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ORIGINAL ARTICLE

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

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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% O_2) pregnancy ± melatonin (M) treatment (5 $\mu g \cdot ml^{-1}.day^{-1}$) 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

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further showed enhanced mesenteric vasoconstrictor reactivity to phenylephrine and the thromboxane mimetic U46619. The effects of hypoxic pregnancy on cardiovascular remodelling and function in the fetal and adult offspring were independent 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 pregnancies with melatonin on the offspring cardiovascular system was unremarkable, 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 potential 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

1 | 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 offspring can be programmed by adverse intrauterine conditions.[2] One of the most common suboptimal pregnancy conditions is chronic fetal hypoxia,[3] and it is well established 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 important 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 cardiovascular dysfunction.[6,10,11] However, in such studies, only high doses of vitamin C proved effective.[6,10,11] This is problematic because excess vitamin C can increase the formation of renal calculi[12] 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 therapy with improved human translational potential. Recent studies have focussed either on increasing oxygen delivery, 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 development, given that it is a small and amphipathic indolamine 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 properties[20-22] and NO-dependent cardioprotective effects.[23-25] Melatonin crosses the placenta[26] and protects placental perfusion and fetal growth in preclinical animal models of adverse pregnancy.[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 cardiovascular dysfunction in hypoxic pregnancy has not been investigated.

Therefore, this study tested the hypothesis that maternal 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 molecular 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 hypoxia 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% O_2, 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.[35,36] The chronic hypoxia 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 PO_2 values between 11 and 13 mmHg.[37-40] This is the PO_2 measured by cordocentesis in human fetuses in pregnancy affected by chronic fetal hypoxia and intrauterine growth restriction.[41] Therefore, this model of hypoxic pregnancy is of human clinical relevance.

Maternal exposure to 10% O_2 isobaric hypoxia can decrease maternal food intake.[34,35] 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 $\mu g \cdot ml^{-1}$. 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.[42] 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 $mg \cdot kg^{-1}$) and xylazine (5 $mg \cdot kg^{-1}$) injected intraperitoneally.[35] 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.

2.5 | Studies in the adult offspring at 4 months of age

At birth, all neonates were sexed and weighed, and each litter was reduced to eight pups (four males and four females) to standardize nutritional intake. The male pups were marked and weighed three times a week until weaning at 21 days of age. At weaning, the mother and female weanlings were culled, and tissues were collected for other studies. The remaining male offspring were group-housed, weighed twice a week, and maintained until 16 weeks of age. At 16 weeks of age, male offspring were randomly assigned to organ dissection or the investigation of peripheral vascular reactivity by *in vitro* wire myography. Those males used for organ dissection were weighed and then anaesthetized with inhalational isoflurane and a mixture of ketamine (40 $mg \cdot kg^{-1}$) and xylazine (5 $mg \cdot kg^{-1}$) injected intraperitoneally. Then, they were killed by exsanguination under general anaesthesia. The heart and descending aorta were dissected. The heart was weighed, then the left ventricle + interventricular septum and right ventricular wall were dissected and weighed. The left ventricle was divided into two halves. The bottom half was snap-frozen in liquid nitrogen and stored at −80°C for subsequent protein expression analysis. The top half of the left ventricle and a section of the descending aorta at the level of the apex of the heart were immersion fixed separately in 4% paraformaldehyde for 5 days and then stored in phosphate-buffered saline until they were processed into paraffin blocks for subsequent histological analysis.

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 inter- and intra-assay coefficients of variation were <12%. The melatonin antiserum showed <1% cross-reactivity with N-acetylserotonin, 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 (×1.25 and ×10 and oil immersion ×100), 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 μm 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 ΣP 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; 1:20 000; 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 (1:50 000; Sigma, code A5441). The horseradish peroxidase-conjugated secondary antibodies were from GE Healthcare UK Ltd (anti-rabbit and -mouse). Membranes were re-probed with antibody recognizing β-actin to control for protein loading and to normalize relative levels of protein expression. The optical density of the immunoreactive bands was analysed using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij), and the ratio protein to β-actin was calculated for each sample.

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, $MgSO_4 \cdot 7H_20$ 1.2, Sigma, KH_2PO_4 1.2, Sigma, $NaHCO_3$ 25.0, Sigma, $CaCl_2$ 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% O_2 and 5% CO_2. 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: $IC = \pi * D * 2(D + d)$, 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 α_1-adrenergic agonist phenylephrine (PE; 10^{-9}–10^{-4} mol/L) and the thromboxane mimetic U46619 (10^{-10}–10^{-6} 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). LabChart was used for data acquisition and analysis (LabChart 6.0, Powerlab 8/30; AD Instruments).

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 pD_2 = -log EC_{50}) and maximal response (K_{max}) 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.

3 | 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.[27] 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

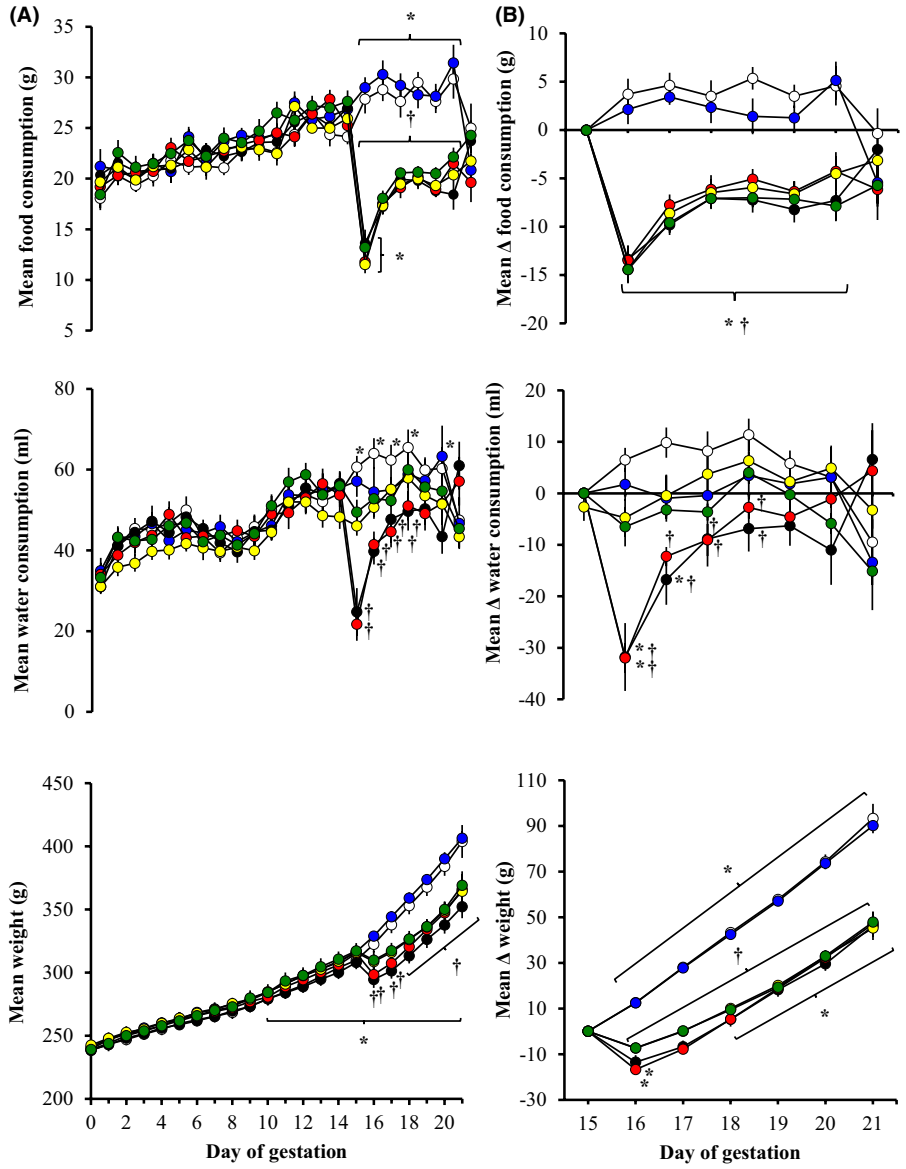

FIGURE 1 Maternal food intake, maternal water consumption and maternal weight gain. Values are mean ± SEM for the absolute values (A) or the change from baseline (B) for maternal food consumption, maternal water consumption and maternal weight gain in normoxic (N; white symbols), hypoxic (H; black symbols), hypoxic + melatonin (HM; red symbols), normoxic + melatonin (NM; blue symbols), pair-fed (PF; yellow symbols) and pair-fed + melatonin (PFM; green symbols) pregnancies ($n = 14$ all groups day 0–20 and $n = 7$ for all groups day 20–22). Significant differences ($p < 0.05$) are: *, vs. baseline expressed as either day 1–5 (A) or day 15 (B); †, vs. normoxic pregnancy (two-way RM ANOVA + post *hoc* Tukey test)

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

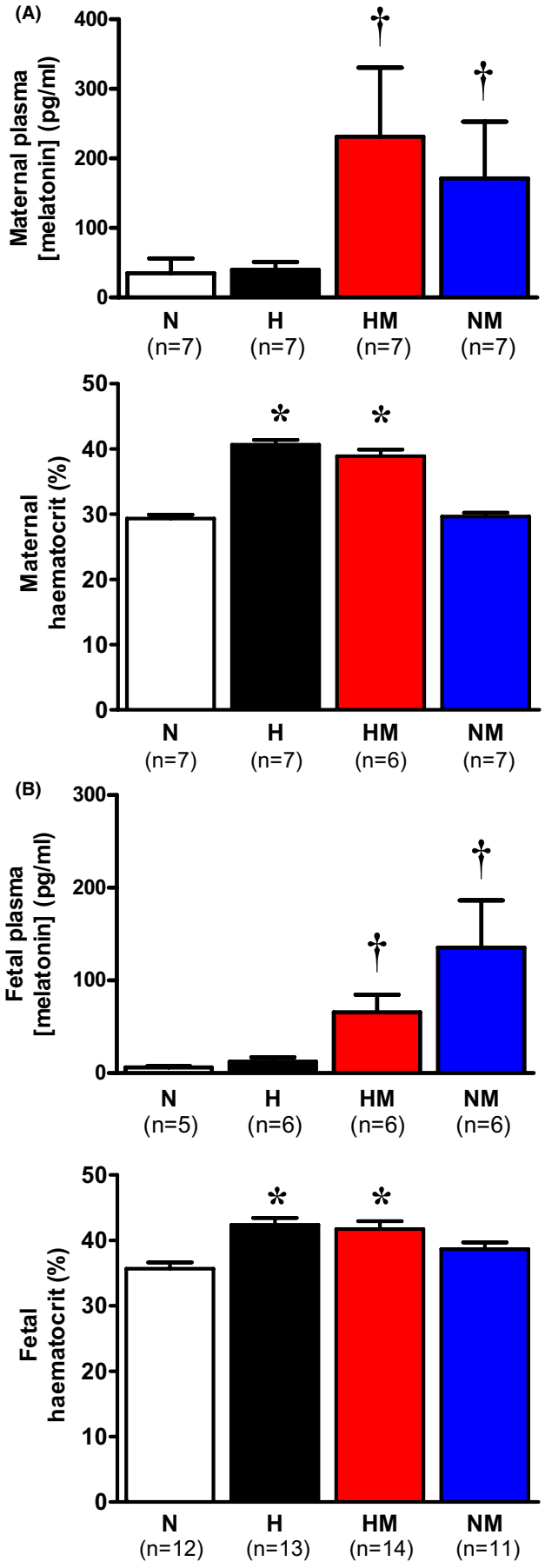

FIGURE 2 Maternal and fetal melatonin and haematocrit. Values are mean ± SEM for maternal (A) and fetal (B) plasma concentration of melatonin and haematocrit in normoxic (N; white bars), hypoxic (H; black bars), hypoxic + melatonin (HM; red bars) and normoxic + melatonin (NM; blue bars) pregnancies at gestational day 20. Significant differences ($p < 0.05$) are: *effect of hypoxia; † effect of melatonin (Two-way ANOVA)

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 (PF fetal aortic wall:llumen area = 0.56 ± 0.11; PF fetal aortic lumen area as a % of total vessel area = 70.5 ± 8.6; PF fetal aortic wall area as a % of total vessel area = 30.8 ± 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 (PF adult cardiac wall:llumen area = 7.2 ± 1.5; PF adult left ventricular lumen area as a % of total heart area = 15.7 ± 1.4; PF left ventricular wall area as a % of total heart area = 85.5 ± 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).

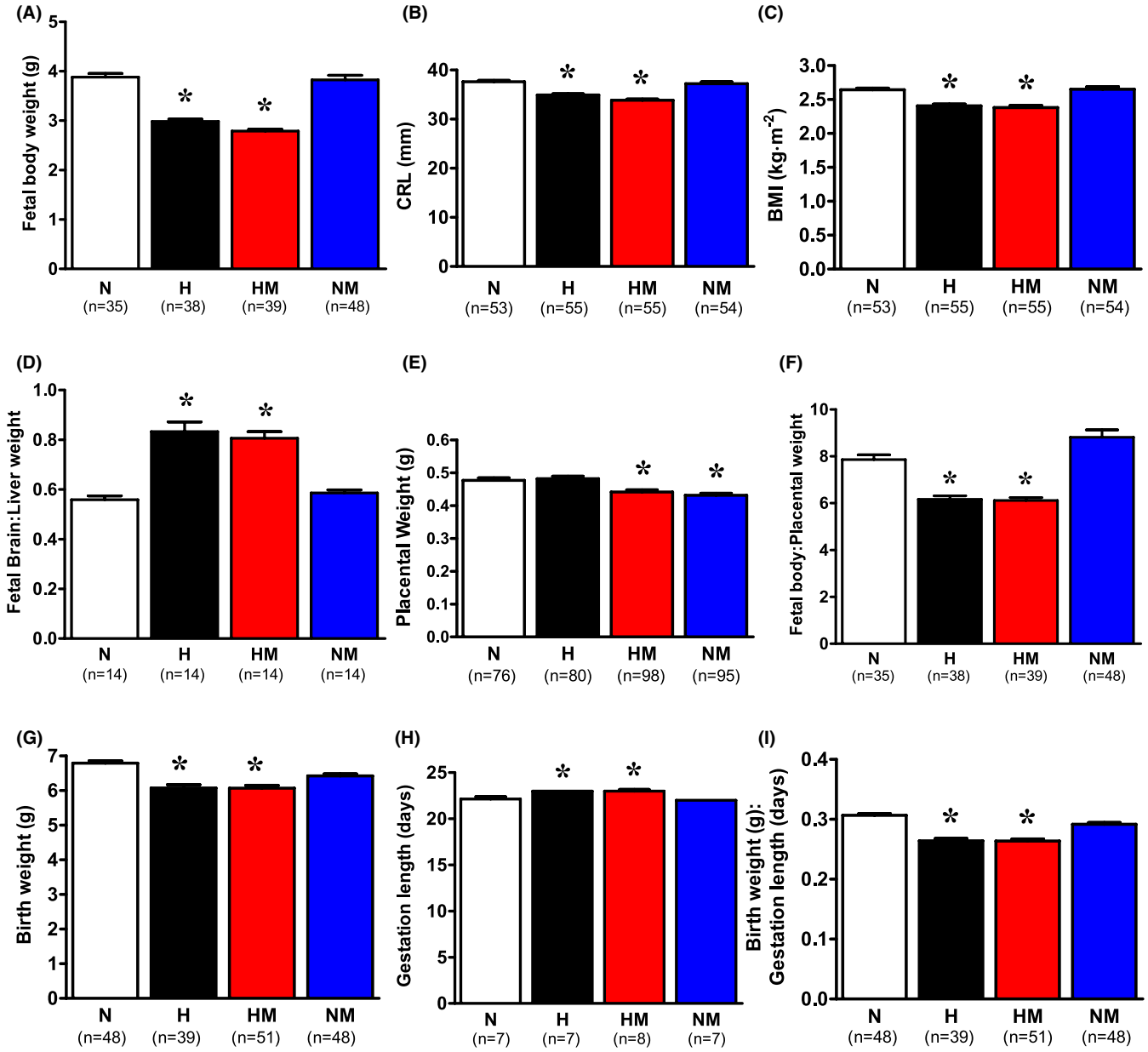

FIGURE 3 Pregnancy characteristics. Values are mean ± SEM for fetal bodyweight (A), fetal crown-rump length, CRL (B), the fetal body mass index, BMI (C), the fetal brain to liver weight ratio (D), the placental weight (E), the fetal to placental weight ratio (F), the birth weight (G), the gestational 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.[87] Significant differences ($p < 0.05$) are: *effect of hypoxia

TABLE 1 Postnatal growth

		N	NM	H	HM	PF	PFM
Postnatal offspring	Fractional growth rate 0–18 weeks of age	0.711 ± 0.004 (n = 8)	0.733 ± 0.004 (n = 8)	0.791 ± 0.008 * (n = 8)	0.710 ± 0.003 (n = 8)	0.810 ± 0.004 * (n = 8)	0.710 ± 0.006 (n = 8)
	Fractional growth rate 0–3 weeks of age	0.415 ± 0.005 (n = 8)	0.410 ± 0.006 (n = 8)	0.441 ± 0.009 * (n = 8)	0.425 ± 0.009 (n = 8)	0.443 ± 0.006 * (n = 8)	0.413 ± 0.007 (n = 8)
	Fractional growth rate 3–8 weeks of age	0.118 ± 0.002 (n = 8)	0.123 ± 0.002 (n = 8)	0.118 ± 0.003 (n = 8)	0.113 ± 0.001 (n = 8)	0.130 ± 0.002 * (n = 8)	0.121 ± 0.002 (n = 8)
	Fractional growth rate 8–16 weeks of age	0.011 ± 0.001 (n = 8)	0.011 ± 0.001 (n = 8)	0.011 ± 0.001 (n = 8)	0.012 ± 0.001 (n = 8)	0.011 ± 0.001 (n = 8)	0.012 ± 0.001 (n = 8)

Note: Values are mean ± SEM for the overall fractional growth rate between (0–18 weeks of age) and for the partial fractional growth rates between 0–3 weeks (weaning), 3–8 weeks and 8–16 weeks in 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).

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 ± 0.34 vs. 1.00 ± 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 ± 0.03 vs. 1.00 ± 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+ 125mM (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 (mm^2)	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 (mm^2)	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 (mm^2)	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 ($g{\cdot}g^{-1}$)	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 (mm^2)	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 (mm^2)	0.637 ± 0.121 ($n = 6$)	0.911 ± 0.194 ($n = 6$)	0.812 ± 0.140 ($n = 6$)	0.634 ± 0.078 ($n = 6$)	0.604 ± 0.149 ($n = 6$)	1.140 ± 0.119 ($n = 6$)
	Left ventricular wall thickness (mm)	0.740 ± 0.037 ($n = 6$)	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 (mm^2)	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 (mm^2)	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 (mm^2)	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 (mm^2)	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 ($n = 8$)	86.2 ± 2.8 ($n = 8$)
	Left ventricular lumen area (mm^2)	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 ($n = 8$)	16.7 ± 1.2 ($n = 8$)
	Left ventricular total area (mm^2)	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).

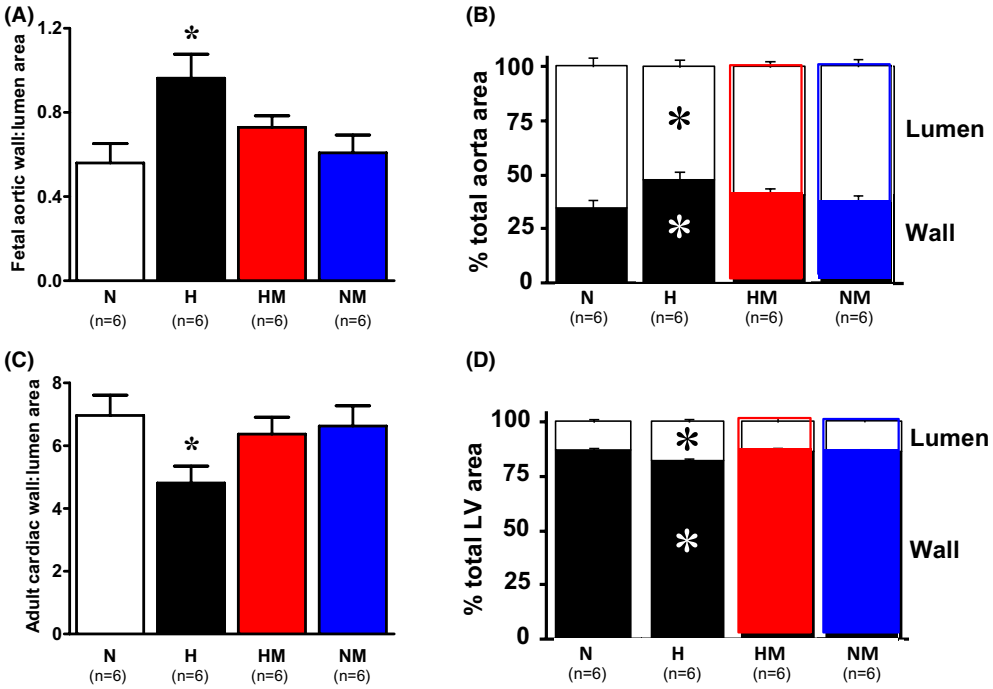

FIGURE 4 Fetal and adult offspring cardiovascular remodelling. Values are the mean ± SEM for the aorta wall to lumen area ratio and the aorta wall and lumen areas expressed as a percentage of total vessel area in fetal offspring (A, B), and the left ventricular (LV) wall to lumen area ratio and the LV wall and lumen areas expressed as a percentage of total LV area in adult offspring (C, D) from normoxic (N; white bars), hypoxic (H; black bars), hypoxic + melatonin (HM; red bars) and normoxic + melatonin (NM; blue bars) pregnancies. There was a significant interaction between hypoxia and melatonin main effects for A-D. Post-hoc significant ($p < 0.05$) differences: *H vs. all (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

TABLE 3 Fetal and adult offspring cardiac protein expression

		N	NM	H	HM	PF	PFM
Fetal hearts (20 days of gestation)	HSP27 (relative to control)	1.00 ± 0.08 ($n = 6$)	1.08 ± 0.20 ($n = 6$)	0.99 ± 0.08 ($n = 6$)	1.02 ± 0.09 ($n = 6$)	1.19 ± 0.15 ($n = 6$)	1.54 ± 0.30 ($n = 6$)
	HSP70 (relative to control)	1.00 ± 0.09 ($n = 6$)	1.06 ± 0.10 ($n = 6$)	0.78 ± 0.11 ($n = 6$)	0.98 ± 0.08 ($n = 6$)	0.87 ± 0.20 ($n = 6$)	1.23 ± 0.12 ($n = 6$)
	4-HNE (relative to control)	1.00 ± 0.11 ($n = 6$)	1.04 ± 0.10 ($n = 6$)	0.76 ± 0.09 ($n = 6$)	0.75 ± 0.06 ($n = 6$)	0.62 ± 0.06 ($n = 6$)	0.88 ± 0.15 ($n = 6$)
Adult offspring hearts (4 months old)	HSP27 (relative to control)	1.00 ± 0.15 ($n = 6$)	1.01 ± 0.01 ($n = 6$)	0.87 ± 0.01 ($n = 6$)	1.01 ± 0.01 ($n = 6$)	0.91 ± 0.03 ($n = 6$)	0.85 ± 0.01 ($n = 6$)
	HSP70 (relative to control)	1.00 ± 0.13 ($n = 6$)	0.94 ± 0.05 ($n = 6$)	0.96 ± 0.12 ($n = 6$)	0.86 ± 0.08 ($n = 6$)	1.24 ± 0.08 ($n = 6$)	1.22 ± 0.16 ($n = 6$)
	4-HNE (relative to control)	1.00 ± 0.11 ($n = 6$)	1.10 ± 0.09 ($n = 6$)	1.08 ± 0.06 ($n = 6$)	1.07 ± 0.02 ($n = 6$)	1.02 ± 0.03 ($n = 6$)	0.97 ± 0.02 ($n = 6$)

Note: Values are mean + SEM for protein expression relative to control of HSP27, HSP70 and HNE in fetal and adult rat offspring hearts from 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).

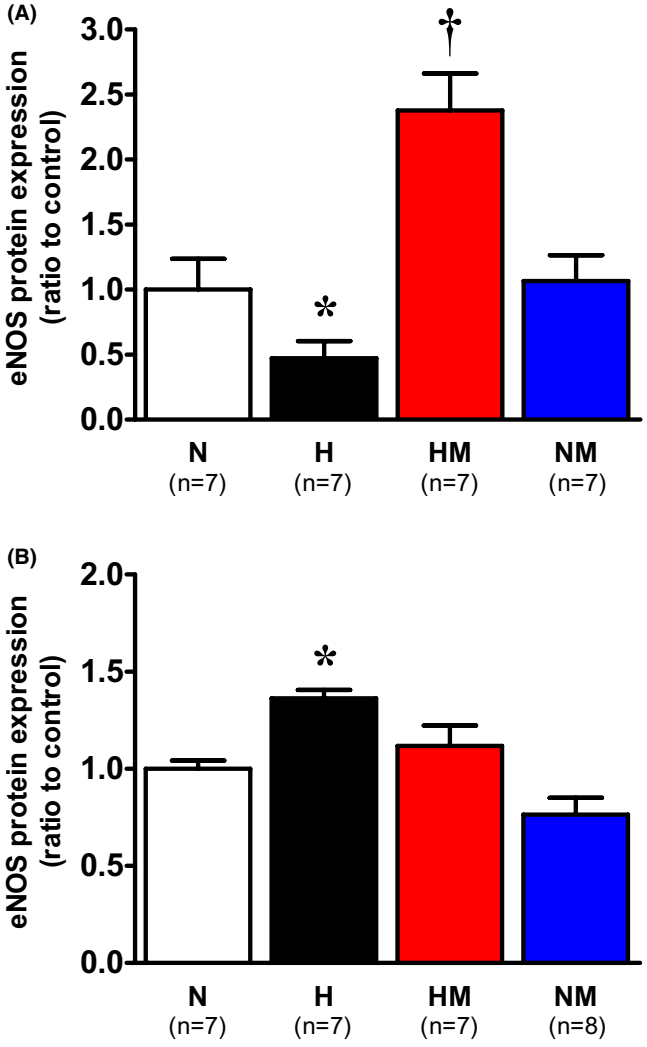

FIGURE 5 Cardiac protein expression of eNOS. Values are mean ± SEM for the protein expression relative to control for eNOS in hearts of fetal (A) and adult (B) offspring from normoxic (N; white bars), hypoxic (H; black bars), hypoxic + melatonin (HM; red bars) and normoxic + melatonin (NM; blue bars) pregnancies. There was a significant interaction between hypoxia and melatonin main effects for A and B. Post-hoc significant ($p < 0.05$) differences: *H vs. N; †HM vs. all; (Two-way ANOVA + Tukey test). Representative Coomassie blue, anti-eNOS and anti-βactin staining images are included in Figure S1

in packed red cell volume.[53] We show that exposure of pregnant dams to 10% O_2 for 5 days in late gestation led to significant increases in maternal and fetal haematocrit 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 in either mother or fetus, and therefore, both treated and untreated pregnancies were exposed to the same degree of hypoxia.

Our data show that basal plasma concentrations of melatonin were higher in the pregnant dam compared

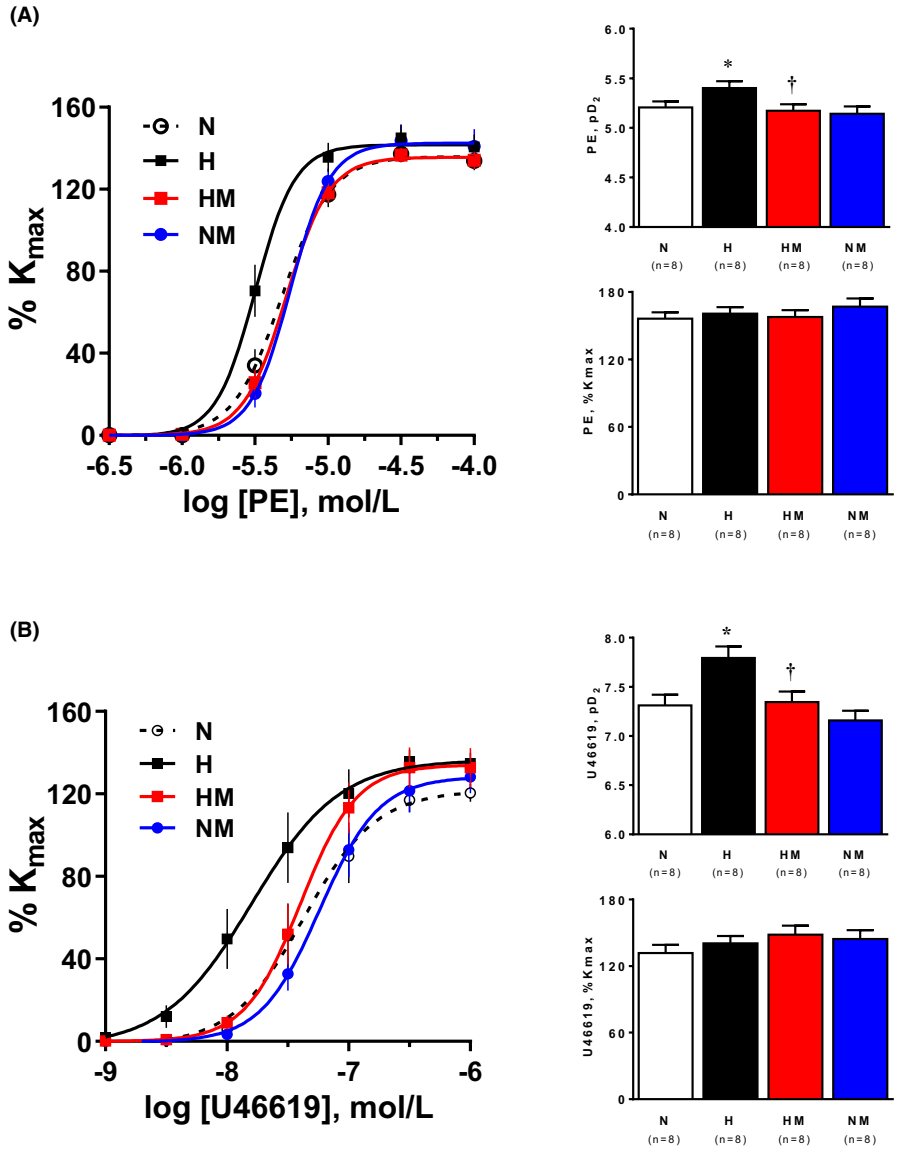

FIGURE 6 Peripheral vascular reactivity. Values are mean ± SEM for the concentration-response curves expressed as percentage of the maximal constriction induced by 125 mM potassium, the vascular sensitivity (pD2) and maximal contraction (%Kmax) in response to phenylephrine (PE, A) and the thromboxane 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)

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.[26,54,55] The dose of melatonin treatment used in this study was based on previous studies showing protective actions of melatonin on fetal development,[27] and it was less than the maximal dose recommended for overcoming jet lag in humans.[42] The resulting concentration of melatonin in maternal plasma following melatonin treatment was similar than that found in other studies using pregnant rats,[56,57] and within the range or lower than those measured in men and women following melatonin intake to avoid jet lag.[58]

The cardiovascular data in the present study show that hypoxic pregnancy induced aortic wall thickening without affecting the cardiac walls in fetal life. Conversely, adult offspring of hypoxic pregnancy showed cardiac wall thinning 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,[4,6,9] ovine hypoxic pregnancy[59] and in chicken embryos incubated under isobaric[60] and hypobaric[61] 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,[62-64] 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.[65-67] The mechanisms underlying aortic wall remodelling in hypoxic fetus are poorly understood but include an increase in vessel tension and pressure-induced load.[68-70]

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.[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.[72] Indeed, NO production by eNOS is of central importance in cardiac protection against ischaemic injury. Cardiac protection is enhanced in animals overexpressing eNOS[73] and reduced in animals deficient of eNOS.[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.[75] Further, chronic hypoxia stimulates periarterial sympathetic nerve development in the chicken embryo.[76] In rats, hypoxic pregnancy leads to enhanced femoral vasoconstrictor responses in newborn pups[77] as well as increased muscle sympathetic nerve activity, sympathetic hyperinnervation, and hypertension in adult progeny.[78] In sheep, chronic hypoxia during pregnancy enhances femoral vasoconstrictor responses to PE in the fetus,[79] the intrauterine growth restriction fetus exhibits a greater reliance on α-adrenergic activation for blood pressure regulation,[80] and hypoxic pregnancy programmes femoral vasoconstrictor hyper-reactivity to various agonists in the adult offspring.[10] Interestingly, the fetal llama, a species adapted to the chronic hypoxia of life at high altitude also shows a greater reliance on α-adrenergic mechanisms for the maintenance of arterial blood pressure regulation under basal and stimulated conditions.[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,[82] as well as its inhibitory effects on thromboxane signalling.[83,84] Alternatively, we have reported that melatonin treatment in the fetus can increase NO bioavailability in the peripheral circulation, offsetting vasoconstrictor influences.[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.[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.[27] Therefore, whilst melatonin treatment may protect placental perfusion in undernourished pregnancy by increasing antioxidant defences,[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.

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REFERENCES

1. Gluckman PD, Hanson MA, Cooper C, et al. Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med*. 2008;359(1):61-73.
2. Fowden AL, Giussani DA, Forhead AJ. Intrauterine programming of physiological systems: causes and consequences. *Physiology*. 2006;21:29-37.
3. Giussani DA. The fetal brain sparing response to hypoxia: physiological mechanisms. *J Physiol*. 2016;594(5):1215-1230.
4. Giussani DA, Davidge ST. Developmental programming of cardiovascular disease by prenatal hypoxia. *J Dev Orig Health Dis*. 2013;4(5):328-337.
5. Darby JRT, Varcoe TJ, Orgeig S, et al. Cardiorespiratory consequences of intrauterine growth restriction: Influence of timing, severity and duration of hypoxaemia. *Theriogenology*. 2020;150:84-95.
6. Giussani DA, Camm EJ, Niu Y, et al. Developmental programming of cardiovascular dysfunction by prenatal hypoxia and oxidative stress. *PLoS One*. 2012;7(2):e31017.
7. Patterson AJ, Xiao D, Xiong F, et al. Hypoxia-derived oxidative stress mediates epigenetic repression of PKCε gene in foetal rat hearts. *Cardiovasc Res*. 2012;93(2):302-310.
8. Al-Hasan YM, Evans LC, Pinkas GA, et al. Chronic hypoxia impairs cytochrome oxidase activity via oxidative stress in selected fetal Guinea pig organs. *Reprod Sci*. 2013;20(3):299-307.
9. Giussani DA, Niu Y, Herrera EA, et al. Heart disease link to fetal hypoxia and oxidative stress. *Adv Exp Med Biol*. 2014;814:77-87.
10. Brain KL, Allison BJ, Niu Y, et al. Intervention against hypertension in the next generation programmed by developmental hypoxia. *PLoS Biol*. 2019;17(1):e2006552.
11. Kane AD, Herrera EA, Camm EJ, et al. Vitamin C prevents intrauterine programming of in vivo cardiovascular dysfunction in the rat. *Circ J*. 2013;77(10):2604-2611.
12. Thomas LD, Elinder CG, Tiselius HG, et al. Ascorbic acid supplements and kidney stone incidence among men: a prospective study. *JAMA Intern Med*. 2013;173(5):386-388.
13. Poston L, Briley AL, Seed PT, et al. Vitamins in Pre-eclampsia (VIP) Trial Consortium. Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebo-controlled trial. *Lancet*. 2006;367(9517):1145-1154.
14. Darby JRT, Saini BS, Soo JY, et al. Subcutaneous maternal resveratrol treatment increases uterine artery blood flow in the pregnant ewe and increases fetal but not cardiac growth. *J Physiol*. 2019;597(20):5063-5077.
15. Darby JRT, Mohd Dollah MHB, Regnault TRH, et al. Systematic review: Impact of resveratrol exposure during pregnancy on maternal and fetal outcomes in animal models of human pregnancy complications-Are we ready for the clinic? *Pharmacol Res*. 2019;144:264-278.
16. Spiroski AM, Niu Y, Nicholas LM, et al. Mitochondria antioxidant protection against cardiovascular dysfunction programmed by early-onset gestational hypoxia. *FASEB J*. 2021;35(5):e21446.
17. Itani N, Skeffington KL, Beck C, et al. Protective effects of pravastatin on the embryonic cardiovascular system during hypoxic development. *FASEB J*. 2020;34(12):16504-16515.
18. Botting KJ, Skeffington KL, Niu Y, et al. Translatable mitochondria-targeted protection against programmed cardiovascular dysfunction. *Sci Adv*. 2020;6(34):eabb1929.
19. Marseglia L, D'Angelo G, Manti S, et al. Potential utility of melatonin in preeclampsia, intrauterine fetal growth retardation, and perinatal asphyxia. *Reprod Sci*. 2016;23(8):970-977.
20. Reiter RJ, Tan DX, Fuentes-Broto L. Melatonin: a multitasking molecule. *Prog Brain Res*. 2010;181:127-151.
21. Rodriguez C, Mayo JC, Sainz RM, et al. Regulation of antioxidant enzymes: a significant role for melatonin. *J Pineal Res*. 2004;36(1):1-9.
22. Gonzalez-Candia A, Veliz M, Carrasco-Pozo C, et al. Antenatal melatonin modulates an enhanced antioxidant/pro-oxidant

ratio in pulmonary hypertensive newborn sheep. *Redox Biol*. 2019;22:101128.
23. Genade S, Genis A, Ytrehus K, et al. Melatonin receptor-mediated protection against myocardial ischaemia/reperfusion injury: role of its anti-adrenergic actions. *J Pineal Res*. 2008;45(4):449-458.
24. Tain YL, Huang LT, Lin IC, et al. Melatonin prevents hypertension and increased asymmetric dimethylarginine in young spontaneous hypertensive rats. *J Pineal Res*. 2010;49(4):390-398.
25. Tain YL, Huang LT, Hsu CN, et al. Melatonin therapy prevents programmed hypertension and nitric oxide deficiency in offspring exposed to maternal caloric restriction. *Oxid Med Cell Longev*. 2014;2014:283180.
26. Serón-Ferré M, Mendez N, Abarzua-Catalan L, et al. Circadian rhythms in the fetus. *Mol Cell Endocrinol*. 2012;349(1):68-75.
27. Richter HG, Hansell JA, Raut S, et al. Melatonin improves placental efficiency and birth weight and increases the placental expression of antioxidant enzymes in undernourished pregnancy. *J Pineal Res*. 2009;46:357-364.
28. Lemley CO, Meyer AM, Camacho LE, et al. Melatonin supplementation alters uteroplacental hemodynamics and fetal development in an ovine model of intrauterine growth restriction. *Am J Physiol Regul Integr Comp Physiol*. 2012;302(4):R454-R467.
29. Chen YC, Sheen JM, Tiao MM, et al. Roles of melatonin in fetal programming in compromised pregnancies. *Int J Mol Sci*. 2013;14(3):5380-5401.
30. Alers NO, Jenkin G, Miller SL, et al. Antenatal melatonin as an antioxidant in human pregnancies complicated by fetal growth restriction–a phase I pilot clinical trial: study protocol. *BMJ Open*. 2013;3(12):e004141.
31. Tare M, Parkington HC, Wallace EM, et al. Maternal melatonin administration mitigates coronary stiffness and endothelial dysfunction, and improves heart resilience to insult in growth restricted lambs. *J Physiol*. 2014;592(12):2695-2709.
32. Van Geijn HP, Kaylor WM Jr, Nicola KR, et al. Induction of severe intrauterine growth retardation in the Sprague-Dawley rat. *Am J Obstet Gynecol*. 1980;137(1):43-47.
33. de Grauw TJ, Myers RE, Scott WJ. Fetal growth retardation in rats from different levels of hypoxia. *Biol Neonate*. 1986;49(2):85-89.
34. Williams SJ, Hemmings DG, Mitchell JM, et al. Effects of maternal hypoxia or nutrient restriction during pregnancy on endothelial function in adult male rat offspring. *J Physiol*. 2005;565(Pt 1):125-135.
35. Camm EJ, Hansell JA, Kane AD, et al. Partial contributions of developmental hypoxia and undernutrition to prenatal alterations in somatic growth and cardiovascular structure and function. *Am J Obstet Gynecol*. 2010;203(5):495.e24-34.
36. Allison BJ, Kaandorp JJ, Kane AD, et al. Divergence of mechanistic pathways mediating cardiovascular aging and developmental programming of cardiovascular disease. *FASEB J*. 2016;30(5):1968-1975.
37. Fletcher AJ, Goodfellow MR, Forhead AJ, et al. Low doses of dexamethasone suppress pituitary-adrenal function but augment the glycemic response to acute hypoxemia in fetal sheep during late gestation. *Pediatr Res*. 2000;47(5):684-691.
38. Fletcher AJ, Gardner DS, Edwards CM, et al. Cardiovascular and endocrine responses to acute hypoxaemia during and following dexamethasone infusion in the ovine fetus. *J Physiol*. 2003;549(Pt 1):271-287.
39. Thakor AS, Richter HG, Kane AD, et al. Redox modulation of the fetal cardiovascular defence to hypoxaemia. *J Physiol*. 2010;588(Pt 21):4235-4247.
40. Fletcher AJ, Edwards CM, Gardner DS, et al. Neuropeptide Y in the sheep fetus: effects of acute hypoxemia and dexamethasone during late gestation. *Endocrinology*. 2000;141(11):3976-3982.
41. Hecher K, Snijders R, Campbell S, et al. Fetal venous, intracardiac, and arterial blood flow measurements in intrauterine growth retardation: relationship with fetal blood gases. *Am J Obstet Gynecol*. 1995;173(1):10-15.
42. Herxheimer A, Petrie KJ. Melatonin for the prevention and treatment of jet lag. *Cochrane Database Syst Rev* 2002;2:CD001520.
43. Adler A, Camm EJ, Hansell JA, et al. Investigation of the use of antioxidants to diminish the adverse effects of postnatal glucocorticoid treatment on mortality and cardiac development. *Neonatology*. 2010;98(1):73-83.
44. Herrera EA, Camm EJ, Cross CM, et al. Morphological and functional alterations in the aorta of the chronically hypoxic fetal rat. *J Vasc Res*. 2012;49(1):50-58.
45. Itani N, Skeffington KL, Beck C, et al. Melatonin rescues cardiovascular dysfunction during hypoxic development in the chick embryo. *J Pineal Res*. 2016;60(1):16-26.
46. Gundersen HJ, Bendtsen TF, Korbo L, et al. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *APMIS*. 1988;96:379-394.
47. Burton GJ, Palmer ME. Eradicating fetomaternal fluid shift during perfusion fixation of the human placenta. *Placenta*. 1988;9:327-332.
48. Richter HG, Camm EJ, Modi BN, et al. Ascorbate prevents placental oxidative stress and enhances birth weight in hypoxic pregnancy in rats. *J Physiol*. 2012;590:1377-1387.
49. Herrera EA, Verkerk MM, Derks JB, et al. Antioxidant treatment alters peripheral vascular dysfunction induced by postnatal glucocorticoid therapy in rats. *PLoS One*. 2010;5:e9250.
50. Skeffington KL, Higgins JS, Mahmoud AD, et al. Hypoxia, AMPK activation and uterine artery vasoreactivity. *J Physiol*. 2016;594(5):1357-1369.
51. Herrera EA, Pulgar VM, Riquelme RA, et al. High altitude chronic hypoxia during gestation and after birth modifies cardiovascular responses in newborn sheep. *Am J Physiol Regul Integr Comp Physiol*. 2007;292:R2234-2240.
52. Zar JH. Data transformations. In: Kurts B, ed. *Biostatistical Analysis*. Prentice-Hall Inc; 1984:236-243.
53. Semenza GL. Hypoxia-inducible factor 1 and cardiovascular disease. *Annu Rev Physiol*. 2014;76:39-56.
54. Yellon SM, Longo LD. Effect of maternal pinealectomy and reverse photoperiod on the circadian melatonin rhythm in the sheep and fetus during the last trimester of pregnancy. *Biol Reprod*. 1988;39(5):1093-1099.
55. McMillen IC, Nowak R. Maternal pinealectomy abolishes the diurnal rhythm in plasma melatonin concentrations in the fetal sheep and pregnant ewe during late gestation. *J Endocrinol*. 1989;120(3):459-464.
56. Okatani Y, Wakatsuki A, Kaneda C. Melatonin increases activities of glutathione peroxidase and superoxide dismutase in fetal rat brain. *J Pineal Res*. 2000;28(2):89-96.
57. Tamura H, Takayama H, Nakamura Y, et al. Fetal/placental regulation of maternal melatonin in rats. *J Pineal Res*. 2008;44(3):335-340.

58. Deacon S, Arendt J. Melatonin-induced temperature suppression and its acute phase-shifting effects correlate in a dose-dependent manner in humans. *Brain Res*. 1995;688(1–2):77-85.
59. Thompson JA, Richardson BS, Gagnon R, et al. Chronic intrauterine hypoxia interferes with aortic development in the late gestation ovine fetus. *J Physiol*. 2011;589(Pt 13):3319-3332.
60. Rouwet EV, Tintu AN, Schellings MW, et al. Hypoxia induces aortic hypertrophic growth, left ventricular dysfunction, and sympathetic hyperinnervation of peripheral arteries in the chick embryo. *Circulation*. 2002;105(23):2791-2796.
61. Salinas CE, Blanco CE, Villena M, et al. Cardiac and vascular disease prior to hatching in chick embryos incubated at high altitude. *J Dev Orig Health Dis*. 2010;1(1):60-66.
62. Skilton MR, Evans N, Griffiths KA, et al. Aortic wall thickness in newborns with intrauterine growth restriction. *Lancet*. 2005;365(9469):1484-1486.
63. Akira M, Yoshiyuki S. Placental circulation, fetal growth, and stiffness of the abdominal aorta in newborn infants. *J Pediatr*. 2006;148(1):49-53.
64. Koklu E, Kurtoglu S, Akcakus M, et al. Increased aortic intima-media thickness is related to lipid profile in newborns with intrauterine growth restriction. *Horm Res*. 2006;65(6):269-275.
65. Arnett DK, Evans GW, Riley WA. Arterial stiffness: a new cardiovascular risk factor? *Am J Epidemiol*. 1994;140(8):669-682.
66. Leeson CP, Kattenhorn M, Morley R, et al. Impact of low birth weight and cardiovascular risk factors on endothelial function in early adult life. *Circulation*. 2001;103(9):1264-1268.
67. Zanardo V, Visentin S, Trevisanuto D, et al. Fetal aortic wall thickness: a marker of hypertension in IUGR children? *Hypertens Res*. 2013;36(5):440-443.
68. Cox RH. Basis for the altered arterial wall mechanics in the spontaneously hypertensive rat. *Hypertension*. 1981;3(4):485-495.
69. Owens GK, Rabinovitch PS, Schwartz SM. Smooth muscle cell hypertrophy versus hyperplasia in hypertension. *Proc Natl Acad Sci U S A*. 1981;78(12):7759-7763.
70. Huang Y, Guo X, Kassab GS. Axial nonuniformity of geometric and mechanical properties of mouse aorta is increased during postnatal growth. *Am J Physiol Heart Circ Physiol*. 2006;290(2):H657-664.
71. Katz AM. Cardiomyopathy of overload. A major determinant of prognosis in congestive heart failure. *N Engl J Med*. 1990;322(2):100-110.
72. Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. *J Mol Cell Cardiol*. 2006;40(1):16-23.
73. Jones SP, Greer JJ, Kakkar AK, et al. Endothelial nitric oxide synthase overexpression attenuates myocardial reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2004;286(1):H276-282.
74. Sharp BR, Jones SP, Rimmer DM, et al. Differential response to myocardial reperfusion injury in eNOS-deficient mice. *Am J Physiol Heart Circ Physiol*. 2002;282(6):H2422-2426.
75. Mulder AL, Miedema A, De Mey JG, et al. Sympathetic control of the cardiovascular response to acute hypoxemia in the chick embryo. *Am J Physiol Regul Integr Comp Physiol*. 2002;282(4):R1156-R1163.
76. Ruijtenbeek K, le Noble FA, Janssen GM, et al. Chronic hypoxia stimulates periarterial sympathetic nerve development in chicken embryo. *Circulation*. 2000;102(23):2892-2897.
77. Williams SJ, Campbell ME, McMillen IC, et al. Differential effects of maternal hypoxia or nutrient restriction on carotid and femoral vascular function in neonatal rats. *Am J Physiol Regul Integr Comp Physiol*. 2005;288(2):R360-367.
78. Rook W, Johnson CD, Coney AM, et al. Prenatal hypoxia leads to increased muscle sympathetic nerve activity, sympathetic hyperinnervation, premature blunting of neuropeptide Y signaling, and hypertension in adult life. *Hypertension*. 2014;64(6):1321-1327.
79. Kim YH, Veille JC, Cho MK, et al. Chronic hypoxia alters vasoconstrictive responses of femoral artery in the fetal sheep. *J Korean Med Sci*. 2005;20(1):13-19.
80. Darby JRT, Varcoe TJ, Holman SL, et al. The reliance on α-adrenergic receptor stimuli for blood pressure regulation in the chronically hypoxaemic fetus is not dependent on postganglionic activation. *J Physiol*. 2021;599(4):1307-1318.
81. Giussani DA, Riquelme RA, Sanhueza EM, et al. Adrenergic and vasopressinergic contributions to the cardiovascular response to acute hypoxaemia in the llama fetus. *J Physiol*. 1999;515(Pt 1):233-241.
82. Paulis L, Simko F. Blood pressure modulation and cardiovascular protection by melatonin: potential mechanisms behind. *Physiol Res*. 2007;56(6):671-684.
83. Leach CM, Thorburn GD. A comparison of the inhibitory effects of melatonin and indomethacin on platelet aggregation and thromboxane release. *Prostaglandins*. 1980;20(1):51-56.
84. Vacas MI, Del Zar MM, Martinuzzo M, et al. Inhibition of human platelet aggregation and thromboxane B2 production by melatonin. Correlation with plasma melatonin levels. *J Pineal Res*. 1991;11(3–4):135-139.
85. Thakor AS, Allison BJ, Niu Y, et al. Melatonin modulates the fetal cardiovascular defense response to acute hypoxia. *J Pineal Res*. 2015;59(1):80-90.
86. Tain YL, Huang LT, Hsu CN. Developmental programming of adult disease: reprogramming by melatonin? *Int J Mol Sci*. 2017;18(2):426.
87. West BT, Welch KB, Linear GAT. *Mixed Models: A Practical Guide Using Statistical Software*. 2nd ed. CRC Press, Taylor & Francis Group, Chapman & Hall; 2014:440 p.

SUPPORTING INFORMATION

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

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