ORIGINAL ARTICLE

Maternal melatonin: Effective intervention against developmental programming of cardiovascular dysfunction in adult offspring of complicated pregnancy

Jeremy A. Hansell1 | Hans G. Richter2 | Emily J. Camm1 | Emilio A. Herrera3 | Carlos E. Blanco4 | Eduardo Villamor5 | Olga V. Patey1 | Mitchell C. Lock6 | Andrew W. Trafford6 | Gina L. J. Galli6 | Dino A. Giussani1,7,8

1Department of Physiology Development and Neuroscience, University of Cambridge, Cambridge, UK
2Facultad de Medicina, Instituto de Anatomía, Histología y Patología, Universidad Austral de Chile, Valdivia, Chile
3Programa de Fisiopatología, Facultad de Medicina, Instituto de Ciencias Biomédicas (ICBM), Universidad de Chile, Santiago, Chile
4National Children’s Research Centre, Our Lady’s Children’s Hospital, Dublin, Ireland
5Department of Pediatrics, School for Oncology and Development Biology (GROW), Maastricht University Medical Center (MUMC+), Maastricht, The Netherlands
6Division of Cardiovascular Sciences, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK
7Cambridge BHF Centre for Research Excellence, Cambridge, UK
8Cambridge Strategic Research Initiative in Reproduction, Cambridge, UK

Abstract

Adopting an integrative approach, by combining studies of cardiovascular function with those at cellular and molecular levels, this study investigated whether maternal treatment with melatonin protects against programmed cardiovascular dysfunction in the offspring using an established rodent model of hypoxic pregnancy. Wistar rats were divided into normoxic (N) or hypoxic (H, 10% O2) pregnancy ± melatonin (M) treatment (5 μg·ml−1·day−1) in the maternal drinking water. Hypoxia ± melatonin treatment was from day 15–20 of gestation (term is ca. 22 days). To control for possible effects of maternal hypoxia-induced reductions in maternal food intake, additional dams underwent pregnancy under normoxic conditions but were pair-fed (PF) to the daily amount consumed by hypoxic dams from day 15 of gestation. In one cohort of animals from each experimental group (N, NM, H, HM, PF, PFM), measurements were made at the end of gestation. In another, following delivery of the offspring, investigations were made at adulthood. In both fetal and adult offspring, fixed aorta and hearts were studied stereologically and frozen hearts were processed for molecular studies. In adult offspring, mesenteric vessels were isolated and vascular reactivity determined by in-vitro wire myography. Melatonin treatment during normoxic, hypoxic or pair-fed pregnancy elevated circulating plasma melatonin in the pregnant dam and fetus. Relative to normoxic pregnancy, hypoxic pregnancy increased fetal haematocrit, promoted asymmetric fetal growth restriction and resulted in accelerated postnatal catch-up growth. Whilst fetal offspring of hypoxic pregnancy showed aortic wall thickening, adult offspring of hypoxic pregnancy showed dilated cardiomyopathy. Similarly, whilst cardiac protein expression of eNOS was downregulated in the fetal heart, eNOS protein expression was elevated in the heart of adult offspring of hypoxic pregnancy. Adult offspring of hypoxic pregnancy...
further showed enhanced mesenteric vasoconstrictor reactivity to phenylephrine and
the thromboxane mimetic U46619. The effects of hypoxic pregnancy on cardio-
vascular remodelling and function in the fetal and adult offspring were in-
dependent of hypoxia-induced reductions in maternal food intake. Conversely,
the effects of hypoxic pregnancy on fetal and postanal growth were similar in
pair-fed pregnancies. Whilst maternal treatment of normoxic or pair-fed preg-
nancies with melatonin on the offspring cardiovascular system was unremark-
able, treatment of hypoxic pregnancies with melatonin in doses lower than those
recommended for overcoming jet lag in humans enhanced fetal cardiac eNOS
expression and prevented all alterations in cardiovascular structure and function
in fetal and adult offspring. Therefore, the data support that melatonin is a po-
tential therapeutic target for clinical intervention against developmental origins
of cardiovascular dysfunction in pregnancy complicated by chronic fetal hypoxia.

**KEYWORDS**
cardiovascular, fetal programming, hypoxia, IUGR, melatonin

## INTRODUCTION

It has been estimated that about one-third of all non-
communicable chronic diseases in humans is linked to
prenatal determinants of adult health and pathology.1
Cardiovascular disease, one of the greatest killers in the
world today, is no exception. It is well documented that
a component of cardiovascular dysfunction in adult off-
spring can be programmed by adverse intrauterine con-
ditions.2 One of the most common suboptimal pregnancy
conditions is chronic fetal hypoxia,3 and it is well estab-
lished that hypoxic pregnancy can increase cardiovascular
risk in the adult progeny.4,5

The hypothesis that oxidative stress and reductions in
nitric oxide (NO) signalling during fetal cardiovascular
development provides a molecular link between chronic
fetal hypoxia and an increased susceptibility of the adult
offspring to cardiovascular dysfunction has gained im-
portant ground in recent years.4,6-9 This line of thinking
gave rise to interventional studies in hypoxic pregnancy
reporting that maternal treatment with antioxidants, such
as vitamin C, could prevent oxidative stress in the fetal
heart and vasculature, enhance NO signalling and thereby
protect the adult offspring from programmed cardiovas-
cular dysfunction.6,10,11 However, in such studies, only high
doses of vitamin C proved effective.6,10,11 This is problem-
atic because excess vitamin C can increase the formation
of renal calculi12 and maternal treatment with high doses
of vitamin C during human pregnancy can increase the
rate of babies born with a low birthweight.13 Therefore,
there is interest in identifying alternative maternal ther-
apy with improved human translational potential. Recent
studies have focussed either on increasing oxygen deliv-
er, to avoid oxidative stress in the first instance,14,15 or
using alternative antioxidant therapy.16-18

Melatonin may be a prime candidate to protect against
oxidative stress and impaired NO signalling during devel-
opment, given that it is a small and amphipathic indol-
amine that displays low toxicity and high efficacy at low
and safe doses.19 Melatonin shows multi-level, intra-
and extra-cellular mechanisms of action, including powerful
direct and indirect antioxidant properties20-22 and NO-
dependent cardioprotective effects.23-25 Melatonin crosses
the placenta26 and protects placental perfusion and fetal
growth in preclinical animal models of adverse preg-
ancy.27-29 Consequently, ongoing clinical trials are testing the
efficacy of melatonin in protecting fetal growth in human
high-risk pregnancy.30 Maternal melatonin treatment also
mitigates coronary stiffness in growth-restricted newborn
lambs.31 However, whether beneficial effects of melatonin
transcend to protect the adult offspring from cardiovas-
cular dysfunction in hypoxic pregnancy has not been
investigated.

Therefore, this study tested the hypothesis that mater-
nal treatment with low doses of melatonin during hypoxic
pregnancy will protect the progeny against cardiovascular
remodelling and dysfunction. The hypothesis was tested
using an established model of hypoxic pregnancy in rats,
combining functional outcomes with cellular and molec-
ular experiments in a longitudinal design at two stages of
life: in the fetus at the end of gestation and in the adult
offspring at 4 months of age. Maternal exposure to hy-
poxia can decrease maternal food intake.32-35 Therefore,
our experimental design included a subset of additional
pregnancies to control for possible hypoxia-induced reductions in maternal food intake.

2 | MATERIALS AND METHODS

2.1 | Animals, breeding and housing

The study was approved by the Cambridge University Animal Welfare and Ethical Review Board. All procedures were carried out under the UK Animals (Scientific Procedures) 1986 Act. Wistar rats (Charles River Limited) were brought into the University of Cambridge Central Biomedical Services and housed in individually ventilated cages (IVC units, 21% O₂, 70–80 air changes per hour) in rooms with controlled humidity (60%), controlled temperature (21°C) and a 12:12 hour light-dark cycle (lights on at 0700 h) with free access to food (maintenance diet, Charles River, UK) and water. After 10 days of acclimatization, virgin female Wistar rats between 10 and 12 weeks of age were paired individually with male Wistar rats, aged minimum 12 weeks in an individual cage. Paper beneath a gridded insert placed at the bottom of the cage was checked daily for the presence of a copulatory plug to indicate mating. The presence of a copulatory plug was taken to be day 0 of pregnancy (term ~22 days). Upon finding a plug, the female was weighed and housed individually in a fresh cage with free access to 200 g VRF1 diet (Special Diet Services) and water. Maternal weight, and food and water consumption were monitored daily.

2.2 | Induction of hypoxia

On day 15 of gestation, pregnant females were randomly assigned to one of four experimental groups: Normoxic (N), Normoxic + Melatonin (NM), Hypoxic (H) and Hypoxic + Melatonin (HM); with n = 7 per group reserved for studies at the end of gestation, and n = 8 per group reserved for studies in the adult offspring at 4 months of age. Pregnant rats assigned to hypoxia were placed inside a transparent hypoxic chamber that could house nine rat cages in a tranquil environment, as previously described in detail. The chronic hypoxic chamber combined a PVC isolator (PFI Plastics Ltd.) with a nitrogen generator (N2MID60, Dominick Hunter Ltd). The percentage of oxygen in the chamber could be controlled with precision over long periods, without changes in ambient carbon dioxide, by altering the inflow and outflow of air and nitrogen in the chamber. The chamber had the same number of air changes as the normoxic individual ventilated cages and was housed in the same room. The chamber contained a battery-operated hygrometer and thermometer for continuous monitoring of humidity and temperature whilst the oxygen concentration of the chamber was monitored throughout with an oxygen analyzer (ICA).

Pregnancies undergoing maternal hypoxia were maintained at an inspired fraction of oxygen of 10% from day 15 of gestation. In experiments in sheep pregnancy, in which the fetus can be catheterized long-term, we have shown that this level of maternal hypoxia results in fetal arterial PO₂ values between 11 and 13 mmHg. This is the PO₂ measured by cordocentesis in human fetuses in pregnancy affected by chronic fetal hypoxia and intrauterine growth restriction. Therefore, this model of hypoxic pregnancy is of human clinical relevance.

Maternal exposure to 10% O₂ isobaric hypoxia can decrease maternal food intake. Therefore, an additional n = 30 dams underwent pregnancy under normoxic conditions but were pair-fed (PF) to the daily amount consumed by hypoxic dams from day 15 of gestation. Half of them received melatonin treatment (PFM) and n = 7 per group were reserved for studies at the end of gestation, and n = 8 per group were reserved for studies in the adult offspring at 4 months of age.

2.3 | Maternal treatment with melatonin

Normoxic, hypoxic and pair-fed pregnancies were randomly assigned for treatment with melatonin in the maternal drinking water. Melatonin was initially dissolved in the minimum volume of ethanol (three drops) and then made up with water to achieve a final concentration of 5 μg·ml⁻¹. The final concentration of ethanol in the solution was <0.01%. The solutions were prepared fresh every other day and were administered using opaque bottles to prevent light-induced degradation. The dose of melatonin was equivalent to the maximal dose recommended for overcoming jet lag in humans. Dams not receiving melatonin in their drinking water were given a vehicle containing three drops of ethanol per 500 ml drinking water.

2.4 | Studies at the end of gestation

On the morning of day 20 of gestation, the dams were weighed. Anaesthesia was induced with inhalational isoflurane and then maintained by a mixture of ketamine (40 mg·kg⁻¹) and xylazine (5 mg·kg⁻¹) injected intraperitoneally. Once anaesthetized, a maternal blood sample (5 ml) was taken by cardiac puncture. The mother was killed under anaesthesia and the pregnant uterus exposed via a mid-line incision. The anaesthetized pups were then isolated and killed via cervical spinal transection. In dams
that had been in the hypoxic chamber from days 15–20 of gestation, this procedure was carried out whilst ventilated with 10% oxygen via a face mask. Fetal blood was collected from the neck incision by capillarity into haematocrit tubes and pooled per litter. Maternal and fetal blood was either centrifuged in a Hawksley centrifuge for determination of haematocrit or centrifuged at 300 g for 5 min, aliquoted and frozen for subsequent analysis of plasma concentrations of melatonin. All fetuses and their associated placentae were isolated and weighed. The fetal sex was noted by measurement of the anogenital distance.\(^{35,36}\) Four male and four female fetuses from each litter underwent detailed biometry including measurement of crown-rump length (CRL). The heart, liver, and brain of one male fetus per litter were dissected and weighed. The placenta and heart from one male fetus per litter were snap-frozen in liquid nitrogen and stored at \(-80°C\) for subsequent protein expression analysis. The thorax of another male fetus per litter was immersion fixed in 4% paraformaldehyde for 5 days, and then stored in phosphate-buffered saline until it was processed into paraffin blocks for histological analysis of the heart and the aorta.

### 2.6 Melatonin assay

Maternal and pooled fetal blood samples were collected in chilled heparinized or ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged at 300 g for 5 min. Plasma aliquots were snap-frozen in liquid nitrogen and maintained frozen at \(-80°C\) until analysis. The concentration of melatonin in both maternal and fetal plasma was determined in all samples at the same time, as before.\(^{27}\) Briefly, melatonin levels were determined using the 2-\([125I]\)iodomelatonin Rat RIA Kit (MP Biomedicals, Eschwege, Germany; code 07L-130102). The sensitivity of the assay was 1.5 pg/ml and the intra- and inter-assay coefficients of variation were <12%. The melatonin antiserum showed <1% cross-reactivity with N-acetylseryotonin, 5-methoxytryptophol and 5-methoxytryptamine and <0.01% with other related amines.

### 2.7 Stereology

All quantitative analysis of fixed tissue obtained from the fetal pups and from the adult offspring was carried out with an Olympus BX-50 microscope with objective lenses (\(\times1.25\) and \(\times10\) and oil immersion \(\times100\)), a motorized specimen stage and microcator using the Computer Assisted Stereology Toolbox (CAST version2.0, Olympus). Paraffin-embedded fetal thoraces and the left ventricles and aorta isolated from the adult offspring were exhaustively sectioned at 5 \(\mu m\) using a Leica RM 2235 microtome (Leica Microsystems). To determine wall and lumen areas, 10 sections were selected at an equal distance apart at the level of the cardiac valves for the fetal and adult hearts, and at a level of the apex of the heart for the descending aorta of the adult offspring. Prepared slides were then stained with Masson’s Trichrome. A point grid was superimposed on each section and areas were quantified as before\(^{43-45}\) using the Cavalieri’s principle.\(^{46}\) Points falling on either the wall or lumen were counted, and areas were calculated as \(A(\text{obj}) = a(p) \times \Sigma P\), where \(A(\text{obj})\) is the estimated area, \(a(p)\) is the area associated with each point and \(\Sigma P\) is the sum of points falling on the relevant area, averaged over the sections. To account for shrinkage due to immersion fixing and paraffin processing, the diameter of erythrocytes present in sections of fetal or adult fixed tissue was measured and compared to the diameter of erythrocytes present in fresh blood from foetal or adult rats of the same age.\(^{47}\) All measurements were corrected using this factor.
2.8 Western blot analysis

Tissue homogenization to obtain protein lysates and subsequent sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotting were performed, as previously.27,48 The specific primary antibodies were rabbit polyclonal to 4-Hydroxynonenal (4-HNE; 1:2000; Abcam, code 210767) and heat shock protein 70 (HSP70; 1:20000; Stressgen, code Spa812C); mouse monoclonal to heat shock proteins 27 (HSP27; 1:1500; Cell Signaling, code 2442), endothelial NOS (eNOS; 1:2500; BD Transduction Labs, code 610297) and β-actin (1:50000; Sigma, code A5441). The horseradish peroxidase-conjugated secondary antibodies were from GE Healthcare (anti-rabbit 1:20000; Stresgen, code Spa812C); mouse monoclonal to Abcam (code 210767) and the heat shock protein 70 (HSP70; 1:1500; Cell Signaling, code A5441). Western blot analysis was used to determine appropriate power calculations to determine minimum sample sizes required to achieve statistical significance.27,35,36,48-50 Wherever possible, scientists measuring outcomes were blinded to treatments. For instance, to minimize bias, all histological analysis was performed using coded slides, with the observer blinded to the treatment groups.

2.9 Ex vivo wire myography

Adult offspring assigned to ex-vivo wire myography were killed via carbon dioxide inhalation and kept on ice until dissection of the vessels. Immediately after euthanasia, the small intestine was removed, placed in ice-cold Krebs buffer (mmol·L⁻¹: NaCl 118.5, Fisher, KCl 4.75, Sigma, MgSO₄·7H₂O 1.2, Sigma, KH₂PO₄ 1.2, Sigma, NaHCO₃ 25.0, Sigma, CaCl₂ 2.5, Sigma, glucose 5.5, Sigma) and a second order branch of the mesenteric artery was dissected. A 2 mm arterial segment was cut and mounted in a four-chamber small-vessel wire myograph (Multi Wire Myograph System 610 M, DMT, Denmark). One jaw of the myograph was connected to a micrometre screw and the other to a force transducer, which was connected to a data acquisition system (Powerlab 8/30, ADInstruments), as before.36,49,50 A digital camera (Olympus) connected to one of the eye-pieces of the microscope displayed a live image of the vessel on a computer screen. The length and diameter of the unstretched vessel segment could then be measured from the digital image and corrected using a known scale factor. The myograph baths were maintained at 37°C and continuously aerated with a gas mixture of 95% O₂ and 5% CO₂. After a stabilization period of 15–20 min, the vessels were stretched to an equivalent physiological transmural pressure of 100 mmHg.36,49,50 The internal circumference (IC) corresponding to this transmural pressure was calculated using the equation: IC = πd²/2(D + d), where D was the diameter (40 μm) of and d was the distance between the wires. After 30 min of incubation under resting tension, a control reference contraction was elicited by raising the K⁺ concentration of the buffer (125 mmol/L) in exchange for Na⁺. After washing the vessels three times with Krebs buffer and waiting for 20 min, cumulative concentration-response curves to the α₁-adrenergic agonist phenylephrine (PE; 10⁻⁹–10⁻⁴ mol/L) and the thromboxane mimetic U46619 (10⁻¹⁰–10⁻⁷ mol/L) were determined in half-log increments. Arteries were given 15–20 min resting and equilibration period between experiments. The responses to phenylephrine and to U46619 were normalized to the response to 125 mM K⁺ of the same vessel (%K+125).

2.10 Data and statistical analyses

Previous data sets with a similar experimental design were used to determine appropriate power calculations to determine minimum sample sizes required to achieve statistical significance.27,35,36,48-50 Wherever possible, scientists measuring outcomes were blinded to treatments. For instance, to minimize bias, all histological analysis was performed using coded slides, with the observer blinded to the treatment groups.

Postnatal fractional growth rate (FGR) was calculated as the change in bodyweight over the period of interest, divided by the number of days within that period. For the wire myography analysis, sensitivity (expressed as pD₂ = -log EC₅₀) and maximal response (Kₘₐₓ) to agonists (PE and U46619) were determined by fitting individual concentration-response data to a non-linear sigmoidal regression curve using Prism 6.0 (GraphPad software), as before.36,50,51 For the molecular biology and histological studies, values given as percentages or ratios were first transformed to arcsin to equalize variance.52 All data are expressed as the mean ± SEM. Comparisons between experimental groups were made using Two-way ANOVA, with repeated measures, as appropriate. If the ANOVA showed a significant interaction between factors, statistical differences between groups were isolated using the Tukey post hoc test (SigmaStat for Windows, Version 2.0; SPSS Inc.). To account for the sex of the offspring and within litter variation, data from one male pup per litter for any one outcome variable were taken. Exceptions were data describing biometry for the fetal study at the end of gestation, such as fetal and placental weights, CRl and BMI. For these outcomes, where more than one values originated from anyone litter, a Mixed Linear Model analysis was used to consider within litter variation (SPSS for Windows, Version 16; SPSS Inc.). For all statistical comparisons, p < 0.05 was considered statistically significant.
RESULTS

3.1 Maternal food intake, maternal water consumption and maternal weight gain

In all pregnancies irrespective of treatment, maternal food and water intake between days 0 and 15 of gestation did not vary significantly and averaged 23.1 ± 0.3 g and 46.3 ± 0.8 ml per day, respectively. In normoxic pregnancies, maternal food intake was significantly increased relative to baseline from day 16. In contrast, hypoxic dams ate significantly less than control pregnancies from days 16–21 of gestation (Figure 1A,B). This equated to a ca. 35% reduction in maternal food consumption relative to normoxic pregnancies (p < 0.05). Relative to normoxic dams, maternal water consumption also fell transiently in hypoxic pregnancies, on days 16 and 17 of gestation, but returned to control values for the remainder of gestation (Figure 1A,B). All dams gained weight through pregnancy and were significantly heavier than their weight at conception from day 10 of gestation onwards. Relative to normoxic dams, hypoxic pregnancies put on significantly less weight during days 16–21 of gestation (Figure 1A,B). The daily profile of maternal food intake in hypoxic dams was successfully replicated in dams undergoing pair-feeding (Figure 1A,B). Maternal treatment with melatonin did not affect maternal food or water consumption, or changes in maternal weight gain in normoxic, hypoxic or pair-fed pregnancies (Figure 1A,B).

To maintain clarity, presentation of the data in graphic form from now on will focus on comparison between normoxic and hypoxic pregnancies with and without maternal treatment with melatonin. Presentation of the data pertaining to additional pair-fed control groups will appear in text or in a table.

3.2 Melatonin concentrations and haematocrit

Plasma concentrations of melatonin measured at day 20 of gestation were higher in the pregnant dam than in the corresponding fetuses (p < 0.05). Hypoxia did not alter maternal or fetal plasma melatonin concentrations (Figure 2A,B). Similarly, pair-feeding did not alter maternal (N, 20.1 ± 4.9 vs. PF, 33.7 ± 8.5 pg/ml; p > .05) or fetal (N, 6.2 ± 1.9 vs. PF, 8.4 ± 4.7 pg/ml; p > 0.05) plasma melatonin concentrations. Relative to untreated pregnancies, maternal treatment with melatonin significantly increased the plasma concentration of melatonin in normoxic and hypoxic mothers and fetuses at day 20 of gestation (p < 0.05; Figure 2A,B).

Similarly, relative to untreated pregnancies, maternal treatment with melatonin significantly increased the plasma concentration of melatonin in pair-fed mothers (255.2 ± 72.2 pg/ml) and fetuses (134.3 ± 36.7 pg/ml) at day 20 of gestation (both p < 0.05). Hypoxia significantly increased maternal and fetal haematocrit at day 20 of gestation (Figure 2A,B). Maternal treatment with melatonin had no effect on maternal or fetal haematocrit in normoxic or hypoxic pregnancies (Figure 2A,B).

3.3 Pregnancy characteristics

Litter size at birth was similar across all groups (N: 13.6 ± 1.0, NM: 13.1 ± 0.4, H: 12.7 ± 1.0, HM: 12.5 ± 0.7, PF: 12.6 ± 1.3, PFM: 12.7 ± 1.1). Relative to fetuses of normoxic pregnancy, fetal bodyweight, crown-rump length (CRL) and body mass index (BMI) at day 20 of gestation were similarly reduced in hypoxic pregnancy with or without melatonin (p < 0.001; Figure 3A–C). Relative to fetuses of normoxic pregnancy, the fetal brain:liver weight ratio was similarly increased in hypoxic pregnancy with or without melatonin (p < 0.001; Figure 3D). Relative to normoxic pregnancy, hypoxic pregnancy did not affect placental weight, but melatonin treatment similarly reduced placental weight in normoxic and hypoxic pregnancy (p < 0.05; Figure 3E). Relative to normoxic pregnancy, the fetal body weight:placental weight ratio was similarly reduced in hypoxic pregnancy with or without melatonin (p < 0.05; Figure 3F). Data derived from the second cohort of animals that were allowed to deliver showed that relative to pups of normoxic pregnancy, birthweight was similarly reduced in hypoxic pregnancy with or without melatonin (p < 0.001; Figure 3G). Conversely, the gestation length was similarly increased in hypoxic pregnancy with or without melatonin (p < 0.01; Figure 3H). Therefore, relative to pups of normoxic pregnancy, the birth weight for gestation length was similarly reduced in hypoxic pregnancy with or without melatonin (p < 0.01; Figure 3I). Relative to untreated normoxic pregnancy, maternal treatment with melatonin in normoxic pregnancy did not affect any outcome (Figure 3A–I).

The pregnancy characteristics in pair-fed groups with and without melatonin treatment have been published as part of another study. In brief, fetuses of pair-fed relative to control pregnancies had significantly lower fetal bodyweight and fetal body:placental weight ratio, higher fetal brain:fetal bodyweight ratio, and lower birth weight [all p < 0.05; 27]. Maternal treatment with melatonin in pair-fed pregnancy did not affect the reduction in fetal bodyweight or the increase in the fetal brain:fetal bodyweight ratio. However, maternal treatment with melatonin in
Pair-fed pregnancy reduced placental weight, which restored the fetal body:placental weight ratio and birth weight to control values \( p < 0.05; 27 \). Maternal treatment with melatonin in control pregnancies also led to significantly lighter placentae \( p < 0.05 \), but it had no effect on other variables. \(^{27} \)

### 3.4 Postnatal growth

Relative to offspring of normoxic pregnancy, offspring of hypoxic pregnancy had a significantly greater fractional growth rate (FGR) between 0 and 18 weeks of age. Partial analysis showed that this effect of hypoxia was in the first 3 weeks of postnatal life, from birth to weaning \( p < 0.01; \) Table 1). Maternal treatment with melatonin in hypoxic pregnancy normalized the postnatal FGR and maternal treatment with melatonin in normoxic pregnancy had no effect (Table 1). The effect of hypoxic pregnancy on postnatal FGR was not independent of an effect of hypoxia-induced reductions in maternal food intake because offspring of pair-fed pregnancies also had a significantly increased FGR relative to offspring of normoxic pregnancies \( p < 0.01; \) Table 1). Maternal treatment with
melatonin in pair-fed pregnancy also normalized the postnatal FGR (Table 1).

### 3.5 Fetal and adult offspring cardiovascular remodelling

The data for the cardiovascular stereology in the fetal and adult offspring are summarized in Table 2 and Figure 4. Relative to fetuses of normoxic pregnancy, fetuses of hypoxic pregnancy had aortic wall thickening, showing an increase in the aortic wall:lumen area \((p < 0.01;\) Figure 4A) with both a significant increase in the wall area and a significant decrease in the lumen area, when expressed as a percentage of the total vessel area \((p < 0.01;\) Figure 4B). These effects of hypoxic pregnancy on fetal aortic remodelling were independent of hypoxic-induced reductions in maternal food intake because values for these variables in fetuses from pair-fed pregnancies were not different \((p > 0.05)\) than from normoxic pregnancies \((\text{PF fetal aortic wall:llumen area} = 0.56 \pm 0.11; \text{PF fetal aortic lumen area as a % of total vessel area} = 70.5 \pm 8.6; \text{PF fetal aortic wall area as a % of total vessel area} = 30.8 \pm 4.9)\).

By adulthood, the aortic wall remodelling in offspring from hypoxic pregnancy normalized (Table 2). However, relative to adult offspring of normoxic pregnancy, these adults of hypoxic pregnancy had left ventricles with thinner walls, showing a decrease in the wall:lumen area ratio \((p < 0.01;\) Figure 4C), with both a significant decrease in the wall area and a significant increase in the lumen area, when expressed as a percentage of the total left ventricular area \((p < 0.01;\) Figure 4D). As with effects in fetal life, these effects of hypoxic pregnancy on cardiac wall remodelling in the adult were independent of hypoxic-induced reductions in maternal food intake because values for these variables in adult offspring of pair-fed pregnancies were not different \((p > 0.05)\) than those of normoxic pregnancies \((\text{PF adult cardiac wall:llumen area} = 7.2 \pm 1.5; \text{PF adult left ventricular lumen area as a % of total heart area} = 15.7 \pm 1.4; \text{PF left ventricular wall area as a % of total heart area} = 85.5 \pm 5.2)\).

The effects of hypoxic pregnancy on the wall:lumen area ratio of the aorta in the fetus and the left ventricle in the adult offspring no longer occurred in hypoxic pregnancy.
treated with melatonin (Figure 4A–D). Maternal treatment with melatonin in normoxic pregnancy had no effects on the aortic wall:lumen area ratio in fetal life, or on the left ventricular wall:lumen area ratio in adult life (Figure 4A–D). However, relative to fetuses of normoxic pregnancy, maternal treatment with melatonin in normoxic pregnancy led to a significant decrease in the left ventricular wall area and thickness ($p < 0.05$; Table 2). Relative to offspring of normoxic pregnancy, offspring from pair-fed pregnancies with or without melatonin showed a significant decrease in the left ventricular wall area in fetal life and a significant decrease in the left ventricular weight in adult life ($p < .05$; Table 2).

FIGURE 3  Pregnancy characteristics. Values are mean ± SEM for fetal bodyweight (A), fetal crown-rump length, CRL (B), the fetal body mass index, BMI (C), the fetal brain to liver weight ratio (D), the placental weight (E), the fetal to placental weight ratio (F), the birth weight (G), the gestational age (H) and the birth weight for gestational age ratio (I) in normoxic (N; white bars), hypoxic (H; black bars), hypoxic + melatonin (HM; red bars) and normoxic + melatonin (NM; blue bars) pregnancies. Comparisons of variables relating to fetal and placental biometry were assessed using a Generalised Mixed Linear Model (SPSS). This statistical technique permits the experimental effects to be assessed on all collected pups and associated placentae considering the size of the litter, thereby negating the need to pool these variables per litter. Significant differences ($p < 0.05$) are: *effect of hypoxia.
3.6 | Fetal and adult offspring cardiac protein expression

The data for the cardiac protein expression in the fetal and adult offspring are summarized in Table 3 and Figure 5. Relative to fetuses of normoxic pregnancy, the fetal cardiac protein expression of eNOS in hypoxic pregnancy was significantly reduced ($p < 0.05$; Figure 5A). This effect of hypoxic pregnancy was independent of hypoxic-induced reductions in maternal food intake because values for cardiac eNOS protein expression in fetuses from pair-fed pregnancies were not different relative to those from normoxic pregnancies ($0.72 \pm 0.34$ vs. $1.00 \pm 0.27$, $p > .05$). Maternal treatment with melatonin in hypoxic pregnancy significantly enhanced the fetal cardiac eNOS protein expression ($p < .01$) and maternal treatment with melatonin in normoxic pregnancy had no effect (Figure 5A).

By contrast, at adulthood, relative to offspring of normoxic pregnancy, the cardiac protein expression of eNOS in hypoxic pregnancy was significantly increased ($p < 0.01$; Figure 5B). As with effects in fetal life, this effect of hypoxic pregnancy on cardiac eNOS protein expression in the adult were independent of hypoxic-induced reductions in maternal food intake because values for cardiac eNOS protein expression in adult offspring of pair-fed pregnancies were significantly reduced rather than enhanced in adult offspring from pair-fed pregnancies relative to those of normoxic pregnancies ($0.45 \pm 0.03$ vs. $1.00 \pm 0.08$, $p < 0.05$). Maternal treatment with melatonin in normoxic or hypoxic pregnancy had no effect on the cardiac protein expression of eNOS in adult offspring (Figure 5B). The cardiac protein expression of HSP27, HSP70 and HNE in hearts of fetal or adult offspring was not affected by any treatment (Table 3).

3.7 | Vascular function in adult offspring

The outer diameter of the unstretched mesenteric arteries was similar between groups (N: 232 ± 16, NM: 216 ± 8, H: 207 ± 16, HM: 211 ± 6, PF: 210 ± 24, PFM: 198 ± 7 μm). In addition, the internal diameter at which the arteries reached an intramural pressure of 100 mmHg (N: 0.48 ± 0.02, NM: 0.45 ± 0.02, H: 0.48 ± 0.03, HM: 0.48 ± 0.03, PF: 0.49 ± 0.03, PFM: 0.43 ± 0.02 mm) and the wall tension in response to K+ 125 mM (N: 7.61 ± 0.52, NM: 7.58 ± 0.75, H: 7.62 ± 0.34, HM: 7.53 ± 0.59, PF: 7.66 ± 0.63, PFM: 7.59 ± 0.33 mN/mm) were similar between groups.

Relative to adult offspring of normoxic pregnancy, mesenteric vessels isolated from adult offspring of hypoxic pregnancies showed enhanced sensitivity to PE
## Table 2: Fetal and adult offspring cardiovascular stereology.

|                      | N                  | NM                 | H                  | HM                 | PF                 | PFM                |
|----------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| **Fetal offspring**  |                    |                    |                    |                    |                    |                    |
| 20 days of gestation |                    |                    |                    |                    |                    |                    |
| Aorta wall area (mm²)| 0.073 ± 0.005 (n = 6) | 0.082 ± 0.004 (n = 6) | 0.083 ± 0.005 (n = 6) | 0.090 ± 0.005 (n = 6) | 0.086 ± 0.005 (n = 6) | 0.084 ± 0.005 (n = 6) |
| Aorta lumen area (mm²)| 0.145 ± 0.019 (n = 6) | 0.147 ± 0.019 (n = 6) | 0.092 ± 0.012 (n = 6) | 0.131 ± 0.013 (n = 6) | 0.129 ± 0.017 (n = 6) | 0.153 ± 0.029 (n = 6) |
| Aorta total area (mm²)| 0.218 ± 0.018 (n = 6) | 0.299 ± 0.017 (n = 6) | 0.176 ± 0.015 (n = 6) | 0.222 ± 0.012 (n = 6) | 0.216 ± 0.020 (n = 6) | 0.236 ± 0.028 (n = 6) |
| Aorta wall thickness (mm)| 0.177 ± 0.003 (n = 6) | 0.166 ± 0.005 (n = 6) | 0.190 ± 0.005 (n = 6) | 0.178 ± 0.005 (n = 6) | 0.177 ± 0.008 (n = 6) | 0.175 ± 0.005 (n = 6) |
| Absolute heart weight (g) | 0.031 ± 0.001 (n = 6) | 0.035 ± 0.003 (n = 6) | 0.028 ± 0.002 (n = 6) | 0.030 ± 0.001 (n = 6) | 0.028 ± 0.002 (n = 6) | 0.028 ± 0.003 (n = 6) |
| Relative heart weight (g.g⁻¹) | 0.810 ± 0.042 (n = 6) | 0.899 ± 0.038 (n = 6) | 0.933 ± 0.064 (n = 6) | 1.007 ± 0.029 (n = 6) | 0.882 ± 0.050 (n = 6) | 0.851 ± 0.036 (n = 6) |
| Left ventricular wall area (mm²)| 4.20 ± 0.18 (n = 6) | 2.83 ± 0.17 * (n = 6) | 4.30 ± 0.29 (n = 6) | 4.01 ± 0.20 (n = 6) | 3.08 ± 0.16 * (n = 6) | 2.93 ± 0.21 * (n = 6) |
| Left ventricular lumen area (mm²)| 0.657 ± 0.121 (n = 6) | 0.911 ± 0.194 (n = 6) | 0.812 ± 0.140 (n = 6) | 0.654 ± 0.078 (n = 6) | 0.604 ± 0.149 (n = 6) | 1.140 ± 0.119 (n = 6) |
| Left ventricular wall thickness (mm)| 0.740 ± 0.087 (n = 6) | 0.561 ± 0.040 * (n = 6) | 0.701 ± 0.040 (n = 6) | 0.704 ± 0.029 (n = 6) | 0.714 ± 0.053 (n = 6) | 0.624 ± 0.054 (n = 6) |
| **Adult offspring**  |                    |                    |                    |                    |                    |                    |
| 4 months old        |                    |                    |                    |                    |                    |                    |
| Aorta wall area (mm²)| 0.953 ± 0.049 (n = 8) | 0.834 ± 0.036 (n = 8) | 1.056 ± 0.065 (n = 8) | 0.959 ± 0.066 (n = 8) | 0.877 ± 0.072 (n = 8) | 0.956 ± 0.074 * (n = 8) |
| Aorta lumen area (mm²)| 2.34 ± 0.07 (n = 8) | 2.30 ± 0.04 (n = 8) | 2.61 ± 0.21 (n = 8) | 2.30 ± 0.08 (n = 8) | 2.48 ± 0.08 (n = 8) | 2.62 ± 0.10 (n = 8) |
| Aorta total area (mm²)| 3.29 ± 0.07 (n = 8) | 3.14 ± 0.07 (n = 8) | 3.66 ± 0.27 (n = 8) | 3.26 ± 0.13 (n = 8) | 3.36 ± 0.12 (n = 8) | 3.57 ± 0.16 (n = 8) |
| Aorta wall thickness (mm)| 0.177 ± 0.003 (n = 8) | 0.166 ± 0.005 (n = 8) | 0.190 ± 0.005 (n = 8) | 0.178 ± 0.005 (n = 8) | 0.177 ± 0.008 (n = 8) | 0.175 ± 0.008 (n = 8) |
| Relative heart weight (%) | 0.220 ± 0.007 (n = 8) | 0.233 ± 0.007 (n = 8) | 0.244 ± 0.007 * (n = 8) | 0.244 ± 0.005 * (n = 8) | 0.225 ± 0.006 (n = 8) | 0.222 ± 0.005 (n = 8) |
| Relative left ventricle + septum weight (%) | 0.168 ± 0.005 (n = 8) | 0.178 ± 0.004 (n = 8) | 0.186 ± 0.005 * (n = 8) | 0.187 ± 0.004 * (n = 8) | 0.192 ± 0.004 * (n = 8) | 0.190 ± 0.002 * (n = 8) |
| Left ventricular wall area (mm²)| 91.3 ± 3.5 (n = 6) | 88.5 ± 3.2 (n = 6) | 82.6 ± 4.4 (n = 6) | 80.4 ± 3.1 (n = 6) | 85.5 ± 2.3 (n = 6) | 86.2 ± 2.8 (n = 6) |
| Left ventricular lumen area (mm²)| 13.7 ± 1.2 (n = 6) | 14.2 ± 1.3 (n = 6) | 18.8 ± 2.4 (n = 6) | 13.1 ± 1.1 (n = 6) | 13.3 ± 0.9 (n = 6) | 16.7 ± 1.2 (n = 6) |
| Left ventricular total area (mm²)| 105.1 ± 3.4 (n = 6) | 102.7 ± 2.8 (n = 6) | 101.6 ± 5.7 (n = 6) | 93.5 ± 3.6 (n = 6) | 98.8 ± 2.4 (n = 8) | 102.9 ± 3.5 (n = 8) |
| Left ventricular wall thickness (μm)| 4466 ± 159 (n = 6) | 4163 ± 154 (n = 6) | 3970 ± 181 (n = 6) | 3894 ± 140 (n = 6) | 4200 ± 145 (n = 8) | 4184 ± 82 (n = 8) |

*Note: Values are mean ± SEM for fetal and adult offspring of Normoxic (N), Normoxic + Melatonin (NM), Hypoxic (H), Hypoxic + Melatonin (HM), Pair-fed (PF), and Pair-fed + Melatonin (PFM) pregnancy. Significant (p < 0.05) differences are: * vs. N (two-way ANOVA + Tukey Test).
and U46619, without an effect on maximal contraction ($p < 0.01$; Figure 6A,B). The effect on vascular sensitivity of hypoxic pregnancy was independent of effects of hypoxia-induced reductions in maternal food intake because mesenteric vessels isolated from adult offspring of pair-fed pregnancies showed similar constrictor sensitivity to PE (PD$_2$, PF: 5.25 ± 0.08 vs. N: 5.31 ± 0.07; $p > 0.05$) and U46619 (PD$_2$, PF: 7.25 ± 0.12 vs. N: 7.29 ± 0.11; $p > 0.05$) compared to adult offspring of normoxic pregnancy.

Maternal treatment with melatonin in hypoxic pregnancy normalized the constrictor sensitivity to PE and U46619 in mesenteric vessels from adult offspring (Figure 6A,B). In contrast, relative to adult offspring from normoxic pregnancy, maternal treatment with melatonin in normoxic pregnancy (Figure 6A,B) or pair-fed pregnancy (PD$_2$, N: 5.31 ± 0.07 vs. PF: 5.00 ± 0.08; $p > 0.05$) and U46619 (PD$_2$, N: 7.29 ± 0.11 vs. 7.19 ± 0.21; $p > 0.05$) had no effect on vascular sensitivity is mesenteric vessels isolated from adult offspring.

4 | DISCUSSION

The data show that late-onset hypoxia in the last quarter of pregnancy in rats promotes significant fetal growth restriction with evidence of fetal brain sparing, thickening of the aortic walls and a fall in cardiac eNOS protein expression in fetal life, accelerated postnatal catch-up growth and dilated cardiomyopathy with constrictor hyper-reactivity to neurogenic and local agonists in the peripheral vasculature at adulthood. These adverse effects of hypoxic pregnancy on cardiovascular outcomes in the fetus and adult offspring are independent of hypoxia-induced reductions in maternal food intake. Conversely, the effects of hypoxic pregnancy on fetal and postnatal growth are similar than those induced by comparable reductions in maternal food intake in normoxic pregnancy. Maternal treatment with melatonin, in doses lower than those recommended to avoid jet lag, protected against the adverse effects of hypoxic pregnancy on the cardiovascular system of the fetal and adult offspring, but it did not affect alterations in fetal or postnatal growth. An increase in cardiac eNOS expression links a molecular mechanism to the protective effects of maternal melatonin in hypoxic pregnancy. Therefore, the data support the hypothesis tested that maternal treatment with low doses of melatonin will protect the progeny against cardiovascular remodelling and dysfunction programmed by developmental hypoxia.

Chronic hypoxia is a major stimulus for HIF-2α synthesis, regulating the expression of several target genes including erythropoietin or *EPO*, which enhances red blood cell production, and this effect is measured by an increase

![FIGURE 4](https://wileyonlinelibrary.com)
We show that exposure of pregnant dams to 10% O₂ for 5 days in late gestation led to significant increases in maternal and fetal hematocrit levels, suggesting HIF-2α activation. Maternal treatment with melatonin did not affect the magnitude of the maternal or fetal erythropoietic response, suggesting that melatonin does not interfere with HIF-2α activation. Maternal treatment with melatonin did not affect the magnitude of the maternal or fetal erythropoietic response, suggesting that melatonin does not interfere with HIF-2α activation.

Our data show that basal plasma concentrations of melatonin were higher in the pregnant dam compared with non-pregnant rats. We show that exposure of pregnant dams to 10% O₂ for 5 days in late gestation led to significant increases in maternal and fetal hematocrit levels, suggesting HIF-2α activation. Maternal treatment with melatonin did not affect the magnitude of the maternal or fetal erythropoietic response, suggesting that melatonin does not interfere with HIF-2α activation. Maternal treatment with melatonin did not affect the magnitude of the maternal or fetal erythropoietic response, suggesting that melatonin does not interfere with HIF-2α activation.

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with the fetus, and that maternal treatment with melatonin resulted in greater levels of circulating melatonin in the mother than in the fetus. Combined, these data confirm that melatonin crosses the placenta, and that basal circulating melatonin in the fetus is of maternal origin. The dose of melatonin treatment used in this study was based on previous studies showing protective actions of melatonin on fetal development, and it was less than the maximal dose recommended for overcoming jet lag in humans. The resulting concentration of melatonin in maternal plasma following melatonin treatment was similar than that found in other studies using pregnant rats and within the range or lower than those measured in men and women following melatonin intake to avoid jet lag. The cardiovascular data in the present study show that hypoxic pregnancy induced aortic wall thickening without an effect on the aortic walls. Maternal treatment with melatonin in hypoxic pregnancy normalised the cardiovascular remodelling in both fetal and adult life. Aortic wall thickening during development complicated by chronic hypoxia has been reported in rodent models of early-onset hypoxic pregnancy, ovine hypoxic pregnancy and in chicken embryos incubated under isobaric and hypobaric chronic hypoxia conditions. Hypoxia-induced fetal aortic wall thickening is particularly relevant in the clinical setting, as it has also been reported in human infants and older children born from pregnancies complicated by fetal growth restriction, and both fetal aortic wall thickening as well as fetal growth restriction have been associated with an increased risk of hypertension, atherosclerosis, peripheral vascular dysfunction, and coronary heart disease in later life. The mechanisms underlying aortic wall remodelling in hypoxic fetus are poorly understood but include an increase in vessel tension and pressure-induced load.

**FIGURE 6** Peripheral vascular reactivity. Values are mean ± SEM for the concentration-response curves expressed as percentage of the maximal constriction induced by 125 mM potassium, the vascular sensitivity (pD2) and maximal contraction (%Kmax) in response to phenylephrine (PE, A) and the thromboxane mimetic U46619 (B) in mesenteric arteries. Groups are normoxic (N; white symbols and bars), hypoxic (H; black symbols and bars), hypoxic + melatonin (HM; red symbols and bars) and normoxic + melatonin (NM; blue symbols and bars) pregnancies. For the line graphs, a log agonist versus response variable slope plot was used. Significant differences (p <0.05): * vs. N, † vs H (Two-way ANOVA + Tukey test)
It has also been reported that sustained load can overwhelm cardiac wall remodelling, switching compensatory hypertrophic growth to dilated cardiomyopathy, which is preeminent of heart failure.\textsuperscript{71} Therefore, maternal treatment with melatonin by normalising aortic wall tension and thickness may also protect against a dilated cardiomyopathy phenotype in later life.

To explore mechanisms of protection against cardiovascular remodelling in fetal and adult life in hypoxic pregnancy treated with melatonin, we investigated changes in the cardiac expression of proteins associated with oxidative stress and NO signalling, as well as programmed alterations in peripheral vascular reactivity. Data in the present study show that the cardiac protein expression of eNOS was significantly reduced in the fetus but enhanced in the adult offspring of hypoxic pregnancy, without affecting the cardiac protein expression of HSP27, HSP70 or 4-HNE in the fetal or adult periods. Maternal treatment with melatonin not only reversed but enhanced cardiac eNOS protein expression in the hypoxic fetus, normalising its levels of protein expression by adulthood. The cardioprotective effects of NO are well established.\textsuperscript{72} Indeed, NO production by eNOS is of central importance in cardiac protection against ischaemic injury. Cardiac protection is enhanced in animals overexpressing eNOS\textsuperscript{73} and reduced in animals deficient of eNOS.\textsuperscript{74} These findings are consistent with impaired cardiac eNOS protein expression in fetal offspring, and a possible compensatory response in adult offspring of hypoxic pregnancy. It is also in keeping with the effects of melatonin enhancing cardiac eNOS protein expression in the fetus, negating the need for a compensatory response in the adult offspring of treated hypoxic pregnancy.

It is well established that hypoxic pregnancy programmes a vasoconstrictor phenotype in the peripheral vasculature of the adult offspring. For instance, sympathetic control of cardiovascular function is well developed by late incubation in the chicken embryo.\textsuperscript{75} Further, chronic hypoxia stimulates periartrial sympathetic nerve development in the chicken embryo.\textsuperscript{76} In rats, hypoxic pregnancy leads to enhanced femoral vasoconstrictor responses in newborn pups\textsuperscript{77} as well as increased muscle sympathetic nerve activity, sympathetic hyperinnervation, and hypertension in adult progeny.\textsuperscript{78} In sheep, chronic hypoxia during pregnancy enhances femoral vasoconstrictor responses to PE in the fetus,\textsuperscript{79} the intrauterine growth restriction fetus exhibits a greater reliance on $\alpha$-adrenergic activation for blood pressure regulation,\textsuperscript{80} and hypoxic pregnancy programmes femoral vasoconstrictor hyper-reactivity to various agonists in the adult offspring.\textsuperscript{10} Interestingly, the fetal llama, a species adapted to the chronic hypoxia of life at high altitude also shows a greater reliance on $\alpha$-adrenergic mechanisms for the maintenance of arterial blood pressure regulation under basal and stimulated conditions.\textsuperscript{81} Consistent with these findings, data in the present study show that late-onset hypoxic pregnancy in rats also programmes constrictor hyper-reactivity to neurogenic and paracrine agonists in the mesenteric circulation of adult offspring. Further, maternal treatment with melatonin in hypoxic pregnancy prevents this programming effect. The protective actions of melatonin against programmed peripheral vascular dysfunction may be due to its known effects as an anti-adrenergic agent,\textsuperscript{82} as well as its inhibitory effects on thromboxane signalling.\textsuperscript{83,84} Alternatively, we have reported that melatonin treatment in the fetus can increase NO bioavailability in the peripheral circulation, offsetting vasoconstrictor influences.\textsuperscript{85} Persistence of this effect into adulthood could provide another pathway explaining the beneficial actions of melatonin on programmed constrictor hyper-reactivity. Accordingly, Tain et al.\textsuperscript{86} have reported that maternal treatment with melatonin can programme increased NO bioavailability in the adult offspring in several animal models of developmental programming. In turn, effects of melatonin offsetting a peripheral vasoconstrictor phenotype via any of the mechanisms above will protect against cardiovascular remodelling in fetal and adult offspring of hypoxic pregnancy.

In contrast with the beneficial effects on cardiovascular structure and function in offspring of hypoxic pregnancy, maternal treatment with melatonin in the present study did not have an effect on fetal growth restriction or postnatal catch-up growth. Broadly, these findings do not support beneficial effects of melatonin on the offspring of hypoxic pregnancy to be simply due to protective effects at the level of placental perfusion. It is interesting that maternal treatment with melatonin restored fetal growth and birth weight in pair-fed normoxic pregnancies, but not in hypoxic pregnancies. The mechanisms mediating the protective effects of melatonin in undernourished pregnancy include an increase in placental antioxidant enzyme expression.\textsuperscript{27} Therefore, whilst melatonin treatment may protect placental perfusion in undernourished pregnancy by increasing antioxidant defences,\textsuperscript{27} this mechanism of protection against fetal growth restriction is not sufficient in hypoxic pregnancy. Combined, past and present data highlight that the effects on fetal and postnatal growth of hypoxic compared with undernourished pregnancy may be mediated via differential pathways.

In conclusion, we show that maternal treatment with melatonin in low doses confers significant protection against programmed adverse effects on cardiovascular structure and function in offspring of hypoxic pregnancy. Mechanisms underlying this protection by melatonin include stimulatory effects on cardiac eNOS protein expression and offsetting programmed constrictor hyper-reactivity...
in the offspring of hypoxic pregnancy. The data support that maternal treatment with melatonin is a plausible therapy with good human translational potential to protect offspring against cardiovascular disease programmed by chronic fetal hypoxia during complicated pregnancy.

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CONFLICT OF INTERESTS
None to declare.

AUTHOR CONTRIBUTIONS
Dino A. Giussani: contributed to concept/design, data analysis/interpretation and drafting/approval of the manuscript. Hans G. Richter, Jeremy A. Hansell, Emily J. Camm, Emilio A. Herrera, Carlos E. Blanco, Eduardo Villamor, Olga V. Patey, Mitchell C. Lock, Andrew W. Trafford and Gina L. Galli: contributed to data acquisition/interpretation and drafting/approval of the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Hans G. Richter https://orcid.org/0000-0002-7224-9388
Emilio A. Herrera https://orcid.org/0000-0002-6342-085X
Mitchell C. Lock https://orcid.org/0000-0002-3594-1455
Andrew W. Trafford https://orcid.org/0000-0002-2770-445X
Dino A. Giussani https://orcid.org/0000-0002-1308-1204

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher’s website.

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