In vivo mitochondria-targeted protection against uterine artery vascular dysfunction and remodelling in rodent hypoxic pregnancy

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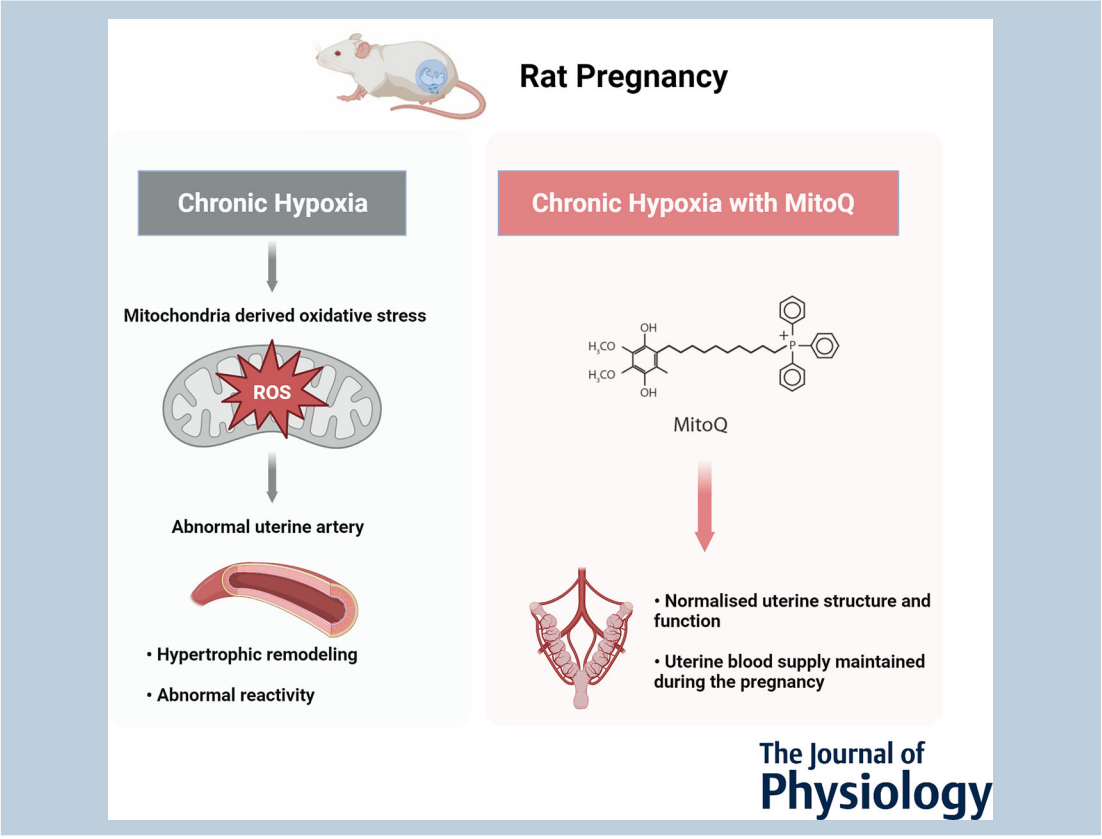

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Abstract Gestational hypoxia adversely affects uterine artery function, increasing complications. However, an effective therapy remains unidentified. Here, we show in rodent uterine arteries that hypoxic pregnancy promotes hypertrophic remodelling, increases constrictor reactivity via protein kinase C signalling, and triggers compensatory dilatation via nitric oxide-dependent mechanisms and stimulation of large conductance Ca^{2+}-activated K^+-channels. Maternal *in vivo* oral treatment with the mitochondria-targeted antioxidant MitoQ in hypoxic pregnancy normalises uterine artery reactivity and prevents vascular remodelling. From days 6–20 of gestation (term ∼22 days), female Wistar rats were randomly assigned to normoxic or hypoxic (13–14% O_2) pregnancy ± daily maternal MitoQ treatment (500 μM in drinking water). At 20 days of gestation, maternal, placental and fetal tissue was frozen to determine MitoQ uptake. The uterine arteries were harvested and, in one segment, constrictor and dilator reactivity was determined by wire myography. Another segment was fixed for unbiased stereological analysis of vessel morphology. Maternal administration of MitoQ in both normoxic and hypoxic pregnancy crossed the placenta and was present in all tissues analysed. Hypoxia increased uterine artery constrictor responses to norepinephrine, angiotensin II and the protein kinase C activator, phorbol 12,13-dibutyrate. Hypoxia enhanced dilator reactivity to sodium nitroprusside, the large conductance Ca^{2+}-activated K^+-channel activator NS1619 and ACh via increased nitric oxide-dependent mechanisms. Uterine arteries from hypoxic pregnancy showed increased wall thickness and MitoQ treatment in hypoxic pregnancy prevented all effects on uterine artery reactivity and remodelling. The data support mitochondria-targeted therapy against adverse changes in uterine artery structure and function in high-risk pregnancy.

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Abstract figure legend Moderate hypoxia (13–14% O_2) during rat pregnancy programmes uterine artery remodelling and dysfunction via mitochondria derived oxidative stress. The mitochondria-targeted antioxidant MitoQ treatment prevents the adverse changes in uterine artery structure and function in high-risk pregnancy.

Key points

- Dysfunction and remodelling of the uterine artery are strongly implicated in many pregnancy complications, including advanced maternal age, maternal hypertension of pregnancy, maternal obesity, gestational diabetes and pregnancy at high altitude.
- Such complications not only have immediate adverse effects on the growth of the fetus, but also they can also increase the risk of cardiovascular disease in the mother and offspring. Despite this, there is a significant unmet clinical need for therapeutics that treat uterine artery vascular dysfunction in adverse pregnancy.
- Here, we show in a rodent model of gestational hypoxia that *in vivo* oral treatment of the mitochondria-targeted antioxidant MitoQ protects against uterine artery vascular dysfunction and remodelling, supporting the use of mitochondria-targeted therapy against adverse changes in uterine artery structure and function in high-risk pregnancy.

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Introduction

Pregnancy imposes a high demand on the maternal vascular system. With advancing gestation, there is a significant increase in uteroplacental blood flow to sustain fetal growth (Duvekot & Peeters, 1994; Longo, 1983; Rosenfeld, 1977). Both the uterus and placenta rely on the uterine artery as the primary source of blood supply and the uterine artery undergoes functional and structural changes during pregnancy to increase uteroplacental perfusion (Mandala & Osol, 2012; Morton et al., 2017; Sanghavi & Rutherford, 2014; Thornburg et al., 2000). These changes include decreased uterine constrictor and myogenic responses, increased vasodilator reactivity and outward hypertrophic growth (Kublickiene et al., 1997; Osol & Cipolla, 1993; Veerareddy et al., 2002; Weiner et al., 1991; White et al., 1998; Xiao et al., 2006). Uterine artery dysfunction and remodelling are strongly implicated in clinical indications of complicated pregnancy including advanced maternal age (Care et al., 2015; Pirhonen et al., 2005), maternal hypertension of pregnancy (Brennan et al., 2014; Myatt & Webster, 2009), maternal obesity, gestational diabetes (Goulopoulou et al., 2014; Stanley et al., 2009) and pregnancy at high altitude (Zamudio, Palmer, Droma et al., 1995). Such complications during pregnancy not only have immediate adverse effects on the mother and fetus, but also they can increase the risk of cardiovascular disease in the adult offspring (Giussani, 2021; Gluckman et al., 2008). Despite this, there is a significant unmet clinical need for therapeutics that treat uterine vascular dysfunction in adverse pregnancy.

Over 140 million people live at altitudes higher than 2500 m, comprising the largest single human group at risk of maternal and fetal complications during pregnancy because of the low atmospheric oxygen availability. Data derived from human clinical studies describe altered uterine blood flow and a greater prevalence of pre-eclampsia in women undergoing pregnancy at high altitude (Keyes et al., 2003; Moore et al., 1982; Palmer et al., 1999; Zamudio, Palmer, Dahms et al., 1995). Several pre-clinical mammalian models have shown that hypobaric and isobaric hypoxic pregnancy has profound effects on uterine artery remodelling and reactivity, impairing the pregnancy-induced increase in uterine perfusion, thereby increasing the risk of adverse outcomes for the mother and offspring (Hu & Zhang, 1997; Hu et al., 1996, 1999; White & Zhang, 2003; White et al., 1998). Data generated by independent laboratories support that oxidative stress may mechanistically link many of the adverse effects of hypoxic pregnancy on the mother and offspring, focusing an interest on potential antioxidant therapy (Botting et al., 2020; Camm et al., 2021; Ganguly et al., 2021; Giussani, 2021; Giussani & Davidge, 2013; Giussani et al., 2012; Hansell et al., 2022; Hu & Zhang, 2021; Kane et al., 2013; Niu et al., 2018; Nuzzo et al., 2018; Richter et al., 2012; Spiroski et al., 2021; Thompson & Al-Hasan, 2012; Tong & Giussani, 2019; Tong et al., 2022).

Mitochondria are a major site of reactive oxygen species (ROS) production; therefore, targeting them should be a very powerful antioxidant therapy. However, conventional antioxidants are ineffective because they cannot penetrate the mitochondria. Part of the problem relates to the difficulty of delivering antioxidants to mitochondria *in situ*. However, Smith & Murphy (2010) have developed a mitochondria-targeted ubiquinone that overcomes this challenge. MitoQ is composed of a lipophilic triphenylphosphonium cation covalently attached to an ubiquinol antioxidant. Lipophilic cations easily move through phospholipid bilayers without requiring a specific uptake mechanism. The triphenylphosphonium cation concentrates MitoQ several hundred-fold within mitochondria, driven by the large mitochondrial membrane potential (Cochemé et al., 2007). Only within the mitochondria, MitoQ is reduced by the respiratory chain to its active ubiquinol form, which is a particularly effective antioxidant that prevents lipid peroxidation and mitochondrial damage (Smith & Murphy, 2010). The benefits of MitoQ have now been reported in a range of studies in chicken embryos, mice, rats and sheep, as well as in Phase II human trials (for review, see Giussani, 2021). By contrast to vitamin C and other conventional antioxidants, MitoQ has no pro-oxidant activity at high doses and chronic administration to mice and humans suggest no toxicity (Botting et al., 2020; Gane et al., 2010; Graham et al., 2009; Rodriguez-Cuenca et al., 2010; Snow et al., 2010). In addition, within the oxidative stress cascade and in contrast to conventional antioxidants, MitoQ does not affect superoxide anion production but instead acts downstream of its generation by preventing lipid peroxidation and mitochondrial damage that is initiated by superoxide (Botting et al., 2020). This is important because the cellular oxidant *milieu* is also an important modulator of vascular resistance, and a main physiological function of superoxide is to readily combine with nitric oxide (NO) and contribute to vasomotion (Chen & Keaney, 2004). The relative levels of superoxide may regulate the local vascular tone, promoting vasoconstriction (increased superoxide and reduced NO levels) or vasodilatation (reduced superoxide and increased NO levels) (Chen & Keaney, 2004). In the late gestation fetus, this vascular oxidant tone is physiologically active (Thakor et al., 2010). During acute fetal hypoxia, increased superoxide levels contribute to fetal peripheral vasoconstriction, which together with fetal carotid chemoreflex activation and fetal endocrine constrictor responses, helps to redistribute blood flow away from the periphery towards the fetal brain; the so-called fetal brain sparing response to acute hypoxia (Giussani, 2021; Thakor et al., 2010). We have previously reported that MitoQ treatment in chronic hypoxic pregnancy in sheep prevents a fetal

origin of cardiovascular dysfunction without affecting the fetal brain sparing to acute hypoxia (Botting et al., 2020; Giussani, 2021; Spiroski et al., 2021). Therefore, in contrast to conventional antioxidants, MitoQ treatment in hypoxic pregnancy prevents the adverse programming effects of hypoxic pregnancy in promoting an increased cardiovascular risk in the offspring, and it does so at the same time as maintaining the fetal capacity to redistribute blood flow away from the periphery towards the fetal brain. Whether the protective effects of MitoQ in hypoxic pregnancy transcend to protect the maternal circulation is only just beginning to be explored.

In a comprehensive and elegant series of studies in uterine arteries isolated from pregnant sheep at high altitude, Hu et al. (2023) have raised the hypothesis that gestational hypoxia impairs the uterine vascular adaptation to pregnancy via an interplay between increased microRNA-210 and decreased TET methylcytosine dioxygenase 2 (TET2), which promotes enhanced mitochondria-derived ROS generation. They showed that TET2 knockdown by small interfering RNAs significantly decreased spontaneous transient outward currents and elevated myogenic tone in uterine arteries. Further, the effect of TET2 knockdown on spontaneous transient outward currents and myogenic tone was negated by *in vitro* treatment with MitoQ (Hu et al., 2023). However, whether MitoQ is effective *in vivo* against uterine artery vascular dysfunction in hypoxic pregnancy is unknown. To further explore the therapeutic efficacy of MitoQ in adverse pregnancy, we tested the hypothesis that maternal *in vivo* oral treatment with MitoQ protects against uterine artery constrictor hyper-reactivity and vascular remodelling in an established rodent model of gestational hypoxia.

Methods

Ethical approval

All experiments were performed in accordance with the UK Home Office guideline under the Animals (Scientific Procedures) Act 1986 and were approved by the University of Cambridge Animal Welfare and Ethical Review Board (AWERB). Experiments were designed and reported in accordance with the ARRIVE guidelines (Kilkenny et al., 2010).

Animal model and experimental design

Wistar rats (Charles River Limited, Margate, UK) were housed in individually ventilated cages (60% humidity, 12:12 h light/dark photocycle at 21˚C) with free access to food (maintenance diet; Charles River) and water. Female Wistar rat pregnancies were established under standard conditions as previously described (Allison et al., 2016; Camm et al., 2010, 2021; Hansell et al., 2022; Herrera et al., 2011; Kane et al., 2013; Niu et al., 2018; Richter et al., 2012). Rats were randomly allocated to one of four groups (n = 10–12 per group): normoxic or hypoxic pregnancy, with or without MitoQ treatment (Fig. 1). MitoQ was obtained locally in a water-soluble form via our collaborators from the Mitochondrial Biology Unit at the University of Cambridge (Cambridge, UK). MitoQ (500 μM) was administered in the maternal drinking water and was prepared fresh every 3 days, using a dose level and regimen we have previously validated to show efficacy in the same rodent model (Nuzzo et al., 2018; Spiroski et al., 2021). Pregnant animals allocated to hypoxic groups were housed in a hypoxic chamber, which combined a PVC isolator (PFI Plastics Ltd, Manchester, UK) with a nitrogen generator (N2MID60; Domnick Hunter Ltd, Gateshead, UK) (Allison et al., 2016; Camm et al., 2010, 2021; Hansell et al., 2022; Herrera et al., 2011; Kane et al., 2013; Niu et al., 2018; Nuzzo et al., 2018; Richter et al., 2012; Spiroski et al., 2021)]. The oxygen concentration was maintained at 13–14% inspired fraction of oxygen by autoregulation of nitrogen and room air at 12–20 air changes per hour, monitored continuously with an oxygen analyser (ICA, London, UK). Induction of hypoxia during pregnancy prior to day 6 of gestation markedly enhances pregnancy loss (Giussani et al., 2012); thus, dams were exposed to hypoxia from day 6 to 20 of gestation. Hypoxia was maintained during husbandry practices by access through an isolator transfer compartment. Daily maternal food and water intake was monitored by calculating the difference in weight of the respective receptacle before and 24h after administration, and cage changes were conducted at comparable intervals to dams maintained in normoxia. Dams allocated to normoxic groups were housed within the room containing the hypoxic isolator with a controlled 12:12 h light/dark photocycle (Allison et al., 2016; Camm et al., 2010, 2021; Giussani et al., 2012; Hansell et al., 2022; Herrera et al., 2011; Kane et al., 2013; Niu et al., 2018; Nuzzo et al., 2018; Richter et al., 2012; Spiroski et al., 2021).

On day 20 of gestation, pregnant rats were weighed and killed humanely by CO_2 inhalation and cervical dislocation. The pregnant uterus was exposed via a mid-line incision and all fetuses and associated placentas were isolated and weighed. Before weighing, the umbilical cord was removed at the insertion site and the placenta and fetus were blotted on paper. The uterine artery was removed and placed into cold Krebs buffer solution consisting of (mM): 118.3 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 11.1 glucose and 0.026 EDTA (pH 7.4). Using a bifocal dissecting microscope (Brunel Microscopes Ltd, Chippenham, UK), the uterine artery was dissected free from surrounding connective tissues and artery segments were prepared for *ex vivo* wire

myography or stereology and histological analyses. The maternal liver, placenta, fetal liver, and fetal brain were snap frozen in liquid nitrogen and stored at −80°C until MitoQ tissue concentrations were quantified.

MitoQ tissue uptake following *in vivo* oral treatment

Maternal liver, placenta, fetal brain and fetal liver were frozen, and samples from each tissue (50 mg) were homogenised in 300 µL of 50 mM of Tris buffer (pH 7.0), extracted with acetonitrile (Sigma-Aldrich, Poole, UK) and dried overnight under a vacuum (Savant SpeedVac; Thermo Fisher Scientific, Waltham, MA, USA). The extracts were reconstituted and the MitoQ content measured using mass spectrometry. Control tissues were spiked with known amounts of MitoQ (1–500 pmol) and the protocol was repeated to generate a standard curve. MitoQ concentration was analysed by liquid chromatography-tandem mass spectrometry using an I-class Acquity UPLC attached to a Xevo TQ-S triple quadruple mass spec (both WatersCorp., Milford, MA, USA), and expressed relative to the internal standard by multiple reaction monitoring using the transition 583 > 441. The results were analysed with MassLynx software (WatersCorp.). The assay could detect as low as 0.1 pmol MitoQ per 100 mg of tissue.

Ex vivo wire myography

A 2 mm segment of the main branch of the uterine artery was mounted onto a four-chamber small-vessel wire myograph (Multi Wire Myograph System 610M; DMT, Hinnerup, Denmark) as previously described (Allison et al., 2016; Hansell et al., 2022; Herrera et al., 2011; Skeffington et al., 2016). The chamber was filled with Krebs buffer solution, bubbled continuously with 95% O_2, 5% CO_2 and maintained at 37°C. Vasoconstrictor responses to potassium chloride (KCl: 16–125 mmol L^{-1}), norepinephrine (10^{-10} to 10^{-4} mol L^{-1}), angiotensin II (10^{-9} to 10^{-5} mol L^{-1}) and the protein kinase C (PKC) activator phorbol 12,13-dibutyrate (PDBu) (10^{-9} to 10^{-5} mol L^{-1}) were generated. Vasodilator responses to sodium nitroprusside (SNP) (10^{-10} to 10^{-4} mol L^{-1}), ACh (10^{-9} to 10^{-5} mol L^{-1}) and the large conductance calcium activated potassium channel opener NS1619 (10^{-9} to 10^{-6} mol·l^{-1}) were also determined. For relaxant responses, vessels were pre-constricted with phenylephrine (10^{-5} mol L^{-1}). To further establish NO-dependent and NO independent effects on endothelial function, additional concentration-dependent responses to ACh were determined following incubation with the endothelial NO synthase inhibitor L-NAME (10^{-5} mol L^{-1}). The contribution of NO to the vascular relaxation induced by ACh was calculated by subtracting

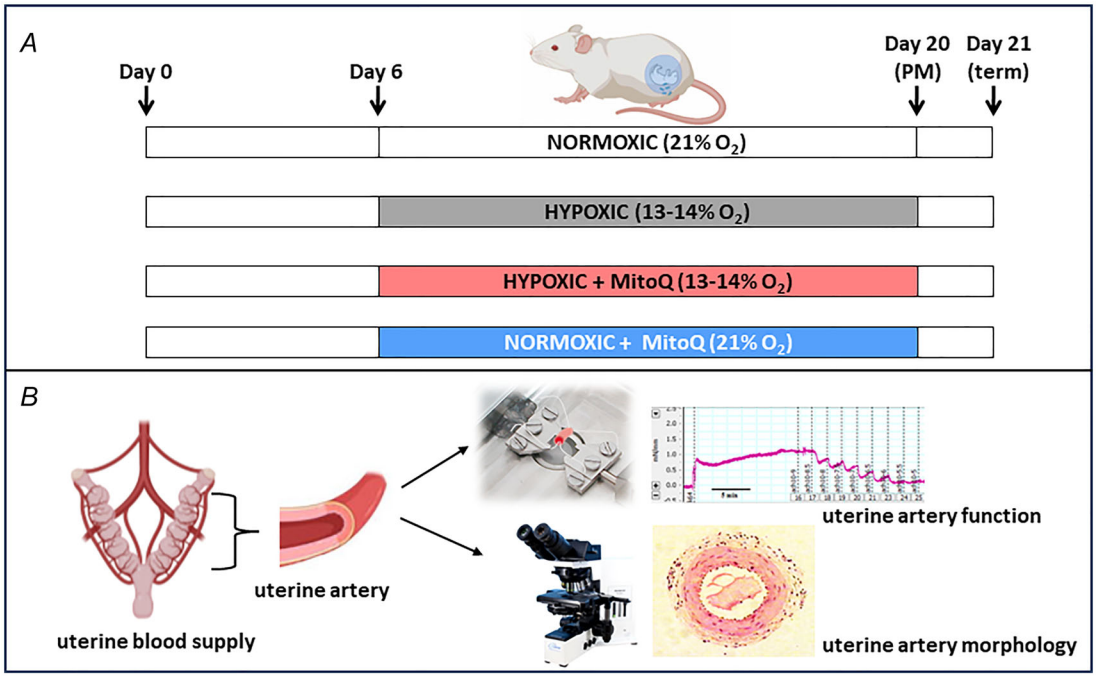

Figure 1. Experimental protocols
A, pregnant dams were singly housed and randomly assigned to either normoxia (N, 21% O_2), hypoxia (H, 13–14% O_2), hypoxia + MitoQ (HQ, 13% O_2) or normoxia + MitoQ (NQ, 21% O_2) treatment from day 6 until day 20 of pregnancy (N = 11–12 per group). MitoQ was administered in the drinking water at a concentration of 500 µM. On day 20, all dams were returned to normoxic conditions and provided normal drinking water. *B*, *post mortem*, the uterine artery was isolated. A segment was used for wire myography and another for stereology and histology.

the area under the curve (AUC) for ACh – the AUC for ACh + LNAME. The contribution of NO-independent mechanisms to the vascular relaxation induced by ACh was calculated by the AUC for ACh + LNAME. The contribution of NO independent mechanisms to the vascular relaxation induced by ACh was calculated by the AUC for ACh + L-NAME (Giussani et al., 2012; Skeffington et al., 2016).

Between constrictor or dilator curves, vessels were washed with Krebs solution and allowed to equilibrate for at least 20 min. Concentration–response curves were analysed using an agonist–response best-fit line. The developed tension of constrictor curves was expressed as a percentage of the maximal KCl-induced contraction. Dilator responses were expressed as percentage of the contraction induced by phenylephrine. Finally, the overall constrictor or relaxant capacity was expressed as the AUC (Giussani et al., 2012; Skeffington et al., 2016).

Stereological analysis of vessel morphology and histology

Uterine artery rings (200–300 μm in diameter) were immersion fixed in 4% paraformaldehyde, embedded in paraffin wax and sectioned at 10 μm (RM 2235 microtome; Leica, Wetzlar, Germany). Ten serial sections per animal were stained with haematoxylin and eosin. Quantitative analysis of the staining was carried out using a BX-50 microscope (Olympus, Tokyo, Japan), fitted with a motorised specimen stage and microcator (Olympus, Tokyo, Japan). All analysis was performed with Computer Assisted Stereology Toolbox (CAST), version 2.0 (Olympus), with the observer blind to the treatment groups. Uterine artery area was determined using a point grid, which was superimposed on the sections and viewed using a 20× objective. Points falling on the wall or lumen of the vessel were counted and the areas were calculated as:

$$A(\text{obj}) = \text{a}(p) \times \sum p$$

where A(obj) is the estimated area, $a(p)$ is the area associated with each point and Σp is the sum of points falling on the relevant area, averaged over the sections.

The lumen diameter was calculated as the maximum perpendicular distance across the lumen and the external diameter of the vessels was measured at the same position as the lumen diameter using CAST. These thicknesses were estimated using a line grid that was superimposed on sections to establish random start points using the method of orthogonal intercepts viewed with a 20× objective (Camm et al., 2010). The density of smooth muscle cells (SMCs) nuclei (an indicator of hyperplasia) in the wall was investigated in five haematoxylin and eosin-stained sections using a 100× objective and the number of SMCs nuclei within a counting frame (617 μm^2) was determined in 10 fields per section. To establish the area of the uterine artery wall occupied by SMCs nuclei, an indicator of hypertrophy, the ratio of the relative wall area/SMCs nuclei number was determined (Camm et al., 2010).

Statistical analysis

Appropriate power calculations derived from previous data sets were performed to determine the minimum sample size required to achieve statistical significance (Allison et al., 2016; Camm et al., 2010, 2021; Giussani et al., 2012; Hansell et al., 2022; Herrera et al., 2011; Kane et al., 2013; Niu et al., 2018; Nuzzo et al., 2018; Richter et al., 2012; Spiroski et al., 2021). The experiments were completed within one experimental season, and the investigators measuring the outcomes were blinded to treatments. Data are expressed as the mean ± SEM and were analysed either using the generalised linear model or two-way analysis of variation (ANOVA) with the Tukey's *post hoc* test when appropriate (SPSS, version 20.0; IBM Corp., Armonk, NY, USA). For all comparisons, $P < 0.05$ was considered statistically significant.

Results

Maternal and offspring biometry and MitoQ tissue uptake

Hypoxic pregnancy with or without maternal treatment with MitoQ increased placental weight without an effect on overall maternal food intake, maternal weight, fetal weight or litter size (Table 1). Maternal treatment with MitoQ in normoxic or hypoxic pregnancy reduced maternal water intake (Table 1). Maternal treatment with MitoQ was taken up in all tissues measured in both normoxic and hypoxic pregnancy (Fig. 2). The uptake of MitoQ in tissues was not significantly different between normoxic compared to hypoxic pregnancy (Fig. 2).

Uterine artery vasoconstrictor function

Relative to normoxic pregnancy, uterine artery constrictor responses to norepinephrine (Fig. 3*A* and *B*), angiotensin II (Fig. 3*C* and *D*) and PDBu (Fig. 3*E* and *F*) were all significantly enhanced in hypoxic pregnancy. Maternal treatment with MitoQ prevented the effect of hypoxic pregnancy on all constrictor agents (Fig. 3). By contrast, maternal treatment with MitoQ in normoxic pregnancy did not have any effect (Fig. 3).

Uterine artery vasodilator function

Relative to normoxic pregnancy, uterine artery relaxant responses to SNP (Fig. 4*A* and *B*), ACh (Fig. 4*C* and

Table 1. Maternal pregnancy and litter characteristics.

	N	H	HQ	NQ
Maternal weight (g)	390.71 ± 6.98 (*n* = 10)	385.03 ± 9.11 (*n* = 11)	377.51 ± 7.56 (*n* = 12)	390.56 ± 9.45 (*n* = 12)
Fetal weight (all) (g)	3.45 ± 0.34 (*n* = 154)	3.39 ± 0.28 (*n* = 172)	3.49 ± 0.29 (*n* = 157)	3.38 ± 0.33 (*n* = 193)
Placental weight (all) (g)	0.53 ± 0.01 (*n* = 120)	0.62 ± 0.01* (*n* = 139)	0.61 ± 0.01* (*n* = 129)	0.52 ± 0.01 (*n* = 155)
Litter size (*n*)	15.09 ± 0.72 (*n* = 11 dams)	15.73 ± 0.78 (*n* = 11 dams)	14.36 ± 0.73 (*n* = 11 dams)	16.25 ± 0.87 (*n* = 12 dams)
Water intake (mL kg day^{-1})	176 ± 8 (*n* = 10 dams)	173 ± 9 (*n* = 10 dams)	125 ± 10 (*n* = 6 dams)	146 ± 9 (*n* = 8 dams)
Food intake (g kg day^{-1})	79 ± 2 (*n* = 10 dams)	70 ± 3 (*n* = 10 dams)	73 ± 3 (*n* = 6 dams)	75 ± 3 (*n* = 8 dams)

Maternal, fetal and placental weights, litter sizes, and maternal water and food intake from normoxic (N), hypoxic (H), hypoxic + MitoQ (HQ) and normoxic + MitoQ (NQ) rodent pregnancy. Values are the mean ± SEM.
* $P < 0.05$ *vs*. N and NQ (Generalised linear model).

D) and NS1619 (Fig. 4*E* and *F*) were all significantly enhanced in hypoxic pregnancy. Deeper analysis of NO-dependent and NO-independent mechanisms contributing to ACh-induced vasorelaxation revealed that hypoxic pregnancy enhanced endothelial relaxation by increasing NO-dependent components (Fig. 4*D*, black bar graph). Maternal treatment with MitoQ prevented the effect of hypoxic pregnancy on all dilator agents (Fig. 4). Maternal treatment with MitoQ in normoxic pregnancy had no effect on SNP or NS1619 but significantly increased the ACh-induced vasorelaxation by increasing NO-dependent mechanisms (Fig. 4*D*, black bar graph).

Uterine artery morphology

Relative to normoxic pregnancy, uterine arteries isolated from hypoxic pregnancy had greater wall thickness and an increased ratio of the wall area to lumen area (Fig. 5*A* and *B*). When expressed as a percentage of the total vessel area, uterine arteries from hypoxic pregnancy had significantly reduced lumen area (Fig. 5*C*, white bar graph) and significantly enhanced wall area (Fig. 5*C*, black bar graph). These effects of hypoxic pregnancy occurred without an effect on uterine SMC nuclear density (N = 12.2 ± 0.9 %, H = 10.7 ± 1.1 %, HQ = 12.8 ± 0.3 %, NQ = 10.4 ± 0.4 %; $P = 0.6156$). Therefore, the relative uterine wall area divided by the estimated cell number, an index of cellular hypertrophy, was significantly increased in hypoxic pregnancy (N = 0.45 ± 0.05, H = 0.65 ± 0.07, HQ = 0.47 ± 0.06, NQ = 0.44 ± 0.05; N *vs*. H, $P = 0.0482$). Maternal treatment with MitoQ prevented all effects in hypoxic pregnancy on uterine vascular remodelling and it had no effect in normoxic pregnancy (Fig. 5). There was a tendency for MitoQ treatment to increase uterine artery diameter in normoxic (0.311 ± 0.028 *vs*. 0.358 ± 0.021 mm, $P = 0.6799$) and hypoxic (0.289 ± 0.036 *vs*. 0.366 ± 0.038 mm, $P = 0.3482$) pregnancy. However, this effect did not reach statistical significance (two-way ANOVA).

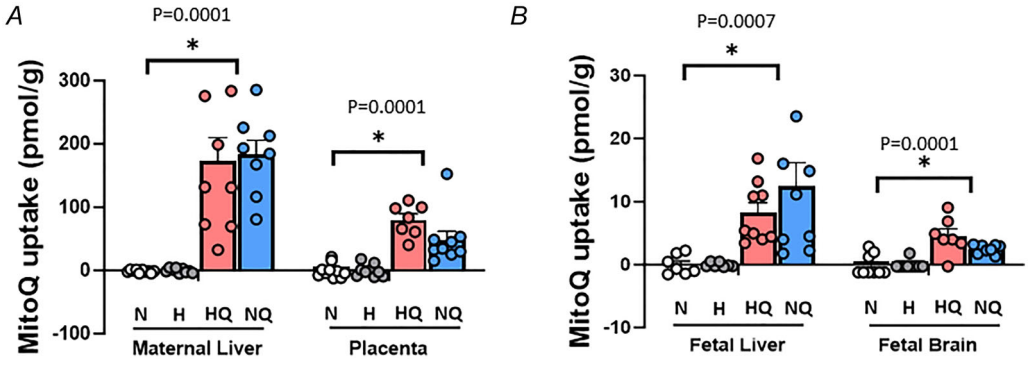

Figure 2. MitoQ uptake in the mother, placenta and fetus
MitoQ uptake by maternal liver and placenta (*A*) and by fetal liver and fetal brain (*B*) at day 20 of gestation in normoxic (N), hypoxic (H), hypoxic + MitoQ (HQ) and normoxic + MitoQ (NQ) rodent pregnancy (*N* = 7–10). *$P < 0.05$ *vs*. N (two-way ANOVA + Tukey's test).

Discussion

The data in the present study show that hypoxic pregnancy in rats increased uterine artery constrictor reactivity to norepinephrine, angiotensin II and the protein kinase C activator PDBu near term. In turn, chronic hypoxia during pregnancy also enhanced uterine artery dilator reactivity to nitroprusside and ACh near term. Deeper vascular experiments and analysis revealed that the enhanced endothelium-dependent reactivity to ACh is mediated via increased NO-dependent mechanisms and activation of the large conductance Ca^{2+}-activated K^{+}-channel (BKCa). Uterine arteries isolated from hypoxic pregnancy near term also showed greater wall thickness with reduced lumen area, as well as an increase in the uterine wall area divided by the estimated cell number, with these findings being consistent with vessel concentric hypertrophy. Maternal *in vivo* treatment with MitoQ during hypoxic pregnancy normalised uterine artery constrictor and dilator reactivity and prevented the uterine artery vascular remodelling. Maternal *in vivo* MitoQ treatment in normoxic pregnancy did not have an effect on uterine artery constriction or remodelling; however, it also enhanced dilator reactivity to ACh via increased NO-dependent mechanisms near term. Maternal treatment with MitoQ in normoxic or hypoxic pregnancy tended to increase uterine artery diameter; however, this effect did not reach statistical significance. Combined, these data indicate that hypoxic pregnancy sensitises uterine vascular bed vasoconstrictor responses mediated via protein kinase C signalling and triggers up-regulation of compensatory dilator mechanisms. Maternal *in vivo* MitoQ treatment prevents the hypoxia-induced increase in uterine artery

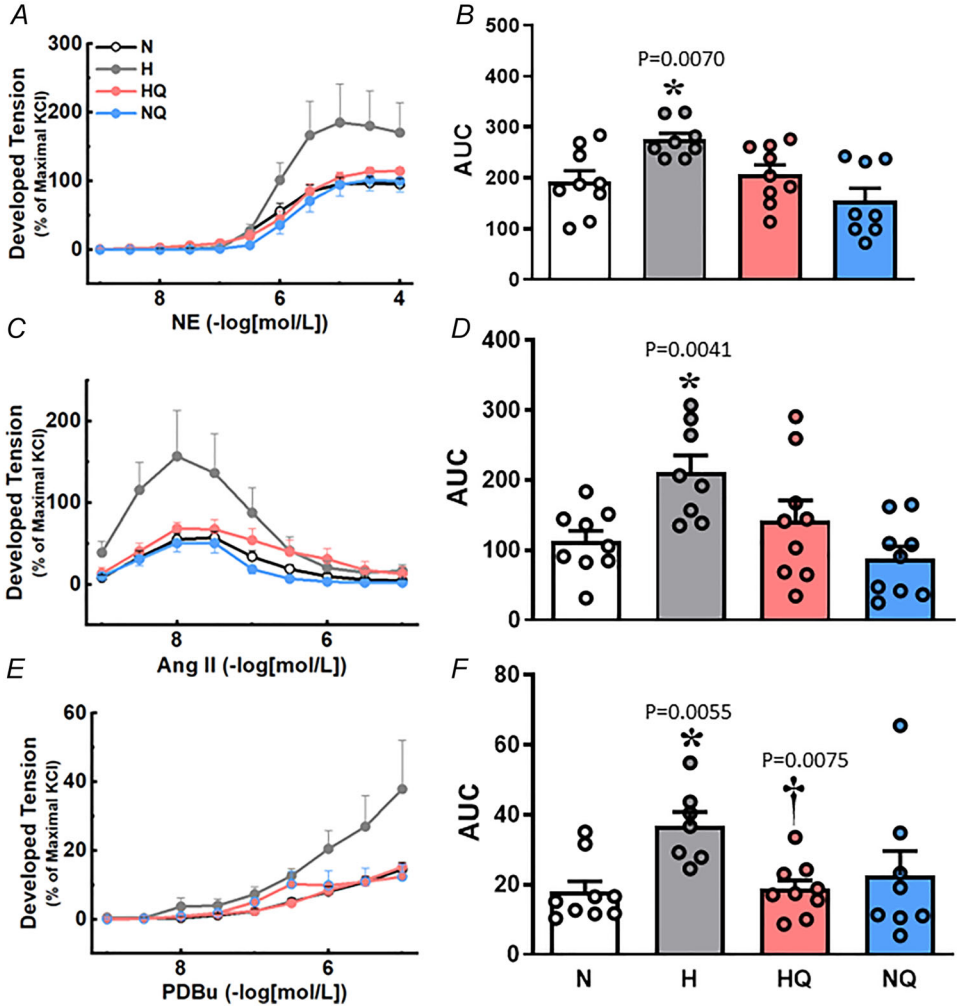

Figure 3. Uterine artery vasoconstrictor function
Concentration response curves and area under the curve (AUC) (mean ± SEM) for constrictor reactivity to norepinephrine (NE) (*A* and *B*), angiotensin II (*C* and *D*) and the protein kinase C activator PDBu (*E* and *F*) in uterine arteries isolated from normoxic (N), hypoxic (H), hypoxic + MitoQ (HQ) and normoxic + MitoQ (NQ) rodent pregnancy (N = 7–9). *$P < 0.05$ *vs*. N. † $P < 0.05$ *vs*. H (two-way ANOVA + Tukey's test).

constrictor reactivity and remodelling, negating the need for enhanced uterine artery dilator compensation. Therefore, the data support the hypothesis that maternal *in vivo* oral treatment with MitoQ protects against uterine artery vasoconstrictor hyper-reactivity and vascular remodelling in pregnancy complicated by gestational hypoxia.

PKC is a ubiquitous enzyme found in almost all cell types including the endothelium and smooth muscle of blood vessels. PKC is activated by α-adrenergic agonists and by angiotensin II (Ringvold & Khalil, 2017). Studies of uterine arteries isolated from pregnant sheep exposed to high altitude during pregnancy reported that the chronic hypoxia-induced depression in α_1-adrenergic receptor constrictor reactivity is mediated, at least in part, by a fall in α_1-adrenergic receptor density, agonist binding affinity and coupling efficiency to inositol trisphosphate receptor synthesis in the uterine artery (Hu et al., 1996, 1999). Further studies in this ovine model reveal that chronic hypoxia enhances the endothelium-dependent relaxation to the calcium ionophore A23187 in precontracted uterine vessels (Xiao et al., 2001). These changes are associated with increased endothelial NO synthase protein expression in the uterine artery of highland sheep (Xiao et al., 2001). Studies of uterine arteries isolated from pregnant guinea pigs exposed to high altitude during

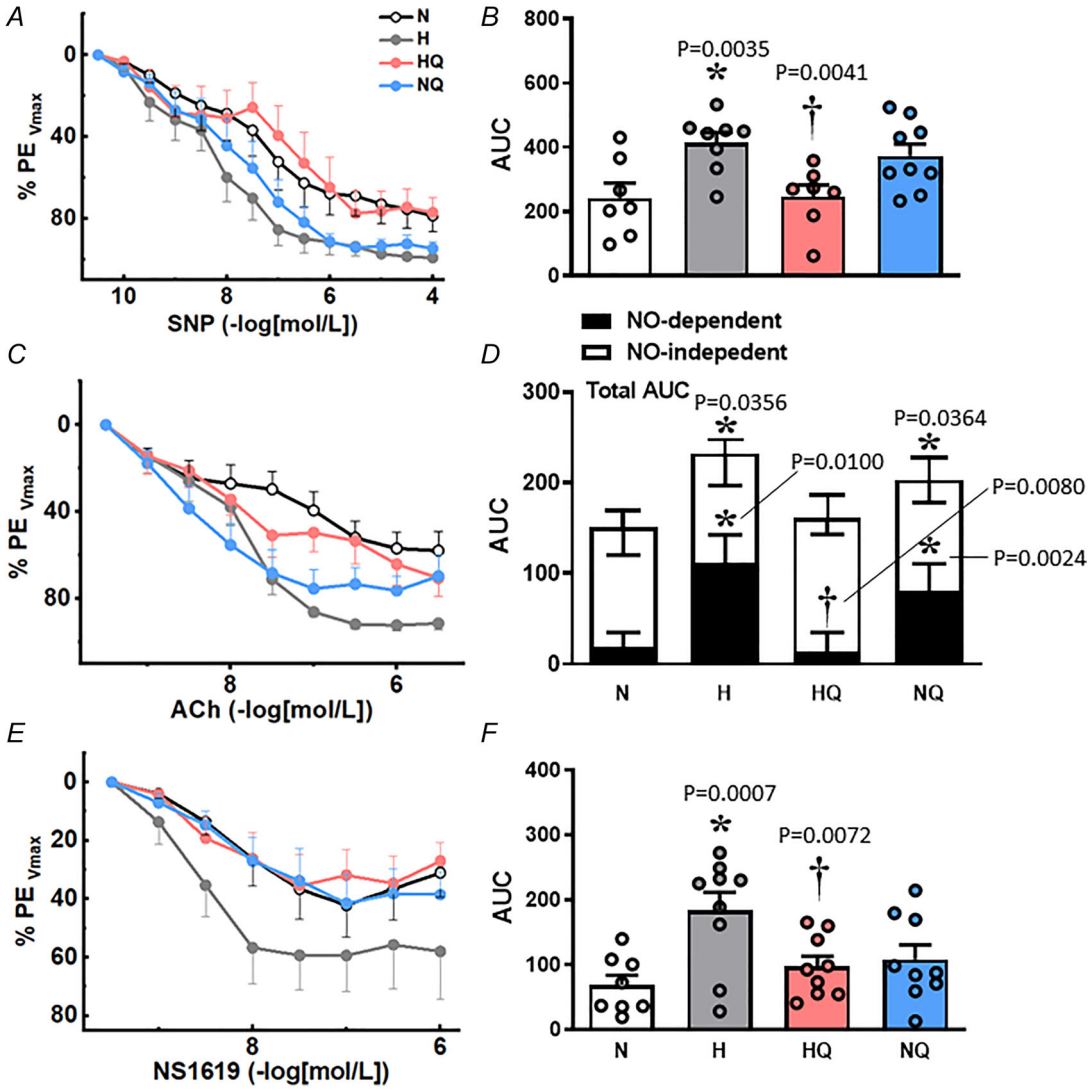

Figure 4. Uterine artery vasodilator function
Concentration–response curves and area under curves (AUC) (mean ± SEM) for reactivity to nitroprusside (SNP) (*A* and *B*), ACh (*C* and *D*) and the Ca^{2+} activated K^+ channel BKCa activator NS1619 (*E* and *F*) in uterine arteries isolated from normoxic (N, white), hypoxic (H, grey), hypoxic + MitoQ (HQ, red) and normoxic + MitoQ (NQ, blue) rodent pregnancy (N = 7–11). *$P < 0.05$ *vs.* N; †$P < 0.05$ *vs.* H (two-way ANOVA + Tukey's test). In (*D*), the positive SEM above the white bar graph is for the total AUC. The negative SEM within the white bar graph is for the NO independent component. The positive SEM above the black bar graph is for the NO-dependent component.

pregnancy demonstrate that chronic hypoxia does not diminish the pregnancy-associated reduction in contractile sensitivity to phenylephrine but enhances basal NO activity in the pregnant mesenteric artery (White et al., 1998, 2000). The data in the present rat study suggest that hypoxic pregnancy enhances constrictor reactivity to the PKC activator PBDu, norepinephrine and angiotensin II and also triggers an increase in endothelium-dependent reactivity mediated in part via increased NO-dependent mechanisms and activation of BKCa in the uterine artery. Furthermore, maternal *in vivo* MitoQ treatment prevents the enhanced constrictor reactivity and normalises dilator responses in uterine arteries from hypoxic pregnant rats. Therefore, although the effects of hypoxic pregnancy on constrictor reactivity in the uterine may vary, past and present data support that hypoxic pregnancy in preclinical mammalian models trigger compensatory up-regulation of dilator mechanisms in the uterine artery that involve enhanced NO signalling and activation of BKCa. In addition, past (Hu et al., 2023) and present data show that MitoQ treatment may not only protect against enhanced myogenic tone, but also may normalise constrictor and dilator reactivity to agonists in the uterine artery during hypoxic pregnancy. Furthermore, maternal MitoQ treatment in normoxic pregnancy enhances NO-dependent uterine artery dilator reactivity. This suggests that, in healthy pregnancy, mitochondria-derived ROS generation limits NO bioavailability and dilator capacity, and that this limitation can be removed with mitochondria-targeted antioxidants.

Uterine artery diameter is a major determinant of uterine blood flow, and sustained changes in uterine artery blood flow are important in vascular growth and remodelling (Mulvany et al., 1996; Pourageaud & Mey, 1997). For example, outward hypertrophic growth occurs with increased flow, whereas inward hypertrophic growth and a decrease in lumenal diameter result from downstream increases in peripheral vascular resistance, and an increase in cardiac afterload (Mulvany et al., 1996; Pourageaud & Mey, 1997). Zamudio, Palmer, Dahms et al. (1995) reported lower near-term uterine artery blood flow in pregnant women residing at a high altitude compared to lowland pregnant women, primarily because of a decrease in vessel diameter resulting from structural remodelling of the uterine artery. Residence at high altitude also increases the incidence of fetal growth restriction and pre-eclampsia (Giussani et al., 2001; Jensen & Moore, 1997; Palmer et al., 1999; Soria et al., 2013), conditions associated

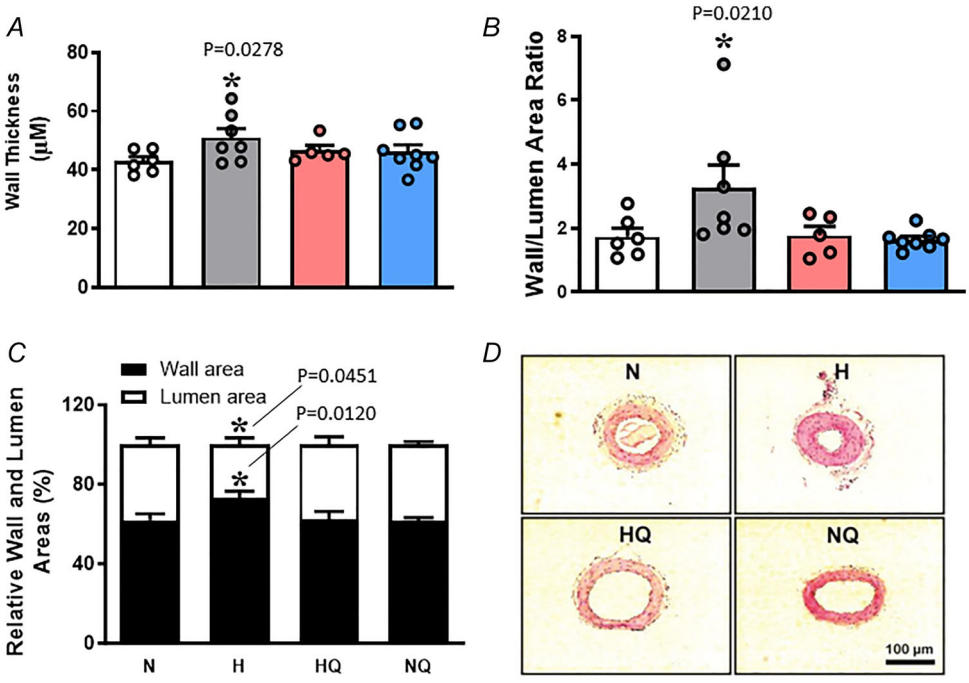

Figure 5. Uterine artery stereology and histology
Data are the mean ± SEM for wall thickness (*A*), wall/lumen area ratio (*B*) and the wall and lumen areas expressed as a percentage of the total vessel area (relative wall and lumen areas) (*C*) for uterine arteries isolated from normoxic (N), hypoxic (H), hypoxic + MitoQ (HQ) and normoxic + MitoQ (NQ) rodent pregnancy. Scaled representative examples of light micrographs are shown in (*D*). Magnification for micrographs is 20×. Scale bar = 100 µm. $^*P < 0.05$ *vs*. N (generalised linear model). For (*C*), an asterisk (*) above the white bar graph represents a significant difference in the white compartmental bar graph between N and H. For (*C*), an asterisk (*) above the black bar graph represents a significant difference in the black compartmental bar graph between N and H.

with reduced uteroplacental blood flow. Pre-eclamptic women demonstrate an absence or reversal of the normal diminution in arterial blood pressure and greater vasoconstrictor response to angiotensin II, suggesting that pre-eclampsia interferes with the normal vascular adjustment to pregnancy (Gant et al., 1977). Similarly, Hu et al. (2017) reported that sheep undergoing pregnancy at high altitude display greater basal arterial blood pressure and uterine vascular resistance and fail to show the expected fall in arterial blood pressure with advancing gestation compared to pregnant sheep at sea level. In addition, we previously reported that pregnant sheep exposed to isobaric chronic hypoxia for the last third of pregnancy failed to show the expected fall in uterine vascular resistance and maternal arterial blood pressure with advancing gestation (Tong et al., 2022). However, an increase in oxidative stress and activation of the unfolded protein response in the placenta was evident. The present study shows that *in vivo* maternal MitoQ treatment in hypoxic pregnancy in rats prevented concentric hypertrophic growth in the uterine artery, consistent with the effects of MitoQ normalising uterine artery constrictor hyper-reactivity and the oxidative stress that mediates uterine artery vascular dysfunction and remodelling.

In rat pregnancy, late-onset hypoxia (10–11% O_2) from day 15 of gestation (term at ∼21 days) induces marked fetal growth restriction (Camm et al., 2010; Hansell et al., 2022), whereas early-onset hypoxia (13–14% O_2) from day 6 of gestation does not (Camm et al., 2010; Giussani et al., 2012). This is because early-onset hypoxia triggers placental adaptations to protect against the adverse effects of chronic hypoxia on fetal growth (Giussani et al., 2012; Nuzzo et al., 2018; Richter et al., 2012). Late-onset hypoxia does not have the same effect because the challenge occurs following placentation and maximal placental growth (Adler et al., 2010; Giussani & Davidge, 2013; Giussani et al., 2012). The data in the present study show that early onset hypoxia in rats leads to uterine vascular dysfunction and remodelling independent of effects on fetal growth. Therefore, the data support that uterine vascular dysfunction and remodelling are direct effect of gestational hypoxia, such that these changes can occur in the absence of fetal growth restriction. Past and present data also highlight that, despite overt uterine concentric hypertrophic growth and a fall in uterine artery lumenal diameter, uterine artery compensatory dilator responses and placental adaptations can act in concert to ameliorate the negative effects of pregnancy complicated by chronic hypoxia on fetal growth (Giussani et al., 2012; Nuzzo et al., 2018; Richter et al., 2012)

In the present study, maternal water intake was slightly reduced by maternal treatment with MitoQ in both normoxic and hypoxic pregnancy. This is a known effect of MitoQ in rodent studies (Nuzzo et al., 2018; Spiroski et al., 2021), probably because of the palatability of oral MitoQ provided in drinking water. Therefore, the dose regimen in the present study was adjusted for efficacy accounting for this effect. However, MitoQ administration in human clinical trials is conducted via tablet (Gane et al., 2010; Snow et al., 2010), resolving any potential issues with taste adversity.

Perspectives

Many adverse conditions during pregnancy, including pregnancy at high altitude, pre-eclampsia, chorioamnionitis, gestational diabetes and maternal obesity, can trigger utero-placental hypoxia (Giussani, 2021; Giussani & Davidge, 2013; Tong & Giussani, 2019). In turn, utero-placental hypoxia has been associated with oxidative stress in the maternal, placental and fetal tissues, triggering adverse consequences on the physiology of the mother and offspring that can increase their cardiovascular risk in later life (Botting et al., 2020; Giussani, 2021; Giussani & Davidge, 2013; Tong & Giussani, 2019). Here, we show that the mitochondria-targeted antioxidant MitoQ can be successfully administered *in vivo* orally in pregnancy and that it can also protect the maternal uterine circulation against vascular dysfunction and remodelling in hypoxic pregnancy. Therefore, maternal MitoQ treatment in adverse pregnancy may not only have beneficial effects on the offspring, but also its protective effects transcend to the maternal uterine circulation in hypoxic pregnancy. Whether maternal oral *in vivo* treatment with MitoQ is similarly protective against uterine vascular dysfunction and remodelling in other types of complicated pregnancy, including advanced maternal age (Care et al., 2015; Pirhonen et al., 2005), maternal hypertension of pregnancy (Brennan et al., 2014; Myatt & Webster, 2009), maternal obesity, gestational diabetes (Goulopoulou et al., 2014; Stanley et al., 2009) and pregnancy at high altitude (Zamudio, Palmer, Droma et al., 1995), awaits subsequent investigations.

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Additional information

Data availability statement

All data are presented within the article itself. Further information and requests for resources and reagents should be directed to the coresponding author.

Competing interests

The authors declare that they have no competing interests.

Author contributions

D.A.G., Y.N., Z.W., J.M. and M.P.M. were responsible for conceptualisation. Z.W., E.J.C., A.M.N., A.S., K.L.S., T.J.A., A.R., T.T., A.L., J.M., M.P.M., Y.N. and D.A.G. were responsible for methodology. Z.W., E.J.C., A.M.N., A.S., K.L.S., T.J.A., A.R., T.T., A.L., J.M., M.P.M., Y.N. and D.A.G. were responsible for data and statistical analysis. Z.W., D.A.G., Y.N. and M.P.M. were responsible for writing the original draft. Z.W., E.J.C., A.M.N., A.S., K.L.S., T.J.A., A.R., T.T., A.L., J.M., M.P.M., Y.N. and D.A.G. were responsible for reviewing and editing. Z.W., E.J.C., A.M.N., A.S., K.L.S., T.J.A., A.R., T.T., A.L., J.M., M.P.M., Y.N. and D.A.G. were responsible for visualisation. D.A.G., Y.N., E.J.C., J.M. and M.P.M. were responsible for supervision. D.A.G., Y.N., E.J.C., J.M. and M.P.M. were responsible for project

administration. D.A.G. and M.P.M. were responsible for funding acquisition.

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Keywords

chronic hypoxia, fetal growth restriction, pre-eclampsia, uterine PI

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

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