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ORIGINAL ARTICLE

Maternal hypomagnesemia alters renal function but does not program changes in the cardiovascular physiology of adult offspring

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Maternal undernutrition is known to adversely impact fetal health and development. Insults experienced *in utero* alter development of the fetus as it adapts to microenvironment stressors, leading to growth restriction and subsequent low birth weight. Infants born small for gestational age have significantly increased risk of developing cardiovascular and renal disease in later life, an effect that is often characterized by hypertension and reduced glomerular number. Maternal magnesium (Mg^{2+}) deficiency during pregnancy impairs fetal growth, however, the long-term health consequences for the offspring remain unknown. Here, we used a mouse model of dietary Mg^{2+} deficiency before and during pregnancy to investigate cardiovascular and renal outcomes in male and female adult offspring at 6 months of age. There were no differences between groups in 24-h mean arterial pressure or heart rate as measured by radiotelemetry. Cardiovascular responses to aversive (restraint, dirty cage switch) and non-aversive (feeding response) stressors were also similar in all groups. There were no differences in nephron number, however, Mg^{2+}-deficient offspring had increased urine flow (in both males and females) and reduced Mg^{2+} excretion (in males only). Despite evidence suggesting that maternal nutrient restriction programs for hypertension in adult offspring, we found that a moderate level of maternal dietary Mg^{2+} deficiency did not program for a nephron deficit, or alter cardiovascular function at 6 months of age. These data suggest there are no long-term adverse outcomes for the cardiovascular health of offspring of Mg^{2+}deficient mothers.

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Key words: Blood pressure, magnesium, nephron number

Introduction

Maternal deficiencies in either macronutrients or micronutrients during pregnancy can adversely affect nutrient transport to the developing embryo, leading to fetal growth restriction and subsequent low birth weight.[1] There is overwhelming evidence to suggest that infants born small for gestational age have a significantly increased risk of developing cardiovascular and renal disease in later life.[2–4] This is often due to adverse intrauterine events that place the developing embryo under stress and restrict growth during gestation.[5] As seen in animal models, this ultimately leads to altered organogenesis and places the offspring at increased risk for developing hypertension[6–8] and impaired renal function[9,10] in adulthood.

A reduction in glomerular number can be a major contributor to the development of cardiovascular disease during later adult life, as suggested by numerous clinical studies.[11–14] A nephron deficit reduces the renal capacity for sodium excretion, leading to sodium retention and increased plasma volume. These changes in renal physiology increase mean arterial pressure (MAP), leading to glomerular hypertension and scarring. This creates a positive feedback loop to further increase blood pressure (BP) through progressive glomerular damage. Although maternal calorie/protein restriction is known to reduce glomerular number and increase adult BP,[15–17] few studies have investigated the role of gestational micronutrient deficiencies in programming renal and cardiovascular function in adult offspring.

Micronutrient deficiencies during pregnancy are well known to adversely impact both maternal and fetal health and lead to poor pregnancy outcomes.[18] Many women of reproductive age do not meet the recommended levels for dietary Mg^{2+} intake, so a significant proportion of women may be Mg^{2+}-deficient either before conception or during pregnancy. One study examining dietary Mg^{2+} intake in a French population reported that over 20% of women (aged 35–60) consumed less than two-third of the recommended daily allowance (320 mg).[19] Low dietary Mg^{2+} intake is also common in younger women, as a recent Australian study of over 450 17-year-old girls found that only 15% met the recommended daily intake, and <50% met the estimated average requirement.[20] These studies suggest that low Mg^{2+} intake in women of reproductive age may be highly prevalent. Although Mg^{2+} levels during pregnancy have not been comprehensively studied, one study in an Indian population reported that ~45% of pregnant women had low serum Mg^{2+} levels (<0.75 mmol/l).[21] We have recently investigated the effect of maternal Mg^{2+} deficiency on fetal and early postnatal outcomes using an animal model.[22] Our results demonstrated that maternal hypomagnesemia caused fetal

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growth restriction, embryonic loss and placental abnormalities. These findings clearly emphasize the important role of Mg^{2+} in placental health and normal embryonic development, however, the long-term consequences for offspring born to Mg^{2+}-deficient mothers have not yet been determined. In the present study, we used this recently described mouse model of maternal Mg^{2+} deficiency[22] to investigate how maternal hypomagnesemia affects cardiovascular and renal outcomes in adult offspring.

Methods

Ethics

All studies were approved by The University of Queensland Anatomical Biosciences Animal Ethics Committee (SBMS/154/12/NHMRC/NHF) and conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC).

Animal treatment

A total of 24 dams and their offspring were treated as per Schlegel *et al.*[22] Briefly, female CD1 mice (7 weeks old) were fed either a control Mg^{2+} diet (0.2%, w/w Mg^{2+}), or a Mg^{2+}-deficient diet (0.02%, w/w, Glen Forrest Stockfeeders, Specialty Feeds, Western Australia) for 4 weeks before mating and throughout gestation ($n = 6$ dams/treatment group). This Mg^{2+}-deficient diet significantly reduces maternal plasma Mg^{2+} concentrations by ~40% compared with controls.[22] Female mice were then mated with CD1 males (fed a standard laboratory chow diet) and allowed to litter naturally. The Mg^{2+}-deficient diet was continued until weaning at postnatal day 21 (PN21), when a subset of offspring was euthanized for determination of glomerular number. All other animals were maintained on a 12:12 h light:dark cycle with food (standard rodent chow) and water *ad libitum*, and were studied at 6 months of age.

Stereological estimation of glomerular number

One to two pups of each sex from each litter were euthanized at PN21 and the right kidney from each pup was collected, weighed, fixed in 4% paraformaldehyde and processed to paraffin. Total glomerular number was estimated using unbiased stereological analysis[23] ($n = 6$/group).

Renal function and electrolyte analysis

A cohort of mice was habituated to metabolic cages on 2 consecutive days for 3 h/day. On the 3rd day, mice were placed in metabolic cages for 24 h with food and water available *ad libitum.* Animal weights, food and water consumption were recorded, and urinary electrolytes measured using a COBAS Integra 400 Plus electrolyte analyzer ($n = 9$–11).

Measurements of BP, heart rate (HR) and activity under basal conditions

Telemetry probes (PA-C10, Data Sciences International, USA) were implanted into a separate group of 6-month-old offspring under isoflurane anesthesia (3–3.5% in O_2) to measure BP, HR and activity[24] ($n = 7$–8). Mice were housed individually following surgery, and data acquisition commenced 10 days after surgery. Data (systolic and diastolic pressures, HR and activity) was captured continuously for 10 s every 15 min for a period of 3 days using Dataquest Advanced Research Technology (Data Sciences International).

Cardiovascular responsiveness to stressors

After measuring BP, HR and activity under basal conditions, we investigated whether the cardiovascular responsiveness to stress was altered by maternal Mg^{2+} deficiency. Responses to stress were assessed by exposing the animals ($n = 7$–8) to aversive (restraint stress, dirty cage switch) and non-aversive stimuli (feeding) that cause changes in cardiovascular parameters, arousal and activity.[25–28] Stressor experiments were performed on 3 consecutive days between 8 am and 12 pm, with continuous data acquisition throughout the experiment and for 60 min before the stressor. For the restraint stress test, each mouse was placed into a well-ventilated clear plastic cylinder (radius ~30 mm, height ~70 mm) for 15 min. For the dirty cage switch test, the experimental mouse was placed into a cage that had been occupied by a different mouse of the same sex and strain for 7 days previously, and data recorded for 90 min. The non-aversive feeding test involved placing a novel food stimulus (~0.5 g of almond) into the home cage, and data were recorded for 10 min.

Tissue collection

Following all experiments, animals were euthanized by cervical dislocation (at PN21) or by CO_2 inhalation (6 months) and organs (heart, kidneys, liver and brain) collected, weighed and either fixed in 4% paraformaldehyde or snap frozen in liquid nitrogen for later analysis. Bone samples were processed by microwave/nitric acid digestion, and Mg^{2+} content measured using inductively coupled plasma atomic emission spectrophotometry. Blood was also collected, centrifuged at 4°C and the plasma stored at −20°C until analysis.

Gene expression

The messenger RNA expression levels of the Mg^{2+} channel TRPM6 in the kidneys of offspring were measured using quantitative PCR (qPCR). Total RNA was isolated using TRIzol and reverse transcribed using a TaqMan reverse transcription reagents kit (Life Technologies). qPCR was performed using Assay on Demand reagents (TRPM6 Mm00463112) and ribosomal 18S as a housekeeping gene. Data were analyzed using the $2^{-\Delta\Delta C_T}$ method and expressed relative to the control group ($n = 6$–11/group).

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Statistics

All data are presented as mean ± S.E.M. Renal data were analyzed using two-way analysis of variance (ANOVA) with a Tukey's multiple comparisons test (Graph Pad Prism); $P < 0.05$ was regarded as being significant. Telemetry data were analyzed by repeated measures two-way ANOVA using sex and maternal diet as factors. Responses to stress were calculated by quantifying the area under the curve during the stressor and comparing with an equivalent amount of time during the baseline period.

Results

Offspring growth and organ weights

In the present study, we monitored animals from weaning (PN21) through 6 months of age, and although we found significant differences between sexes, there were no differences in body weights between treatment groups. Animals were not weighed on the day of birth. There was a significant increase in liver weight in the Mg^{2+}-deficient offspring at 6 months of age, however, there were no other differences in organ weights between groups (Table 1).

Renal function, gene expression and nephron number

Urinary Mg^{2+} excretion was reduced in Mg^{2+}-deficient male (but not female) offspring when compared with control offspring (Fig. 1a). This was not associated with altered renal expression of the TRPM6 Mg^{2+} channel (Fig. 1b), nor were there any differences in plasma and bone Mg^{2+} levels between groups (Fig. 1c and 1d). Although males had significantly more nephrons than females, there was no difference in nephron number between control and Mg^{2+}-deficient groups at PN21 (Fig. 2a). Both male and female Mg^{2+}-deficient offspring showed a significant ($P < 0.05$) increase in urine flow at 6 months of age (Fig. 2b), but there were no statistically significant differences in water consumption between

Table 1. *Body and organ weights from 6-month-old offspring*

Organ (6 months)	Control males (n = 6–8)	Mg^{2+}-deficient males (n = 6)	Control females (n = 6–7)	Mg^{2+}-deficient females (n = 6–12)	Source of variation (P-values)		
					Interaction	Sex	Diet
Body weight (g)	52.7 ± 1.8	53.8 ± 1.4	47.0 ± 1.2	50.7 ± 0.9	0.36	<0.01*	0.10
Heart weight (mg)	257 ± 14	265 ± 20	172 ± 7	188 ± 5	0.68	<0.0001*	0.31
Kidney weight (mg)	715 ± 27	752 ± 31	378 ± 5	421 ± 14	0.89	<0.0001*	0.066
Liver weight (mg)	2483 ± 176	3133 ± 205	1785 ± 58	2143 ± 120	0.34	<0.0001*	<0.01*
Brain weight (mg)	471 ± 10	480 ± 9	462 ± 32	480 ± 11	0.79	0.82	0.44

* $P < 0.05$.

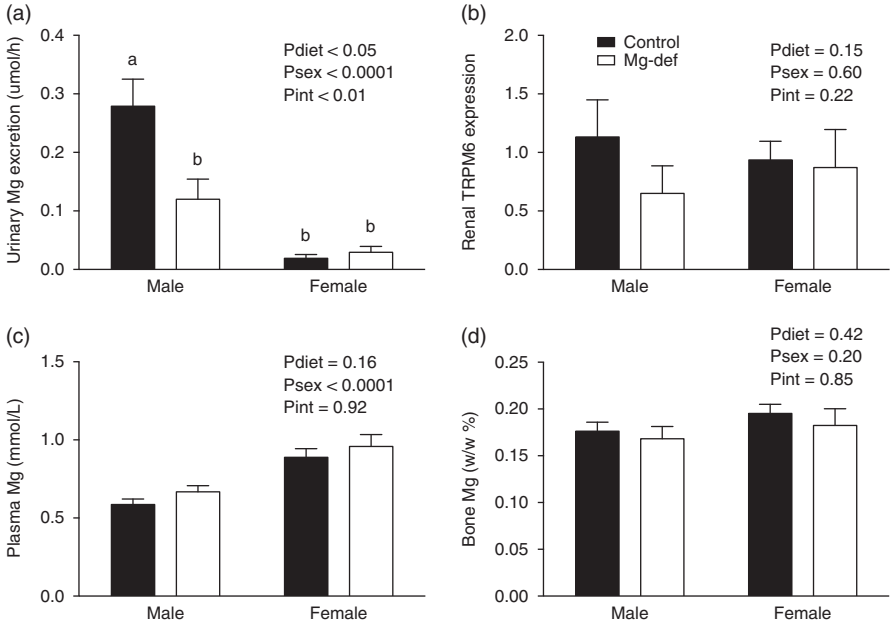

Fig. 1. Mg^{2+} handling in 6-month offspring. (*a*) Mg^{2+} urinary excretion. (*b*) Renal TRPM6 expression. (*c*) Plasma Mg^{2+}. (*d*) Bone Mg^{2+} (w/w, %). n = 6–12 offspring/group per sex. [a,b]Different letters indicate significant differences ($P < 0.05$) between groups.

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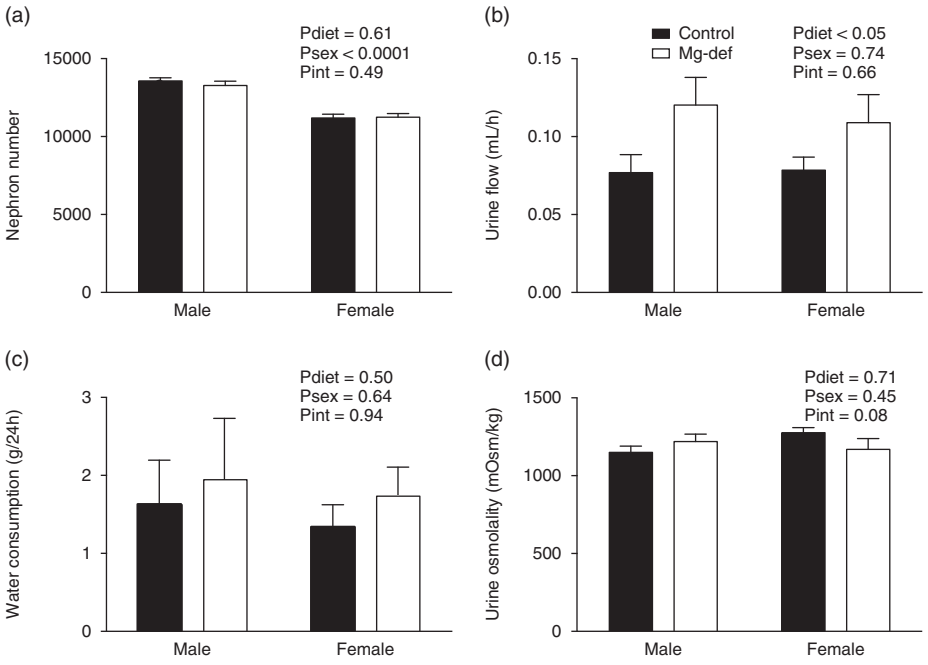

Fig. 2. Renal physiology in offspring. (*a*) Nephron number at postnatal day 21, (*b*) urinary flow, (*c*) water consumption per 24 h and (*d*) urine osmolality at 6 months of age. $n = 6$–12 offspring/group per sex.

Table 2. *Urinary Na, K, Cl excretion, Na/K excretion ratio and plasma Na, K, Cl from 6-month-old offspring*

Parameters	Control males ($n = 7$–9)	Mg^{2+}-deficient males ($n = 7$–11)	Control females ($n = 7$–11)	Mg^{2+}-deficient females ($n = 7$–11)	Source of variation (P-values) Interaction	Sex	Diet
Na excretion (μmol/h)	6.0 ± 1.0	8.0 ± 1.0	7.0 ± 1.0	9.0 ± 2.0	0.94	0.57	0.30
K excretion (μmol/h)	10.0 ± 1.0	10.0 ± 1.0	10 ± 2.0	10.0 ± 2.0	0.83	0.77	0.13
Cl excretion (μmol/h)	6.0 ± 0.8	8.0 ± 0.9	7.0 ± 0.9	8.0 ± 1.0	0.91	0.48	0.14
Na/K excretion ratio	0.6 ± 0.06	0.5 ± 0.05	0.7 ± 0.08	0.6 ± 0.06	0.70	0.063	0.43
Plasma Na (mmol/l)	131.3 ± 2.5	135.5 ± 3.1	136.5 ± 5.35	139.5 ± 6.1	0.90	0.32	0.44
Plasma K (mmol/l)	8.7 ± 0.3	8.5 ± 0.3	10.87 ± 1.41	8.2 ± 0.3	0.13	0.22	0.069
Plasma Cl (mmol/l)	103.0 ± 2.5	103.3 ± 3.9	106.5 ± 3.3	111.0 ± 4.5	0.58	0.14	0.51

treatment groups (Fig. 2c). All offspring had similar urinary osmolar excretion (Fig. 2d), and plasma/urinary levels of Na, K and Cl (Table 2).

Basal MAP, HR and activity

Analysis of radiotelemetry data revealed no differences in basal MAP or HR between groups during either the day or night periods (Fig. 3). All groups showed normal circadian variations in MAP, HR and activity (Fig. 3). A collective 24-h analysis of radiotelemetry data across all 3 days also showed no treatment-related differences in basal MAP (control males, 116 ± 3 mmHg; control females, 113 ± 2 mmHg; Mg^{2+}-deficient males, 118 ± 1 mmHg; Mg^{2+}-deficient females, 117 ± 2 mmHg), HR (control males, 539 ± 16 bpm; control females, 621 ± 12 bpm; Mg^{2+}-deficient males, 549 ± 10 bpm; Mg^{2+}-deficient females, 583 ± 15 bpm) or activity (control males, 5 ± 1 AU; control females, 8 ± 1 AU; Mg^{2+}-deficient males, 6 ± 1 AU; Mg^{2+}-deficient females, 9 ± 1 AU). Separate analysis of the day and night periods also found no statistically significant differences in these parameters.

Cardiovascular responses to stressors

Restraint stress caused an immediate increase in MAP (Δ23–32 mmHg) and HR (Δ88–163 bpm) above baseline levels. The dirty cage switch also caused an immediate elevation in MAP (Δ16–21 mmHg), HR (Δ140–217 bpm) and activity (Δ25–31 AU) in all groups. The almond-feeding test (non-aversive stimulus) caused an immediate and modest increase in MAP in control (Δ19–20 mmHg) and Mg^{2+}-deficient (Δ12–14 mmHg) offspring ($P = 0.061$). HR (Δ95–138 bpm)

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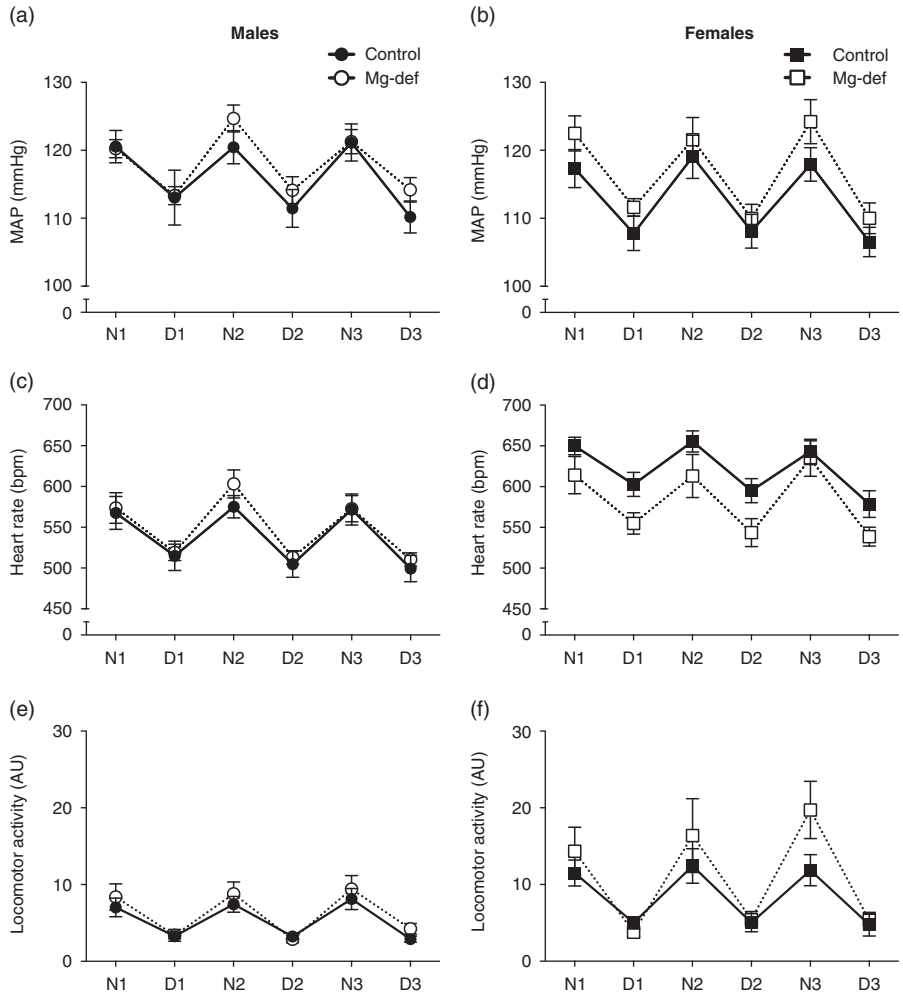

Fig. 3. Mean arterial pressure (MAP) in (*a*) male and (*b*) female offspring at 6 months of age. Heart rate in (*c*) male and (*d*) female offspring in 6-month-old offspring. Activity in (*e*) male and (*f*) female offspring in 6-month-old offspring. Data were recorded for 10 s every 15 min and are expressed as a 12 h average of all sampled data across 3 consecutive day (D) and night (N) periods. $n = 5–8$ offspring/group per sex.

and activity (Δ9–12 AU) were also elevated across all groups. There were no significant differences in ΔMAP or ΔHR between groups in response to any of the above stressors (Table 3).

Discussion

This study has for the first time examined the long-term cardiovascular and renal outcomes of offspring born to Mg^{2+}-deficient mothers. Maternal hypomagnesemia altered renal physiology by increasing urinary flow (in offspring of both sexes) and decreasing Mg^{2+} excretion (in male offspring only). However, this alteration in urinary Mg^{2+} excretion was not associated with altered expression of the Mg^{2+}-permeable ion channel TRPM6 in the kidney. Furthermore, we found no differences in nephron number between treatment groups. The basal BPs and HRs of adult offspring were unaffected by maternal Mg^{2+} deficiency, nor were there any differences in cardiovascular responses to stress. Our current findings demonstrate that maternal Mg^{2+} deficiency causes minor alterations in renal function but does not cause high BP during later adult life.

Many prenatal dietary deficiencies result in renal dysfunction.[9,10] In the present study, Mg^{2+}-deficient offspring had ~30–40% increase in urinary flow rate compared with controls. This was accompanied by an increase in water intake of ~15–25% in Mg^{2+}-deficient offspring. Although this was not statistically different to the control group, the high variability in the water intake data may have prevented the detection of significant changes. In addition, mice consume only small volumes of water over a 24-h period (when compared with larger species such as rats), which may contribute to the lack of accuracy in measuring changes in this parameter, and the subsequent variability in the data. Other models of maternal dietary restriction have demonstrated that the offspring of dams receiving a low-protein diet had an increase in urinary flow rates that was associated with an increase in water intake.[29] An increase in water intake is thus a likely explanation for the increase in urinary flow in our model

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Table 3. *Mean arterial pressure (MAP) and heart rate (HR) in 6-month-old offspring subject to cardiovascular reactivity/stress tests*

Parameters	Control males (n = 7–8)	Mg^{2+}-deficient males (n = 7–8)	Control females (n = 7–8)	Mg^{2+}-deficient females (n = 7–8)	Source of variation (P-values) Interaction	Sex	Diet
			Restraint stress				
MAP (ΔAUC)	512 ± 47	440 ± 67	350 ± 90	322 ± 56	0.74	<0.05*	0.46
HR (ΔAUC)	2533 ± 473	2202 ± 316	1285 ± 712	1360 ± 568	0.71	0.063	0.81
			Dirty cage swap				
MAP (ΔAUC)	1440 ± 140	1399 ± 98	1522 ± 122	1400 ± 268	0.41	0.73	0.91
HR (ΔAUC)	10,331 ± 894	11,458 ± 561	9272 ± 489	9211 ± 1941	0.60	0.15	0.63
			Almond feeding				
MAP (ΔAUC)	299 ± 35	216 ± 21	261 ± 12	229 ± 37	0.38	0.67	0.061
HR (ΔAUC)	1833 ± 213	1529 ± 334	1427 ± 175	1578 ± 249	0.43	0.41	0.67

Stressor measurements were calculated by quantifying the area under the curve (AUC) during the stressor (restraint stress, 15 min; dirty cage swap, 60 min; almond feeding, 10 min) and normalized to an equivalent amount of time during baseline (pre-stressor).

* $P < 0.05$.

of maternal Mg^{2+} deficiency. An alternative explanation is that different responses to the metabolic cage-induced stress led to differences in water consumption between groups. However, as we found no differences in cardiovascular responsiveness to stressors, a differential response to metabolic cage stress seems unlikely. Furthermore, if the increased urinary flow rate occurred in the absence of increased water consumption, we would expect to have found a hypovolemic reduction in BP. Instead, radiotelemetry measurements of BP revealed no differences between groups. Taken together, this makes an increase in water intake, which is the most likely explanation for the increased urinary flow in Mg^{2+}-deficient offspring.

In addition to the increased urinary flow rate, male offspring from Mg^{2+}-deficient dams also showed reduced urinary Mg^{2+} excretion. However, this was not associated with increased levels of Mg^{2+} in either plasma or bone. Studies have reported that fractional Mg^{2+} excretion and urinary output of Mg^{2+} is reduced in offspring from diabetic dams;[30,31] a similar phenotype to male offspring from our Mg^{2+}-deficient group. Changes in Mg^{2+} excretion also have been reported clinically, as Mughal *et al.*[32] found altered Mg^{2+} handling in children born to mothers with insulin-dependent diabetes mellitus. However, programmed alterations in Mg^{2+} homeostasis due to prenatal or perinatal Mg^{2+} deficiency have not been previously reported. In the current study, we found that the change in Mg^{2+} excretion in males was not due to altered TRPM6 expression in the kidney, despite this channel being primarily responsible for renal Mg^{2+} reabsorption and the regulation of Mg^{2+} excretion.[33] Nonetheless, these changes in Mg^{2+} excretion in males may be a programmed adaptation by which fetuses exposed to reduced Mg^{2+} levels *in utero* develop a compensatory and permanent reduction in Mg^{2+} excretion that is retained during later life in order to maintain normal Mg^{2+} homeostasis. However, the mechanism(s) responsible for these changes in the renal handling of Mg^{2+} remain unknown.

Given the association between maternal dietary nutrient restriction and reduced nephron number,[10,15,17] we determined nephron number in male and female offspring and were surprised to find that it was unaltered by maternal Mg^{2+} deficiency. Although numerous models of both mild and severe prenatal nutrient deficiencies have demonstrated reduced glomerular number in offspring,[10,17] in our model moderate maternal Mg^{2+} deficiency did not cause changes in total glomerular number. At 6 months of age there were marked differences in body and organ weights between male and female pups. Interestingly, despite similar kidney weights at P21[22] males had a greater glomerular number than females. This is consistent with previous studies, both animal and human, that have demonstrated glomerular number is significantly linked to gender.[34–36] In addition, we saw no changes in 24-h MAP and HR in Mg^{2+}-deficient offspring. These findings were quite unexpected, as maternal deficiencies in other trace elements such as zinc[37] and iron[38,39] are known to reduce nephron number and increase BP in the adult offspring. The absence of any programmed outcomes from maternal Mg^{2+} deficiency during gestation and lactation is particularly surprising. We have previously shown that different degrees of maternal dietary Mg^{2+} deficiency leads to graded fetal growth restriction, and the offspring born to mothers who were moderately Mg^{2+}-deficient were mildly growth restricted during early postnatal development.[22] Such growth restriction is often associated with adverse impacts on the developing kidneys and cardiovascular system. Although this previous study showed mild growth restriction in moderately Mg^{2+}-deficient offspring, in the present study we did not assess birth weight, placental transport of Mg^{2+}, or Mg^{2+} plasma levels at PN21 in the same animals that were used for cardiovascular studies at 6 months of age; this may be considered a limitation to our current study. We were unable to detect even small (<5 mmHg) changes in basal 24-h BP, HR or activity through the use of radiotelemetry, the 'gold-standard' and highly sensitive method for measuring cardiovascular parameters

in mice.[24] As demonstrated by Woods *et al.*,[40] there is evidence that the degree of dietary restriction can be correlated with the adverse programmed outcome. Therefore, it is possible that a more severe dietary Mg^{2+} deficiency during gestation and development may affect BP in later life. However, the effects of severe Mg^{2+} deficiency could not be explored in this model due to the high neonatal mortality of offspring born to severely Mg^{2+} deficient mothers.[22]

In addition to measuring BP under basal conditions, we also investigated whether the cardiovascular responsiveness to aversive and non-aversive stress was affected by maternal Mg^{2+} deficiency. Clinical research shows that increased cardiovascular reactivity to psycho-emotional stress is a predictor for the development of hypertension and heart disease.[41,42] Altered cardiovascular reactivity can also be developmentally programmed, as rodent models have shown that maternal stress and exposure to prenatal corticosteroids can alter cardiovascular reactivity in postnatal offspring.[43,44] Despite these studies linking maternal perturbations and altered cardiovascular reactivity, in the present study offspring from Mg^{2+}-deficient dams did not show any changes in cardiovascular reactivity in response to aversive and non-aversive stimuli. Overall, this suggests that moderate maternal Mg^{2+} deficiency does not put offspring at an increased risk of developing cardiovascular disease during later adult life. Nonetheless, we cannot discount the possibility of other programmable phenotypes (e.g. altered metabolic outcomes) that result from maternal Mg^{2+} deficiency, or the impact of a 'second-hit' (such as poor diet or lifestyle) that may exacerbate or unmask existing conditions or predispositions to disease.

Maternal nutrition plays an important role in the health outcomes of offspring in both the early postnatal period and later in adulthood. Although we have previously demonstrated that maternal Mg^{2+} deficiency caused fetal and postnatal growth restriction, the present study shows that this did not cause a deficit in glomerular number, or any changes in BP, HR or cardiovascular reactivity. This infers that babies born to women with moderate levels of Mg^{2+} deficiency during pregnancy are unlikely to suffer from adverse cardiovascular health for this reason alone.

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Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the Australian guides on the care and use of laboratory animals (Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, NH&MRC), and were approved by The University of Queensland Anatomical Biosciences Animal Ethics Committee.

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