Perinatal iron deficiency and a high salt diet cause long-term kidney mitochondrial dysfunction and oxidative stress

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Aims

Perinatal iron deficiency (ID) alters developmental trajectories of offspring, predisposing them to cardiovascular dysfunction in later life. The mechanisms underlying this long-term programming of renal function have not been defined. We hypothesized perinatal ID causes hypertension and alters kidney metabolic function and morphology in a sex-dependent manner in adult offspring. Furthermore, we hypothesized these effects are exacerbated by chronic consumption of a high salt diet.

Methods and results

Pregnant Sprague Dawley rats were fed either an iron-restricted or replete diet prior to and throughout pregnancy. Adult offspring were fed normal or high salt diets for 6 weeks prior to experimentation at 6 months of age. Blood pressure (BP) was assessed via indwelling catheters in anaesthetized offspring; kidney mitochondrial function was assessed via high-resolution respirometry; reactive oxygen species and nitric oxide were quantified via fluorescence microscopy. Adult males, but not females, exhibited increased systolic BP due to ID ($P=0.01$) and high salt intake ($P=0.02$). In males, but not in females, medullary mitochondrial content was increased by high salt ($P=0.003$), while succinate-dependent respiration was reduced by ID ($P<0.05$). The combination of perinatal ID and high salt reduced complex IV activity in the cortex of males ($P=0.01$). Perinatal ID increased cytosolic superoxide generation ($P<0.001$) concomitant with reduced nitric oxide bioavailability ($P<0.001$) in male offspring, while high salt increased mitochondrial superoxide in the medulla ($P=0.04$) and cytosolic superoxide within the cortex ($P=0.01$). Male offspring exhibited glomerular basement membrane thickening ($P<0.05$), increased collagen deposition ($P<0.05$), and glomerular hypertrophy (interaction, $P=0.02$) due to both perinatal ID and high salt. Female offspring exhibited no alterations in mitochondrial function or morphology due to either high salt or ID.

Conclusion

Perinatal ID causes long-term sex-dependent alterations in renal metabolic function and morphology, potentially contributing to hypertension and increased cardiovascular disease risk.

Keywords

Anaemia • Developmental programming • Hypertension • Mitochondria • Nitric oxide
• Pregnancy • Renal

1. Introduction

Cardiovascular disease (CVD) is a leading cause of mortality, responsible for approximately a third of all deaths worldwide.¹ The aetiology of CVD is complex, and the developmental origins of health

and disease (DOHaD) hypothesis has identified new risk factors, which are dictated by the quality of the foetal and early postnatal environment. The DOHaD theory, first articulated by the late Sir David Barker, posits that stressors, such as nutrient deficiencies and hypoxaemia, can alter the growth trajectories of the developing

organism and in turn predispose the offspring to chronic disease in later life.²

Iron deficiency (ID) is the most common nutritional deficiency worldwide. It occurs when iron demands chronically exceed intake, resulting in depletion of tissue iron stores and eventually leading to reduced circulating haemoglobin (Hb). During gestation and early childhood, increased iron demands due to growth and blood volume expansion contribute to the increased risk of ID and anaemia.³ Globally, an estimated 38% of pregnant women are anaemic,⁴ and the prevalence of latent ID in pregnancy, in which overt anaemia is not yet manifest, is undoubtedly higher. Even in many developed countries where prenatal iron supplementation is common, approximately one in four pregnant women still develop anaemia,⁴ emphasizing the need to better understand long-term implications of ID on offspring health outcomes.

In addition to increasing the risk of foetal death, preterm birth and intrauterine growth restriction,^{5,6} ID during pregnancy is associated with long-term neurocognitive,⁷ metabolic,⁸ and cardiovascular complications.^{9,10} However, the mechanisms underlying these adverse long-term outcomes remain unclear. We recently showed that prenatal ID anaemia causes hypoxia, mitochondrial dysfunction, and increased generation of reactive oxygen species (ROS) in the term rat foetus.^{11,12} These adverse outcomes were organ-specific and sex-specific, such that kidneys of male foetuses exposed to prenatal ID were most affected,^{11,12} suggesting that renal development is particularly sensitive to programming effects. Indeed, adult offspring born to pregnancies complicated by perinatal ID exhibit hypertension and enhanced blood pressure (BP) responsiveness to high salt intake,^{8,9,13} implicating intrinsic changes in renal function.¹⁴ However, the long-term effects of perinatal ID on kidney function have not been studied. The objective of this study was to determine whether perinatal ID causes long-term kidney mitochondrial dysfunction and oxidative stress within the kidney, which has been described in other models of hypertension and salt sensitivity.^{15,16} Moreover, we sought to determine whether these outcomes would be exacerbated by chronic consumption of a high salt diet.

2. Methods

Please see [Supplementary material online](#) for detailed methods.

2.1 Animals and treatments

The protocols described herein were approved by the University of Alberta Animal Care Committee in accordance with guidelines established by the Canadian Council of Animal Care, consistent with NIH guidelines. Female Sprague-Dawley rats were purchased from Charles River (Saint-Constant, QC, Canada) and housed at the University of Alberta animal care facility, which maintained a 12 h light/dark cycle and ambient temperature of 23°C. Rats had *ad libitum* access to food and water throughout the study.

All purified diets fed to dams in this study were based on the AIN93-G diet and were otherwise identical with the exception of the amount of added ferric citrate. Two-weeks prior to pregnancy, female rats were randomly assigned to the iron restricted group, in which they were fed a purified diet low in iron (containing 3 mg/kg elemental iron); the remaining females were assigned to the control group, in which they were fed a purified control diet (containing 37 mg/kg elemental iron). After 2 weeks on their respective diets, female rats were bred by co-housing overnight with age-matched males fed a standard rodent chow (5L0D; PicoLab). Upon confirmation of pregnancy, dams in the iron restricted group were

fed a moderately iron restricted diet (containing 10 mg/kg elemental iron), and those in the control group were maintained on the control diet. Within 12 h of giving birth, all dams were fed the standard rodent chow, and pup weights were recorded, and litters were standardized to eight offspring (four males and four females). Offspring were weaned from their dams on postnatal day (PD) 21 and fed standard rodent chow.

At four and a half months of age (6 weeks prior experimentation), offspring from each litter were fed either a high salt diet (HS, 5.0% NaCl w/w) or a normal salt diet (NS, 0.27% NaCl w/w). Body weights, food intake, and water consumption were monitored weekly during this 6-week treatment period.

2.2 Haemodynamic assessments

Direct haemodynamic assessments were performed in spontaneously breathing adult offspring under isoflurane anaesthesia (in medical grade oxygen at a flow rate of 1 mL/min; 3–5% for induction, 1.5% for maintenance) on a 37°C warming bed (heated by a circulating water bath). Catheters connected to a pressure transducer were implanted into the left femoral artery for direct BP measurements, and a catheter was implanted into the left femoral vein for drug delivery. Following completion of surgery, rats were given a minimum of 15 min to achieve steady haemodynamics, and baseline parameters were recorded for the next 5–10 min. After baseline recordings, haemodynamic responses to the pan-nitric oxide (NO) synthase inhibitor L-Nitro-G-Arginine Methyl ester (L-NAME; 60 mg/kg IV) were assessed over 15 min. Isoflurane anaesthesia was then increased to 5% and rats were euthanized by exsanguination and subsequent excision of the heart.

2.3 High-resolution respirometry

Respirometry was performed using the Oroboros Oxygraph-O2k system (Innsbruck, Austria). Kidneys were separated into cortical and medullary components under a microscope, and approximately 20 mg of tissue were homogenized in 2 mL of MiRO5 buffer. The remainder of homogenate was flash frozen and stored at -80°C for citrate synthase (CS) activity assay (see Section 2.4). The titration protocol used herein (see [Supplementary material online](#)) allows evaluation of the mitochondrial function at three different respiratory states [LEAK (respiration in the presence of substrate, but in the absence of ADP; includes a variety of processes and is not limited to electron leak, thus it is expressed capitalized to note the distinction), oxidative phosphorylation (OXPHOS), and electron transfer (ET)] as previously described.^{12,17}

2.4 Mitochondrial content

CS activity was measured using a UV/Vis spectrophotometer (Ultrospec 2100 Pro; Biochrom, Cambridge, MA, USA) heated to 37°C by a cell holder and circulating water bath as previously described.¹⁸ Medullary and cortical tissue homogenates were thawed and underwent an additional cycle of homogenization with a conical glass homogenizer for 10 s. Absorbance at 412 nm was monitored to assess reduction of 0.1 mmol/L 5,5-dithiobis-2-nitrobenzoic acid (ϵ : 13.6 mL cm⁻¹ μ M⁻¹).¹⁸

2.5 Fluorescence microscopy

The following reagents were used for experimentation: dihydroethidium (DHE; for assessment of cytosolic superoxide), 4-amino-5-methylamino-2', 71-difluorofluorescein diacetate (DAF-FM; for assessment of cytosolic nitric oxide), and MitoSox Red (for assessment of mitochondrial superoxide). Cryosectioned kidney sections mounted on slides were incubated with either DHE (20 μ mol/L for 30 min), DAF-

FM (20 µmol/L for 30 min) or MitoSox (5 µmol/L for 10 min) and were subsequently mounted in HBSS (DHE and MitoSox) or Vectashield Antifade Mounting Medium with DAPI. Fluorescence was visualized for both cortical and medullary regions of the kidney sections using an Olympus IX81 fluorescent microscope.

2.6 Renal morphology assessments

Kidneys were fixed and embedded as previously described.¹¹ Jones silver stain counterstained with alcoholic Eosin Y and Harris haematoxylin was used for glomerular basement membrane (GBM) thickness assessments. Masson's trichrome staining was performed for the assessment of interstitial and glomerular collagen deposition.

2.7 Statistical analyses

In all data sets, *n* values reflect the number of litters (i.e. treated dams are the experimental unit); instances where more than one offspring per litter were used to obtain a value (e.g. neonatal weights and Hb levels), the litter average was treated as a single data point. In total, data was collected from 136 adult offspring from a total of 30 litters; 76 animals were allocated to haemodynamics experiments and 60 to mitochondrial function and oxidative stress measurements. Data were analysed by unpaired Student's *t*-test, or two-way ANOVA for the main effects of perinatal ID and salt treatment, with a Sidak's *post hoc* test. All male and female offspring data were analysed separately. Data are presented as mean ± SEM. *P* < 0.05 was considered significant. Grubb's test was used to identify statistical outliers, which were excluded from analysis. All statistical analyses were conducted using GraphPad Prism 8.0 software.

3. Results

3.1 Pregnancy outcomes and offspring growth

Iron restriction prior to and throughout pregnancy resulted in a 34% decrease in maternal Hb levels by gestational day (GD)21, one day prior to full term (Table 1). ID dams gained 14% less weight during pregnancy and had a 19% reduction in litter size (Table 1) compared with control (CTL) dams, despite no differences in food intake during pregnancy (CTL 580.4 ± 23.7 g vs. ID 541.6 ± 16.3 g, *P* = 0.21). On PD1, male and female pups born to ID dams had approximately 40% decreases in circulating Hb and approximately 11% lower body weights (Table 1). In these offspring, the growth restriction persisted in the pre-weaning phase, such that they were approximately 27% smaller than their CTL counterparts on PD21 (male CTL 49.8 ± 1.5 g vs. ID 36.4 ± 3.2 g, *P* = 0.006; female CTL 49.2 ± 1.7 g vs. ID 35.4 ± 2.9 g, *P* = 0.003). Prior to beginning the 6-week HS treatment, adult male perinatal ID offspring remained approximately 10% smaller than their CTL counterparts, with no initial differences in size when comparing the groups allocated to NS and HS treatments (ID effect *P* = 0.003, HS effect *P* = 0.61, interaction *P* = 0.20; data not shown). In contrast, female offspring exhibited no differences in body weight between groups (ID effect *P* = 0.42, HS effect *P* = 0.53, interaction *P* = 0.56; data not shown). Notably, the smaller male perinatal ID offspring ate and drank less than their control counterparts during the 6-week treatment period (Supplementary material online, Table S1). The HS diet increased water intake by greater than two-fold in both male and female offspring, despite HS not altering food intake (Supplementary material online, Table S1).

Table 1 Pregnancy and neonatal outcomes

Parameters	Treatments		P-value
	CTL	ID	
Maternal weight gain GD0–GD21 (g)	227.2 ± 7.5	195.5 ± 9.0	0.01
Litter size (no. pups)	18.1 ± 0.9	14.6 ± 0.6	0.003
Maternal Hb (g/dL)			
GD0	15.3 ± 0.2	11.8 ± 0.6	<0.001
GD7	14.7 ± 0.2	11.7 ± 0.5	<0.001
GD14	14.0 ± 0.2	10.7 ± 0.5	<0.001
GD21	12.5 ± 0.3	8.3 ± 0.4	<0.001
PD1 Hb (g/dL)			
Males	11.2 ± 0.4	6.8 ± 0.5	<0.001
Females	11.0 ± 0.3	6.3 ± 0.5	<0.001
PD1 body weight (g)			
Males	6.49 ± 0.14	5.76 ± 0.15	0.004
Females	6.13 ± 0.20	5.54 ± 0.14	0.02

All groups *n* = 8–11 offspring from separate litters. Data are expressed as mean ± SEM and analysed by unpaired *t*-test. *P* values are bold where significant.

CTL, control; GD, gestational day; Hb, haemoglobin; ID, perinatal iron deficient group; PD, postnatal day.

At the end of the 6 weeks salt treatment period, male perinatal ID offspring remained approximately 10% smaller than their CTL counterparts, with shorter crown-rump lengths and reduced abdominal girth (Table 2). Male offspring had increased relative (i.e. normalized to body weight) right ventricular (RV) weight due to both perinatal ID and HS, whereas only HS causes an increase in relative left ventricle and septum (LVS) weight (Table 2). No differences in relative kidney weights in male offspring were observed (Table 2). In contrast, female offspring exhibited no alterations in size or weight, or differences in organ weights due to perinatal ID or HS, with the exception of a trend for increased RV weight due to perinatal ID (*P* = 0.06; Table 2).

3.2 Haemodynamics

At 6 months of age, there was an overall effect of perinatal ID and HS on systolic BP (SBP) in male offspring (Figure 1A). Furthermore, HS increased diastolic BP (DBP) and mean arterial pressure (MAP) in male offspring, whereas perinatal ID increased MAP but not DBP (*P* = 0.09; Supplementary material online, Table S2). In contrast, there was no effect of perinatal ID or HS on haemodynamic parameters in female offspring (Supplementary material online, Table S2), albeit the interaction between perinatal ID and HS in SBP approached significance (*P* = 0.06, Figure 1A).

In male offspring, intravenous administration of pan-NOS inhibitor L-NAME caused a gradual rise in SBP, culminating in a 20–42% increase over baseline, which was least pronounced in the perinatal ID offspring fed the HS diet (Figure 1B). The magnitude of SBP rise was reduced by perinatal ID and HS (Figure 1B), resulting in similar SBP between groups after L-NAME treatment (Figure 1C). In female offspring, the L-NAME-mediated rise in SBP ranged from 6% to 17% and was mitigated by the effects of HS—an effect that was most pronounced in the perinatal ID offspring (Figure 1B).

3.3 Mitochondrial content and function

Mitochondrial respiration in renal tissues is expressed both as flux per unit homogenate mass, as well as flux control ratio (FCR; see Supplementary material online), where FCR is indicative of qualitative

Table 2 Body measurements and organ weights of 6-month-old offspring

Parameters	Treatments				P-value		
	CTL	HS	ID	HS	ID	HS	Interaction
Male							
Body weight (g)	757.7 ± 24.7	699.4 ± 23.5	640.5 ± 19.3	638.5 ± 10.8	<0.001	0.17	0.19
CR length (cm)	21.5 ± 0.3	21.1 ± 0.2	20.3 ± 0.3	20.5 ± 0.1	<0.001	0.74	0.26
Abdominal girth (cm)	26.0 ± 0.5	24.6 ± 0.4	23.9 ± 0.4	23.9 ± 0.4	0.003	0.14	0.14
LVS (mg/g)	1.62 ± 0.03	1.79 ± 0.06*	1.75 ± 0.05	1.83 ± 0.07	0.11	0.02	0.36
RV (mg/g)	0.41 ± 0.01	0.44 ± 0.02	0.46 ± 0.02	0.51 ± 0.03	0.002	0.03	0.80
Kidneys (mg/g)	5.93 ± 0.23	6.34 ± 0.12	6.45 ± 0.36	6.21 ± 0.20	0.42	0.72	0.16
Female							
Body weight (g)	434.5 ± 21.8	422.6 ± 12.7	391.8 ± 17.3	410.9 ± 12.8	0.13	0.84	0.38
CR length (cm)	17.6 ± 0.3	17.4 ± 0.2	17.3 ± 0.2	17.4 ± 0.2	0.51	0.88	0.64
Abdominal girth (cm)	21.0 ± 0.6	20.3 ± 0.3	19.8 ± 0.4	20.4 ± 0.4	0.27	0.92	0.16
LVS (mg/g)	1.90 ± 0.06	1.94 ± 0.06	1.90 ± 0.06	1.94 ± 0.07	0.96	0.56	0.99
RV (mg/g)	0.46 ± 0.02	0.48 ± 0.02	0.51 ± 0.03	0.52 ± 0.03	0.06	0.54	0.87
Kidneys (mg/g)	5.80 ± 0.26	6.48 ± 0.17	6.11 ± 0.38	6.21 ± 0.27	0.94	0.19	0.33

All groups $n=8-11$ offspring from different litters. Data are expressed as mean $\pm$ SEM and are analysed by two-way ANOVA with Sidak's *post hoc* test where * denotes $P < 0.05$ vs. NS group. *P* values are bold where significant.

CR, crown-rump; CTL, control; HS, high salt diet; ID, perinatal iron deficiency group; LVS, left ventricle and septum; NS, normal salt diet; RV, right ventricle.

Figure 1 Systolic blood pressure (SBP) and systemic nitric oxide (NO) signalling interrogated by NO synthase inhibitor L-NAME in 6-month-old offspring fed a normal salt (NS) or high salt (HS) diet, born from control (CTL) or iron deficient (ID) dams. (A) Baseline SBP, (B) percentage increase in SBP with the addition of L-NAME, and (C) SBP in the presence of L-NAME. All groups $n=8-11$. Data are analysed by two-way ANOVA with Sidak's *post hoc* test where * denotes $P < 0.05$ vs. NS.

Figure 2 Renal medullary respirometry data expressed as flux per unit mass of tissue from 6-month-old offspring fed a normal salt (NS) or high salt (HS) diet, born from iron deficient (ID) or control (CTL) dams. Measurement of function is made under two states, oxidative phosphorylation capacity (OXPHOS; coupled to ATP production) and electron transfer capacity (ET; uncoupled). (A) NADH (N-) pathway OXPHOS, (B) N- and Succinate (S-) pathway OXPHOS, (C) NS-pathway ET (D) S-pathway ET, and (E) complex IV activity. Males $n = 4$ in ID-NS, 6–8 in all others; females $n = 6$ –8 in all groups. Data are analysed by two-way ANOVA with Sidak's *post hoc* test where * and ** denote $P < 0.05$ and $P < 0.01$ vs. NS, respectively.

changes in mitochondrial respiratory control and independent of mitochondrial density.¹⁷ Within the medulla and cortex, LEAK respiration (oxygen flux in the absence of ADP unavailable for performing biochemical work)¹⁷ was not affected by either ID or HS in male or female offspring (Supplementary material online, Figure S1). The magnitude of LEAK respiration was low and indicative of well coupled mitochondria in all groups (Supplementary material online, Figures S2 and S3). Moreover, addition of cytochrome c resulted in similar fractional increases in N-pathway respiration between groups (Supplementary material online, Table S3), indicating integrity of the outer mitochondrial membrane was similar in tissues among treatment groups.¹⁷ Notwithstanding, all coupled oxidative phosphorylation (OXPHOS; in the presence of saturating ADP) and electron transfer (ET; non-coupled respiration after the addition of uncoupler) capacities presented herein reflect measurements taken after the addition of exogenous cytochrome c, avoiding possible bias resulting from damage to the outer mitochondrial membrane during tissue preparation.

In adult male offspring, medullary oxygen flux per unit mass through the NADH- and Succinate- (N- and S-) pathways as well as complex IV activity were increased by HS (Figure 2A–E), while perinatal ID reduced flux through the S-pathway (Figure 2B–D). FCR in the medulla of male offspring are unaffected by ID and HS (Supplementary material online, Figure S2). Within the cortex, ID and HS did not alter oxygen flux per unit mass for any parameter (Figure 3A–E), nor did it alter FCR for N- and S-pathways (Supplementary material online, Figure S3A–D). An

interaction between perinatal ID and HS was observed in the cortex of male offspring, where complex IV FCR was reduced by HS only in ID offspring (Supplementary material online, Figure S3E); a similar trend was observed in flux per mass (interaction $P = 0.07$, Figure 3E). There were no effects of perinatal ID or HS in oxygen flux per unit mass in either the renal medulla (Figure 2) or cortex (Figure 3) in adult female offspring, nor when data were expressed as FCR (Supplementary material online, Figures S2 and S3). Mitochondrial content of the renal medulla, as assessed by CS activity assay, was increased by HS in male, but not female offspring (Figure 4A). Within the cortex, no alterations in mitochondrial content were observed in either male or female offspring due to perinatal ID or HS (Figure 4B).

3.4 Kidney superoxide and NO levels

In male offspring, there was an overall effect of HS on mitochondrial superoxide within the medulla (Figure 5A), but there was no effect of either ID or HS within the cortex (Figure 5A and D). Perinatal ID caused an increase in cytosolic superoxide levels in both the renal medulla (Figure 5B) and cortex (Figure 5E) in adult male offspring, and HS caused a further increase within the cortex (Figure 5B and E). NO bioavailability in the medulla and cortex of male offspring was reduced by ID, with a trend for further decreases by HS in the medulla ($P = 0.08$; Figure 5C and F). In female offspring, mitochondrial superoxide was not altered by perinatal ID or HS within either the cortex or medulla of the kidney (Figure 5A and D). Females exhibited a subtle but significant interaction effect for

Figure 3 Renal cortical respirometry data expressed as flux per unit mass of tissue from 6-month-old offspring fed a normal salt (NS) or high salt (HS) diet, born from iron deficient (ID) or control (CTL) dams. Measurement of function is made under two states, oxidative phosphorylation capacity (OXPHOS; coupled to ATP production) and electron transfer capacity (ET; uncoupled). (A) NADH (N-) pathway OXPHOS, (B) N- and Succinate (S-) pathway OXPHOS, (C) NS-pathway ET (D) S-pathway ET, and (E) complex IV activity. Males $n = 4$ in ID-NS, 6–8 in all others; females $n = 6$ –8 in all groups. Data are analysed by two-way ANOVA with Sidak's *post hoc* test.

cytosolic superoxide levels due to perinatal ID and HS within the medulla and cortex (Figure 5B and E). No reductions in NO levels were seen in females due to perinatal ID in either the medulla or cortex, and there was a trend for decreased NO in the cortex due to HS ($P = 0.06$; Figure 5C and F). Representative fluorescence images are shown in [Supplementary material online Figures S4 and S5](#).

3.5 Renal morphology

In male offspring, GBM thickness was increased by both perinatal ID and HS (Figure 6A). Maximal glomerular cross-sectional area in male offspring was increased by the combination of perinatal ID and HS, but not by either factor alone (Figure 6B). Finally, interstitial and glomerular collagen deposition in male offspring was increased by both perinatal ID and HS (Figure 6C). In contrast, female offspring exhibit no alterations in GBM thickness, maximal glomerular cross-sectional area, or collagen deposition due to either perinatal ID or HS (Figure 6A–C). Representative histological images are shown in [Supplementary material online, Figure S6](#).

4. Discussion

Here, we show that perinatal ID and a HS diet in adulthood cause sex-specific alterations in haemodynamics, and kidney morphology and metabolic function. Specifically, adult male perinatal ID offspring exhibit increased BP concomitant with renal structural changes, alterations in

medullary mitochondrial function in flux per mass, and dysregulation of ROS and NO levels, whereas female offspring are largely unaffected. In addition, male offspring exhibit an interaction effect for reduced complex IV activity in the cortex with the combination of both perinatal ID and HS. Taken together, these findings suggest that the developing kidney is susceptible to long-term programming by perinatal ID, and female offspring are protected from these long-term effects.

Maternal iron restriction during pregnancy caused anaemia in the dams and offspring at birth, as well as reduced weight gain in the dams during pregnancy, which is consistent with smaller litters and foetal growth restriction. Notably, male and female offspring exhibited similar levels of anaemia and growth restriction at birth, suggesting the degree of insult on the offspring was similar in both sexes. Although all rats were fed iron replete diets after giving birth, offspring born to iron restricted dams exhibit reduced Hb levels until shortly after weaning.^{8,9,19} This exposure therefore reflects a 'perinatal' rather than exclusively 'prenatal' insult which encompasses the entire window of kidney development in the rat.²⁰ By six months, an age which corresponds to approximately 18–25 years in humans,²¹ male perinatal ID offspring remained growth restricted (based on body weights, lengths, and abdominal girth) compared with their control counterparts, yet differences were no longer evident in female offspring. Thus, based on growth patterns alone, male and female offspring exhibit different susceptibilities to the effects of perinatal ID.

The observation that perinatal ID resulted in elevated BP in adult male offspring is consistent with previous findings from our group^{8,9,13} and

others.^{10,22–25} The introduction of a HS diet to the adult offspring was intended to mimic the excess consumption of dietary sodium, a modifiable risk factor for hypertension and CVD.²⁶ Approximately 89% of the US population²⁷ and 58% of the Canadian population²⁸ exceed daily recommended salt intake, which can negatively impact both renal and vascular function.²⁶ In the present study, HS diet induced a greater than two-fold increase in water intake (without affecting food intake), thus imposing stress to the cardiovascular system by increasing renal filtration and pressure natriuresis, as reflected in increased SBP in both CTL and perinatal ID male offspring. However, the interaction between the perinatal ID and HS treatments was not significant in male offspring, suggesting the salt-sensitivity of BP was not more pronounced in programmed offspring, in contrast to our previous reports.^{8,9,13} The explanation for this may reside in the fact that BP assessments in the present study were done entirely under isoflurane anaesthesia, which can blunt sympathetic outflow²⁹ and thus attenuate underlying salt-sensitivity.³⁰

Renal injury was observed in male offspring due to both perinatal ID and HS. Glomerular basement membrane thickness and collagen deposition were increased due to both perinatal ID and HS, which were likely secondary to increased BP. Furthermore, the combination of perinatal ID and HS resulted in enlargement of glomerular cross-sectional area, which may indicate compensatory hypertrophy to maintain GFR with a reduced nephron complement.²⁵ Elevations in LVS mass occur

secondary to increased BP,³¹ which may support the patterns of cardiac hypertrophy observed in the HS-treated offspring. In contrast, the effect of perinatal ID on LVS size did not reach statistical significance, despite these offspring being hypertensive and exhibiting renal injury, which may indicate that the study was not powered to detect this difference. Alternatively, it may be that LV effects will become manifest as the hypertension progresses with age; indeed, Rueda-Clausen *et al.* reported that LV weights in offspring exposed to prenatal hypoxia, which shares a common aetiology to perinatal ID,¹¹ showed no overt differences in LV size at 4 months of age, but pronounced effects at 12 months of age³² when hypertension becomes apparent in this model.³³ RV hypertrophy was also noted in adult male perinatal ID offspring, potentially reflecting increased resistance within the pulmonary circulation.³⁴ Although this remains to be investigated, adult offspring exposed to prenatal hypoxia develop signs of pulmonary hypertension in rat models,³² drawing further parallels between these models of developmental stress.

In our first study focusing on BP outcomes in programmed female offspring, we found that there was no effect of perinatal ID on BP. For reasons unclear to us, BPs assessed under anaesthesia, even in control female offspring, were higher than anticipated, which could arguably mask elevated BP in the programmed females. However, left ventricular weights and histological assessments of renal morphology were similar in all groups, suggesting no overt cardiovascular phenotype in female perinatal ID offspring. Although reports utilizing tail cuff plethysmography have shown perinatal ID increases BP in offspring of both sexes, the effects are less pronounced in females.^{10,23–25} HS also had little effect on BP in female offspring, consistent with a lack of effect of perinatal ID and HS on LVS weights. Interestingly, the effect of perinatal ID approached significance in RV weights, suggesting the increased pulmonary resistance occurring in offspring is not sex-dependent. These patterns are consistent with those in adult offspring exposed to prenatal hypoxia, in which males exhibited a greater propensity for systemic hypertension (in later life),³³ whereas both males and females are susceptible to pulmonary hypertension.³²

The BP effects in male offspring attributed to perinatal ID and HS occur concomitantly with marked alterations in renal mitochondrial function, ROS generation, and ultimately NO signalling. Mitochondrial dysfunction within the kidney has been implicated in chronic kidney disease,³⁵ and has been observed in models characterized by renal dysfunction³⁶ and sensitivity to HS diet,¹⁵ suggesting proper kidney function relies on a delicate balance of cellular energy metabolism. While perinatal ID had no effect on mitochondrial content, the medullae of male offspring exhibited reductions in oxygen flux per mass through the succinate-dependent pathway. In contrast, HS caused marked increases in medullary mitochondrial content in male offspring (irrespective of perinatal exposure), despite having little impact on mitochondrial content or function within the cortex. This is consistent with previous reports describing mitochondrial biogenesis in the wake of increased medullary osmolality and Na^+ concentrations.³⁷ Our results show that the medulla is highly sensitive to both perinatal stressors and a chronic HS diet. Interestingly, in female offspring, neither perinatal ID nor HS diet caused alterations in mitochondrial content or function in the medulla or cortex, which suggests fundamental differences in the way males and females respond to excess sodium intake.³⁸

The mitochondrial function data described above are supported by the patterns of renal superoxide in adult offspring. Male perinatal ID offspring exhibit increased cytosolic superoxide within the medulla and cortex, consistent with dysfunction in complexes II and IV,^{39,40} respectively. Within the medulla, HS caused an increase in mitochondrial

Figure 5 Mitochondrial superoxide (MitoSOX), cytosolic superoxide (DHE), and cytosolic NO (DAF-FM) mean fluorescent intensities (MFI) in 6-month-old offspring fed a normal salt (NS) or high salt (HS) diet, born from iron deficient (ID) or control (CTL) dams. (A) Medulla MitoSOX, (B) Medulla DHE, (C) medulla DAF-FM, (D) cortex MitoSOX, (E) cortex DHE, and (F) cortex DAF-FM. Males $n = 5-10$; females $n = 6-8$ in all groups. Data are analysed by two-way ANOVA with Sidak's *post hoc* test where * denotes $P < 0.05$ vs. NS.

content roughly proportional to increased mitochondrial superoxide levels. In contrast, female offspring had no alterations in mitochondrial content or function, coincident with minimal alterations in mitochondrial and cytosolic superoxide levels due to ID or HS. Although a significant interaction between perinatal ID and HS was observed in DHE staining in both medulla and cortex of female offspring, the magnitude of differences were small, and therefore, the physiological relevance is likely minor. Vieyra-Reyes et al.⁴¹ have shown adult female perinatal ID offspring exhibit increased antioxidant enzyme activity, which could explain why perinatal ID females are less prone to oxidative stress, including from major non-mitochondrial sources such as NADPH oxidases.⁴²

Oxidant status within the kidney plays a direct role in the bioavailability of NO, which is known to regulate sodium and fluid homeostasis.⁴³ We report that male perinatal ID offspring exhibit >50% reductions in NO levels within both the medulla and cortex, in agreement with elevated superoxide levels scavenging NO.⁴³ Low levels of NO may contribute to the hypertension in male offspring by promoting salt-retention, either via direct actions on tubular sodium reuptake,⁴³ or via reducing medullary blood flow.^{43,44} Indeed, L-NAME treatment ultimately normalized BP levels in male offspring, suggesting loss of NO may underlie the baseline differences between control and perinatal ID offspring. Although NO can modulate medullary blood flow, tubuloglomerular feedback and natriuresis,^{43,44} contributing to hypertension⁴⁵ and salt sensitivity,⁴⁶ we cannot discount intrinsic differences in systemic vascular NO signalling that contribute to programming of BP regulation in perinatal ID offspring. Therefore, the programming of vascular function in perinatal ID male offspring treated with a HS diet should be studied. Investigations in female offspring may also provide important insights,

and may uncover clues as to why oxidant status and NO signalling remain largely unaffected. Increased antioxidant capacity and actions of sex-hormones are potential mechanisms by which female offspring exposed to perinatal ID maintain normal oxidant status, NO signalling, and normal cardiovascular and renal function.

This study has limitations. First, as discussed above, the use of anaesthesia may have influenced haemodynamic assessments, particularly if programmed offspring have altered responsiveness to the BP lowering effect of isoflurane.⁴⁷ Because of this potential confounder, BP data was complemented with measures of ventricular mass and renal morphology, which exhibit pathophysiological changes secondary to hypertension.⁴⁸ Second, certain aspects of the rat model used in this study limit the extrapolation of results to humans. For example, as an altricial species, rats are born immature and undergo comparatively greater growth and development in the immediate postnatal period compared with humans. The extent to which iron supply and demand are mismatched dictate the severity of ID, and whether this is similar in rats and humans during periods of kidney development is not clear. Nevertheless, the rat model of perinatal ID employed herein has notable strengths (e.g. development of maternal anaemia mirrors that in humans, shared hemochorial placentation), and provides a platform to study the impact of maternal effects (e.g. liver hypoxia¹¹ and altered lipid metabolism⁴⁹) vs. offspring effects (e.g. renal hypoxia¹¹ and mitochondrial dysfunction¹²) in the long-term programming of offspring health. Finally, while not a limitation *per se*, the present study characterizes renal pathology in adult offspring but it does not mechanistically link these effects to previously observed foetal kidney hypoxia,¹¹ oxidative stress and mitochondrial dysfunction.¹² It is possible that the mitochondrial dysfunction and

Figure 6 Renal morphological parameters in 6-month-old offspring fed a normal salt (NS) or high salt (HS) diet, born from iron deficient (ID) or control (CTL) dams. (A) Glomerular basement membrane thickness, (B) maximum glomerular cross-sectional area, and (C) interstitial and glomerular collagen deposition. Male $n = 5-8$; female $n = 6-10$. Data are analysed by two-way ANOVA with Sidak's *post hoc* test where * denotes $P < 0.05$ vs. NS.

oxidative stress observed in foetuses persist into adulthood and contribute to renal dysfunction in later life. The alternative is that alterations in foetal energy metabolism may cause persistent renal structural and functional changes, which in turn promote *de novo* cellular stress in adulthood. More studies are needed to assess the link between foetal and adult kidney oxidative stress and mitochondrial dysfunction in perinatal ID offspring.

In summary, the results presented herein show that perinatal ID causes long-term alterations in mitochondrial function and oxidative stress in the kidneys, which have been implicated in hypertension and cardiovascular dysfunction. Together with our previous findings in the foetal kidney,^{11,12} these results suggest that the developing male kidney is particularly vulnerable to hypoxia-like insults.⁵⁰ Though beyond the scope of the current study, the involvement of mitochondrial dysfunction and increased ROS levels points to the use of mitochondrial targeted therapeutics to improve energetics in the developing and adult kidney. Irrespective of the mechanism, these findings speak to the importance of early interventions to prevent the programming of cardiovascular and renal function in adulthood and improve long-term outcomes.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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