NO pathway in pregnancy. Endogenous synthesis of NO involves the oxidation of L-arginine by nitric oxide synthase (NOS) enzymes; L-citrulline can be converted to L-arginine *in vivo*, providing an alternative means of stimulating this pathway. In the 'alternative NO pathway', inorganic nitrate (derived either from oxidation of endogenous NO or from dietary sources) is reduced in the body to nitrite via bacterial nitrate reductase (NaR) enzymes. Nitrite can then be reduced to NO within the circulation and tissues via a host of nitrite reductase (NiR) enzymes, generating NO. In pregnancy, enhancing NO synthesis by either pathway aims to improve uteroplacental blood flow, and hence fetal growth.

Abbreviations cGMP, 3',5'-cyclic GMP; FGR, fetal growth restriction; NO, nitric oxide; NOS, nitric oxide synthase; PDE-5, phosphodiesterase type 5; ROS, reactive oxygen species; SC, sildenafil citrate; sGC, soluble guanylate cyclase.

Introduction

Fetal growth restriction (FGR), the failure of a fetus to achieve its genetic growth potential, affects around 5% of pregnancies (Bamfo & Odibo, 2011), and carries significant risks in terms of both short-term survival and long-term offspring health. The incidence of stillbirth is increased ~4-fold in FGR pregnancies (Gardosi *et al.* 2013), along with increased rates of neonatal mortality and morbidity (McIntire *et al.* 1999). In addition, there are significant lifetime risks associated with being born small; these infants are more likely to develop metabolic disease and behavioural disorders in adult life compared to infants within the normal birth weight range (Barker, 2006). Despite these risks, there are currently no treatments for FGR, and premature delivery remains the only option. The pharmaceutical industry has not invested in developing treatments for pregnancy complications, at least in part due to potential risks of fetal toxicity (Fisk & Atun, 2008). As such, there is a clear need to identify therapeutic approaches that may enhance fetal growth in a safe and effective manner.

Importance of maternal diet for fetal growth

A host of factors can impact on fetal growth, including maternal and fetal genetic factors, as well as the maternal environment. Maternal stressors, including the presence of chronic disease (e.g. chronic hypertension; Ananth *et al.* 1995), infection (Romero *et al.* 1989) or psychological stress (Rondo *et al.* 2003), are associated with impaired fetal growth. Maternal diet is also key and, importantly,

modifiable. The role of maternal micronutrient status, including sufficiency of folate, iron and zinc, is well established as being critical for optimal fetal development, and widespread supplementation of these micronutrients exists in developed countries. Supplementation strategies such as this aim primarily to prevent adverse outcomes, and can do so effectively, as has been the case for prevention of neural tube defects by periconceptional folate supplementation (MRC Vitamin Study Research Group, 1991). With regards to other micronutrients, emerging evidence continues to inform how best to treat pregnant women, in terms of timing, dose and duration of supplementation, to improve pregnancy outcomes and reduce disease risks. For example, maternal vitamin D status has emerged as another potential key factor in determining pregnancy outcomes. Maternal vitamin D deficiency is associated with increased risk of FGR (Chen *et al.* 2015) and other adverse pregnancy outcomes (Wei *et al.* 2012), and supplementation in pregnancy is now also widely recommended.

As well as micronutrient deficiencies, maternal diets high in refined or processed foods are associated with poor pregnancy outcomes (Brantsaeter *et al.* 2009). The mechanistic basis for these associations is currently poorly understood. However, animal models using maternal dietary manipulations to induce FGR and other pregnancy complications, including high fat–high sugar feeding (Sferruzzi-Perri *et al.* 2013), caloric restriction (Lederman & Rosso, 1980) and macro- or micronutrient deficiencies (Cottrell *et al.* 2012; Liu *et al.* 2013), have contributed significantly to our understanding of pathways by which a

poor maternal diet can affect maternal health, placental development and fetal growth. Conversely, diets high in vegetables and fruit (Brantsaeter *et al.* 2009) and probiotic foods (Brantsaeter *et al.* 2011) have been associated with reduced risk of FGR and other pregnancy complications, likely to be due at least in part to provision of micronutrients but also potentially via other bioactive compounds, as discussed in more detail below.

Impaired uteroplacental vascular function in FGR

Although the mechanisms underlying FGR are incompletely understood, impairments in uteroplacental vascular function and blood flow are consistently shown to be involved (Kingdom *et al.* 1997; Shibata *et al.* 2008; Ghosh & Gudmundsson, 2009). Several lines of evidence suggest that these impairments may arise due to reduced trophoblast invasion and poor placentation in early pregnancy, leading to increased vascular resistance and decreased blood flow across the placenta (Chaddha *et al.* 2004). As a result, placental damage occurs, caused by ischaemia–reperfusion and/or increased production of reactive oxygen species (ROS), and placental capacity for nutrient and gas exchange is reduced.

Nitric oxide (NO) is critically involved in maintaining a low-resistance/high flow uteroplacental vasculature in healthy pregnancies (Poston *et al.* 1995; Kublickiene *et al.* 1997), and reductions in NO bioavailability have been associated with FGR in humans (Casanello & Sobrevia, 2002; Schiessl *et al.* 2006; Krause *et al.* 2013). Endogenously, NO is derived from oxidation of the amino acid L-arginine by nitric oxide synthase (NOS) enzymes, of which there are three isoforms (neuronal, inducible and endothelial; nNOS, iNOS and eNOS, respectively). During pregnancy, there is marked upregulation of maternal NO production via both nNOS and eNOS in vascular tissues (Xu *et al.* 1996). In fetoplacental tissues, eNOS-derived NO plays a critical role throughout pregnancy, being involved in implantation, placental angiogenesis and the regulation of fetoplacental vascular tone (Krause *et al.* 2011). In animal models, experimental inhibition of endogenous NO synthesis (by either pharmacological or genetic manipulations) has been shown to lead to reductions in uteroplacental blood flow and cause FGR (Molnar *et al.* 1994; Salas *et al.* 1995; Kulandavelu *et al.* 2006, 2012, 2013). Given this central role of NO in pregnancy, there has been significant effort aimed toward enhancing NO production and/or signalling in obstetric research over the past 20 years.

Approaches to target the NO pathway

Efforts to enhance NO signalling in pregnancy have largely involved approaches that aim to either stimulate endogenous production of NO or prolong NO actions.

In terms of dietary approaches, supplementation with functional amino acids (i.e. those which participate in and/or regulate metabolic pathways; Wu, 2013) has shown some promise. Maternal L-arginine supplementation, increasing availability of substrate for NOS enzymes, has been effective in several animal models of FGR at increasing fetal growth (Vosatka *et al.* 1998; Lassala *et al.* 2010). However, beneficial effects have not been universally shown (Satterfield *et al.* 2013). Similarly, some studies in pregnant women have reported beneficial effects of L-arginine supplementation on maternal NO levels and fetal growth (Germain *et al.* 2004; Sieroszewski *et al.* 2004; Xiao & Li, 2005; Singh *et al.* 2015). However, a recent randomised controlled trial demonstrated no significant improvement in birth weight or fetal outcomes following maternal L-arginine supplementation in a group of women presenting with severe vascular FGR (Winer *et al.* 2009). The limited efficacy of this approach may be explained in part by the fact that L-arginine undergoes rapid metabolism by arginase in the liver and gastrointestinal tract, resulting in its limited availability for use by NOS enzymes (Cynober, 2007). Given this limitation, L-citrulline administration has been posited as an alternative approach to L-arginine supplementation for increasing endogenous NO synthesis (Romero *et al.* 2006). *In vivo*, L-citrulline is converted to L-arginine in the kidney. In pregnant sheep, maternal administration of L-citrulline results in a sustained increase in L-arginine in both maternal and fetal compartments, greater than that achieved by L-arginine (Lassala *et al.* 2009). Maternal L-citrulline administration has also recently been shown to increase fetal weight and placental efficiency in a rodent model of FGR (Bourdon *et al.* 2016) and to enhance expression of angiogenic and growth factor genes within the placenta (Tran *et al.* 2017). In terms of efficacy, an important consideration regarding approaches that aim to enhance endogenous NO production is the oxygen dependence of NOS enzymes. Thus, amino acid supplementation with L-arginine or L-citrulline may be ineffective under conditions of hypoxia, where enzyme function is reduced.

The canonical NO signalling pathway in the cardiovascular system involves the activation of soluble guanylate cyclase (sGC) and increased production of 3',5'-cyclic GMP (cGMP), leading to smooth muscle relaxation and vasodilatation. Phosphodiesterase type 5 (PDE-5) catalyses the hydrolysis of cGMP, curtailing the effects of NO signalling (Fig. 1). A number of additional signalling pathways have been identified for NO (and other bioactive nitrogen oxides); these actions are beyond the scope of this review, but have been covered extensively recently (Omar *et al.* 2016). Though not a dietary approach, inhibition of PDE-5 using sildenafil citrate (SC) to prolong the actions of NO has shown significant promise in experimental models of FGR, in terms of improving

vascular function (Wareing *et al.* 2005) and fetal growth (Satterfield *et al.* 2010; Stanley *et al.* 2012; Dilworth *et al.* 2013). Furthermore, several small clinical trials have verified the safety of maternal SC administration in pregnancy (Lacassie *et al.* 2004; Molelekwa *et al.* 2005), and in one study, SC treatment was reported to increase fetal abdominal circumference growth in severe early-onset FGR (von Dadelszen, 2011). Given these promising data, the first large-scale randomised controlled trial is currently underway to assess the efficacy of maternal SC therapy to improve pregnancy outcomes in severe early-onset FGR (the STRIDER trial; Sildenafil Therapy In Dismal prognosis Early-onset intra-uterine growth Restriction; (Ganzevoort *et al.* 2014). Trial outcomes are expected to be reported from 2017 onwards.

Alternative NO production via the nitrate–nitrite–NO pathway

In addition to the production of NO via NOS enzymes, as described above, there is now a wealth of evidence demonstrating that NO can be generated via an alternative, NOS-independent pathway (see Abstract Figure). Inorganic nitrate (NO_3^-) and nitrite (NO_2^-) anions, derived either from the oxidation of endogenously produced NO or from dietary sources, can be recycled *in vivo* to generate NO and other important bioactive nitrogen oxides. The importance of these anions has received growing attention in recent years, particularly as

supplementation studies using dietary nitrate have shown significant promise in cardiovascular medicine (Lundberg *et al.* 2008).

Bioactivation of dietary nitrate (found in abundance in green leafy vegetables and beetroot; Lidder & Webb, 2013) involves firstly its reduction to nitrite, predominantly via bacterial nitrate reductase enzymes in the oral cavity. Nitrite then enters the systemic circulation, where it is further reduced to NO by tissue nitrite reductases. A number of enzymes possessing nitrite reductase activities have been identified to date, including haemoglobin, myoglobin and xanthine oxidoreductase (Lundberg *et al.* 2008). Importantly, the reduction of nitrite to NO is enhanced under hypoxic conditions, where NO production from oxygen-dependent NOS activity would be limited. Thus, this ‘alternative NO pathway’ may be particularly important in diseased or damaged organs where perfusion is compromised and tissue oxygenation reduced.

In non-pregnant adults, supplementation with dietary nitrate in the form of beetroot juice significantly lowers blood pressure and improves vascular function (Webb *et al.* 2008; Kapil *et al.* 2010). Beneficial effects of dietary nitrate supplementation have also been shown in terms of enhancing exercise performance and blood flow in both humans and animal models (Ferguson *et al.* 2013; Jones, 2014), as well as reducing myocardial damage caused by acute ischaemia–reperfusion injury (Webb *et al.* 2004; Salloum *et al.* 2015).

A dietary approach to target the nitrate–nitrite–NO pathway in pregnancy

Given the beneficial effects of dietary nitrate supplementation in non-pregnant humans and animals, we hypothesised that this approach could be used to increase NO bioavailability and improve vascular function in complicated pregnancies. Moreover, it is likely that a dietary intervention, such as beetroot juice supplementation, may be more appealing to pregnant women and potentially have fewer off-target effects compared with pharmacological approaches, and therefore may encourage compliance.

Our preclinical findings thus far have shown that maternal dietary nitrate supplementation (using beetroot juice) increases both maternal and fetal nitrate and nitrite concentrations and improves maternal uterine artery vascular function *ex vivo* in a mouse model of FGR associated with marked vascular dysfunction, the eNOS knockout mouse (Cottrell *et al.* 2015). Our ongoing studies using this approach aim to understand how the beneficial effects of nitrate supplementation are conferred in this model, and to establish the mechanisms involved in nitrate/nitrite bioactivation in uteroplacental tissues of pregnant animals.

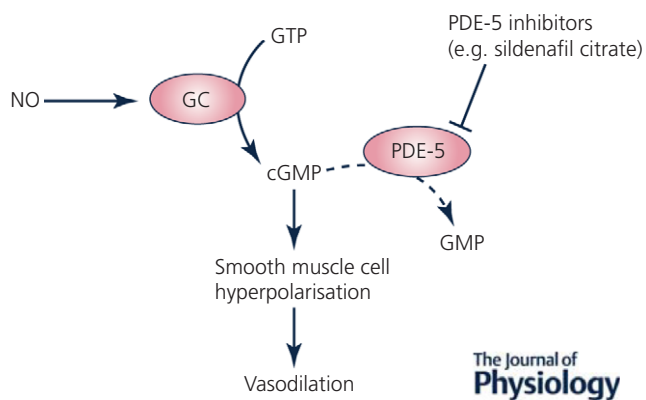


Figure 1. Canonical NO–cGMP signalling pathway in vascular tissues

Nitric oxide (produced from endogenous NOS pathways, or via reduction of dietary nitrate) binds a prosthetic haem group on sGC to activate the enzyme, leading to synthesis of the second messenger cGMP. Increased intracellular cGMP leads ultimately to smooth muscle cell hyperpolarisation and vasodilatation. Activity through this pathway is attenuated via the actions of PDE-5, which degrades cGMP. Agents such as sildenafil citrate, which inhibits the PDE-5 enzyme, can enhance NO–cGMP signalling by prolonging the elevation of intracellular cGMP.

Recently, a similar approach to target the nitrate–nitrite–NO pathway in pregnancy has been used in an alternative animal model, in which endogenous NO synthesis was inhibited using the NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME). In this preeclampsia-like model, maternal treatment with sodium nitrite (bypassing the need for nitrate to nitrite conversion) attenuated the maternal hypertension induced by L-NAME, and significantly improved fetal outcomes (Goncalves-Rizzi *et al.* 2016). Thus, although currently under-explored, there appears to be significant promise in targeting the alternative NO pathway in pregnancy to improve both maternal and fetal outcomes in compromised pregnancies. However, it should be noted that in both of the preclinical models described here, endogenous NO production was the primary deficiency; it remains to be determined whether targeting the alternative NO pathway will be effective in other models of FGR, or in more complex pregnancy pathologies. Nonetheless, it is of interest that maternal green leafy vegetable intake has been shown by several groups to be significantly associated with improved pregnancy outcomes and birth weight (Rao, 2001; Ramón, 2009; McCowan, 2010). Green leafy vegetables provide a rich source of folate and iron; however it is also plausible that some of the beneficial effects attributed to these foods could be due to increased provision of dietary nitrate.

Given our promising preliminary evidence in animal models that maternal dietary nitrate supplementation in pregnancy is well tolerated, and associated with improvements in uteroplacental vascular function (as discussed above), we have recently begun a feasibility trial utilising this dietary approach in pregnant women. Drawing on evidence from cardiovascular medicine, we are currently targeting women with hypertension (who are at increased risk of delivering FGR babies) to take part in a short-term (1 week) study involving dietary nitrate supplementation in the form of beetroot juice. This is a double blind, placebo-controlled trial, and aims to investigate the effects of this intervention on maternal cardiovascular function and uteroplacental vascular resistance, as well as determining whether this dietary supplementation approach is acceptable to pregnant women. This trial (ClinicalTrials.gov, NCT02520687) is currently in progress, and we expect results will be reported in 2017. If effective, this approach could be expanded in future studies to intervene in a number of pregnancy complications associated with impaired vascular function and limited NO bioavailability, including FGR and preeclampsia.

Conclusions and future research challenges

Finding effective and acceptable treatments for pregnancy complications remains a significant challenge in obstetrics.

There is considerable scope for the development of dietary interventions in pregnancy to reduce disease risk and/or severity. Future research in this field requires a better understanding of mechanisms by which specific dietary components impact on maternal physiology and fetoplacental development. Progress will require epidemiological datasets as well as preclinical experimental models, in order to understand the biology underpinning population-based association studies, and to translate these findings into novel therapeutic strategies.

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Additional information

Competing interests

The authors declare that no competing interests exist.

Author contributions

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Translational importance

Many pregnancy complications, including fetal growth restriction, are associated with impairments in maternal and uteroplacental vascular function. Interventions aimed at targeting the nitric oxide (NO) pathway remain a key area of interest in obstetric research, though at present no therapeutics are available for treating pregnancy complications. A wealth of evidence generated in non-pregnant individuals has identified the importance of the alternative NO pathway, whereby dietary nitrate (derived from foods such as green leafy vegetables and beetroot) is transformed in the body to NO and other bioactive nitrogen oxides, conferring beneficial effects on vascular function. Supplementation with dietary nitrate is a potential strategy for enhancing NO in complicated pregnancies, with the additional benefits of being easy and inexpensive to administer, and potentially more appealing to pregnant women compared with pharmacological approaches.