—Original Article—

Fetal Loss and Hyposulfataemia in Pregnant NaS1 Transporter Null Mice

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Abstract. Sulfate is important for growth and development, and is supplied from mother to fetus throughout pregnancy. We used NaS1 sulfate transporter null (*Nas1*^{-/-}) mice to investigate the role of NaS1 in maintaining sulfate homeostasis during pregnancy and to determine the physiological consequences of maternal hyposulfataemia on fetal, placental and postnatal growth. We show that maternal serum (≤ 0.5 mM), fetal serum (< 0.1 mM) and amniotic fluid (≤ 0.5 mM) sulfate levels were significantly lower in pregnant *Nas1*^{-/-} mice when compared with maternal serum (≈ 2.0 mM), fetal serum (≈ 1.5 mM) and amniotic fluid (≈ 1.7 mM) sulfate levels in pregnant *Nas1*^{+/+} mice. After 12 days of pregnancy, fetal reabsorptions led to markedly reduced (by $\geq 50\%$) fetal numbers in *Nas1*^{-/-} mice. Placental labyrinth and spongiotrophoblast layers were increased (by $\approx 140\%$) in pregnant *Nas1*^{-/-} mice when compared to pregnant *Nas1*^{+/+} mice. Birth weights of progeny from female *Nas1*^{-/-} mice were increased (by $\approx 7\%$) when compared to progeny of *Nas1*^{+/+} mice. These findings show that NaS1 is essential to maintain high maternal and fetal sulfate levels, which is important for maintaining pregnancy, placental development and normal birth weight.

Key words: Placenta, Pregnancy, Sulfate

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Sulfate (SO_4^{2-}) is vital for many processes in fetal growth and development, including neurogenesis and steroid metabolism [1–3]. The ratio of sulfonated (inactive) to unconjugated (active) steroids has important implications for many of the steroid-responsive events that regulate placental and fetal growth [4]. For example, placental estradiol-3-sulfate is taken up by the fetal brain, where it is desulfonated by steroid sulfatase to estradiol, which acts as a potent stimulator of fetal adrenocorticotropin secretion and the hypothalamus-pituitary-adrenal axis [5]. In addition, sulfonation of structural components, such as heparan sulfate proteoglycans (HSPGs), is essential for the maintenance of normal structure and function of placental and fetal tissues [6]. Together, these findings demonstrate an important role for sulfate in maintaining steroid and glycosaminoglycan homeostasis in the developing fetus.

SO_4^{2-} is obtained from the diet and from the intracellular metabolism of methionine and cysteine [6]. Circulating SO_4^{2-} levels are maintained by the NaS1 sulfate transporter which is expressed in the kidney where it mediates sulfate reabsorption [7]. During pregnancy, maternal blood sulfate levels increase by approximately 2-fold which provides an essential supply to the placenta and developing fetus [8–11]. This increase in circulating sulfate level is correlated with increased maternal renal sulfate reabsorption [9, 12]. Since the fetus has a relatively low capacity to form sulfate from methionine and cysteine [13, 14], most of its sulfate must come from the maternal circulating sulfate pool. These findings are relevant to the decreased fecundity of NaS1 knockout (*Nas1*^{-/-}) mice which exhibit both hyposulfataemia and mid-gestational mis-

carriage [15]. Fetal loss also occurs in heparan (*Hs3t1*^{-/-}) and estrogen (*Sult1e1*^{-/-}) sulfotransferase null mice [16, 17], indicating that sulfonation plays a critical role in maintaining pregnancy.

Since NaS1 is not expressed in the placenta or fetus [7, 18], we used pregnant *Nas1*^{-/-} mice as a model for investigating the physiological consequences of maternal hyposulfataemia on placental, fetal and postnatal growth and development. From the matings of *Nas1*^{-/-} female $\times$ *Nas1*^{+/+} male mice and *Nas1*^{+/+} female $\times$ *Nas1*^{-/-} male mice, which generates *Nas1*^{+/+} progeny in all litters, we show that pregnant *Nas1*^{-/-} mice have reduced maternal serum and amniotic fluid sulfate levels throughout pregnancy, negligible fetal serum sulfate levels, increased fetal reabsorptions, decreased fetal/placental weight ratios, enlarged placental spongiotrophoblast and labyrinth layers and increased post-natal growth in offspring.

Materials and Methods

Animals

All procedures were carried out in accordance with the University of Queensland Animal Ethics Office. We previously generated *Nas1*^{-/-} mice in which the *Nas1* gene was disrupted by targeted mutagenesis [15]. Male and virgin female mice with a mixed genetic background (129Sv and C57BL/6) were mated overnight using the breeding strategy: *Nas1*^{-/-} female $\times$ *Nas1*^{+/+} male; and *Nas1*^{+/+} female $\times$ *Nas1*^{-/-} male. This strategy produces *Nas1*^{+/+} progeny in all litters, and compares placental, fetal and post-natal phenotypes between maternal hyposulfataemic *Nas1*^{-/-} and normosulfataemic *Nas1*^{+/+} mice. Male *Nas1*^{-/-} mice have normal fertility [15] and are therefore unlikely to reduce fecundity in our study. The presence of a copulatory plug in the morning was designated as embryonic day 0.5 (E0.5). Female mice were singly housed in conventional conditions and fed a standard rodent chow (no. AIN93G; Glen Forrest Stockfeeders, Glen Forrest, Western Australia) and

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water *ad libitum*. Females were randomly selected on E7.5, E12.5 and E18.5 of pregnancy to be euthanized for analysis of fetal and placental tissues. All procedures were carried out in accordance with the University of Queensland Animal Ethics Office.

Biochemical analyses

Adult mouse blood was collected from the submandibular vein using an animal bleeding lancet (MEDIpoint). Fetal blood was collected immediately following decapitation. Amniotic fluid was collected by needle aspiration. Serum and amniotic fluid sulfate was measured using a modified turbidometric assay as previously described [19].

Histological analyses

Tissues were dissected into approximately 50 volumes of buffered 10% formaldehyde and fixed for 3 days prior to paraffin embedding. Embedded tissue was sectioned, stained with H&E, and examined by light microscopy.

In situ hybridization

Tissues were dissected into 4% paraformaldehyde in PBS and fixed for 1 day at 4°C. Samples were processed, sectioned and probed with a spongiotrophoblast-specific marker (*Pr17a2*) as previously described [20]. The labyrinth, spongiotrophoblast and decidua/mesometrial triangle areas were outlined from digital images of the spongiotrophoblast-stained placental sections using a digitizing pen and measured using Image Pro Plus software (Media Cybernetics). A stage micrometer was used to calibrate the measurements.

Quantitative real-time PCR (qRT-PCR) analysis of placental gene expression

Total RNA was isolated from the placentae of female *Nas1^{+/+}* and *Nas1^{-/-}* mice at 0900 h, using previously described methods [15]. We examined the mRNA levels of 2 placental sulfate transporter genes (*Slc13a4* and *Slc26a2*) and 2 sulfotransferase genes (*Sult1e1* and *Hs3st1*). Total RNA (2 µg) was reverse transcribed by using random hexamers and an Omniscript RT kit (Qiagen) as recommended by the manufacturer. PCR was performed in quadruplicate with 5 µl cDNA (from 25 ng RNA) and 10 µl mastermix containing Fast SYBR Green Master Mix (Applied Biosystems) and 200 nM forward and reverse primers (Supplementary Table 1) in a 7500 FAST real time PCR system (Applied Biosystems, Scoresby, Australia). The thermal cycling protocol was: 50°C for 2 min; 94°C for 2 min; 45 cycles of 94°C for 1 sec, 60°C for 10 sec, and 72°C for 15 sec. RNA expression levels and absolute threshold cycle values (Ct values) of each gene were normalised to those of ribosomal RNA with the manufacturer's software (Applied Biosystems). Amplification specificity was confirmed by melting curve analysis and agarose gel electrophoresis.

Behavioural testing

Same-litter groups of male or virgin female mice were housed 2–5 per cage (25 × 42 × 12 cm) at a constant temperature (23 ± 1°C) with a 12 h light/dark cycle (lights on at 0600 and off at 1800 h). To facilitate adaptation, mice were transported to the behavioural

studies facility at least 24 h prior to testing. Experiments were conducted between 0800 and 1300 h with the lighting level adjusted to 100 lux. Other than the brief testing periods, mice had access to food and water *ad libitum*. One group of mice was tested sequentially in the marble bury and open field tests with a one week interval. A second group of mice was tested in the elevated plus maze. Behavioural testing was performed as previously described [21].

Statistical analyses

Data were analysed using GraphPad Prism (Version 5.01, GraphPad Software, La Jolla, CA, USA). Where data were normally distributed, the statistical significance of differences between *Nas1^{+/+}* and *Nas1^{-/-}* groups was assessed by an unpaired Student's *t*-test, with *P* < 0.05 considered significant. Differences in fetal, placental and maternal weights, and placental areas, were analysed by the Dunnett Multiple comparison test, with *P* < 0.05 considered significant.

Results

Biometry

Fetal numbers and reabsorptions: Previously, we reported reduced litter sizes in progeny from female *Nas1^{-/-}* mice ($n = 4.1 \pm 3.9$) when compared to *Nas1^{+/+}* ($n = 10.7 \pm 2.5$) mice [15]. In order to characterize this further, here we determined the number of fetuses and reabsorption sites in pregnant *Nas1^{-/-}* and *Nas1^{+/+}* mice at embryonic days E7.5, E12.5 and E18.5 (Fig. 1A, B). At E7.5, we found similar numbers of fetuses and reabsorption sites in pregnant *Nas1^{-/-}* and *Nas1^{+/+}* mice. However, fetal numbers were significantly reduced (by ≥50%) in *Nas1^{-/-}* mice at E12.5 (median=4, *P*=0.003) and E18.5 (median=3, *P*=0.015) when compared with pregnant *Nas1^{+/+}* mice (median=8). These findings were supported by a significantly increased number of fetal reabsorption sites in pregnant *Nas1^{-/-}* mice at E18.5 (Fig. 1B).

Maternal, fetal and placental weights: The increase in body weight of pregnant *Nas1^{-/-}* mice at E12.5 (by ≈21%) and E18.5 (by ≈50%) was significantly less than pregnant *Nas1^{+/+}* mice at E12.5 (by ≈31%) and E18.5 (by ≈79%) (Fig. 1C), which corresponds to the reduced number of fetuses found in *Nas1^{-/-}* mice (Fig. 1A). At E12.5 and E18.5, there were slight (but non-significant) decreases in fetal weights and increased placental weights in pregnant *Nas1^{-/-}* mice when compared with *Nas1^{+/+}* mice (data not shown). As an indicator of placental efficiency, we determined the fetal:placental weight ratios. No difference was observed at E12.5 but a significant reduction was observed at E18.5 (by ≈25%, *P*=0.005) in *Nas1^{-/-}* mice (8.9 ± 0.5 , *n*=20) when compared to *Nas1^{+/+}* mice (11.8 ± 0.4 , *n*=42) (Fig. 1D).

Fetal and placental morphology

The important roles of sulfonation in brain and bone development [3, 22], led us to compare these tissues in fetuses from pregnant *Nas1^{-/-}* and *Nas1^{+/+}* mice. Histological analyses of fetal brain and bone at E12.5 revealed no gross structural differences in *Nas1^{-/-}* and *Nas1^{+/+}* mice (data not shown). As placental weights appeared to be slightly increased in pregnant *Nas1^{-/-}* mice, we ana-

Fig. 1. Maternal, fetal and placental biometry data from the matings of *Nas1^{+/+}* female × *Nas1^{-/-}* male mice (filled circles) and *Nas1^{-/-}* female × *Nas1^{+/+}* male mice (open circles). (A) Fetal and (B) reabsorption site numbers, range, median and P values showing a reduced number of fetuses and an increased number of fetal reabsorptions in *Nas1^{-/-}* mice at E12.5 and E18.5. n=4–10 per gestational time point. (C) Maternal *Nas1^{-/-}* body weights (% increase, n=5–11, mean, SD) were reduced at E12.5 and E18.5 when compared to *Nas1^{+/+}* mice. (D) Ratio of fetal to placental weights in *Nas1^{+/+}* (n=19–20, filled bars) and *Nas1^{-/-}* (n=41–42, unfilled bars) mice, showing a decreased placental efficiency in *Nas1^{-/-}* mice at E18.5. Mean, SE and P values from a multiple comparisons test.

lyzed the morphology of the labyrinth and spongiotrophoblast layers as well as the maternal components of the placenta, the decidua/mesometrial triangle. Spongiotrophoblast staining allowed for the clear demarcation of the placental layers (Fig. 2A,B). The decidua/mesometrial triangle area was similar in size between pregnant *Nas1^{-/-}* ($7.2 \pm 0.9 \text{ mm}^2$) and *Nas1^{+/+}* ($8.4 \pm 1.6 \text{ mm}^2$) mice, whereas labyrinth and spongiotrophoblast areas were increased by ≈ 2.4 -fold in placentas from pregnant *Nas1^{-/-}* mice ($5.2 \pm 1.7 \text{ mm}^2$, $3.2 \pm 1.0 \text{ mm}^2$) when compared to pregnant *Nas1^{+/+}* mice ($2.2 \pm 0.9 \text{ mm}^2$, $1.3 \pm 0.5 \text{ mm}^2$) (Fig. 2C–E).

Serum and amniotic fluid sulfate levels

In order to determine if body sulfate levels were altered in pregnant *Nas1^{-/-}* mice, we measured circulating sulfate levels in pregnant *Nas1^{-/-}* and *Nas1^{+/+}* mice at three time points after conception (Fig. 3A). Day 7.5 was chosen as the earliest time point to study sulfate levels because this gestational age is before signs of reabsorptions at E12.5 (Fig. 1B). Serum sulfate levels were significantly ($P < 0.05$) increased (by ≈ 2 -fold) in pregnant *Nas1^{+/+}* mice, with levels peaking at E12.5 ($2.3 \pm 0.8 \text{ mM}$), but remained low (0.5 mM) throughout pregnancy in *Nas1^{-/-}* mice (Fig. 3A). Serum sulfate levels (Fig. 3B) were markedly lower (by ≈ 30 -fold) in E18.5 fetuses from *Nas1^{-/-}* mice ($0.05 \pm 0.01 \text{ mM}$) when compared to

Fig. 2. Enlarged labyrinth and spongiotrophoblast (ST) layers in E12.5 placentae from pregnant *Nas1^{-/-}* mice when compared to pregnant *Nas1^{+/+}* mice. (A and B) Representative sections from the center of placentae probed with a spongiotrophoblast-specific riboprobe (*Pr17a2*). Bar, 1 mm. (C) Labyrinth and (D) spongiotrophoblast areas were enlarged in placentae from maternal *Nas1^{-/-}* mice when compared to maternal *Nas1^{+/+}* mice, whereas (E) decidua/mesometrial triangle area were similar in placentae from pregnant *Nas1^{-/-}* and *Nas1^{+/+}* mice. Mean $\pm$ SD, n=9 per group. ** $P < 0.001$, * $P < 0.01$ and NS, non-significant, when compared to placentae from pregnant *Nas1^{+/+}* mice.

Nas1^{+/+} mice ($1.53 \pm 0.20 \text{ mM}$). Amniotic fluid sulfate levels (Fig. 3C) were significantly lower in *Nas1^{-/-}* mice when compared to *Nas1^{+/+}* mice at E12.5 (by ≈ 3 -fold) and at E18.5 (by ≈ 9 -fold).

Placental gene expression

Previously, we showed an altered hepatic transcriptional profile of sulfate transporters and sulfotransferases in *Nas1^{-/-}* mice, which we proposed to be a compensatory response to hyposulfataemia [23]. In this study, we quantitated the mRNA levels of two sulfate transporters (*Slc13a4* and *Slc26a2*) and two sulfotransferases (*Sult1e1* and *Hs3st1*), which are normally expressed in mouse placenta [16, 17, 24–25], from E12.5 and E18.5 placentae of pregnant *Nas1^{-/-}* and *Nas1^{+/+}* mice. No significant differences were found for *Slc13a4*, *Slc26a2*, *Sult1e1* and *Hs3st1* mRNA levels (Supplementary Table 2).

Postnatal growth

Our previous findings of growth retardation in *Nas1^{-/-}* mice [15], led us to investigate the consequences of fetal hyposulfataemia (Fig. 3B) on post-natal growth. As an indicator of growth, we measured the body weights and tail lengths of progeny from *Nas1^{-/-}* and *Nas1^{+/+}* mice. At birth, body weights were slightly increased (by $\approx 7\%$, $P < 0.05$) in male ($1.59 \pm 0.04 \text{ g}$, n=25) and female ($1.54 \pm 0.05 \text{ g}$, n=23) progeny from maternal *Nas1^{-/-}* mice when compared to male ($1.47 \pm 0.02 \text{ g}$, n=31) and female ($1.45 \pm 0.02 \text{ g}$, n=38) progeny from maternal *Nas1^{+/+}* mice (Fig. 4). At 1 week of age, body weights of male and female mice were similar between groups, and were not significantly different in male mice up to 16

Fig. 3. Serum and amniotic fluid (AF) sulfate levels in maternal *Nas1*^{+/+} and *Nas1*^{-/-} mice (n=4 to 5 per group). Sulfate levels remain low in (A) maternal and (B) fetal serum, and (C) amniotic fluid from progeny of *Nas1*^{-/-} female x *Nas1*^{+/+} male mice (open bars) when compared to progeny of *Nas1*^{+/+} female x *Nas1*^{-/-} male mice (filled bars). Data are means $\pm$ SE. *P<0.05, **P<0.01 and ***P<0.001 when compared to *Nas1*^{+/+} mice.

weeks. From 5 to 16 weeks of age, body weights were increased (by $\approx$ 10%, P<0.05) in the female progeny of maternal *Nas1*^{-/-} mice when compared to female progeny from maternal *Nas1*^{+/+} mice (Fig. 4B). Tails lengths of male and female offspring were similar between groups from 1 to 16 weeks of age (data not shown).

Behavioural studies of progeny from *Nas1*^{-/-} and *Nas1*^{+/+} mice

To determine the consequences of maternal and fetal hyposulfataemia on post-natal behaviour, we conducted behavioural testing on the adult progeny of maternal *Nas1*^{-/-} and *Nas1*^{+/+} mice. No significant differences were found between groups in the elevated plus maze, open field and marble bury tests (data not shown).

Discussion

In this study, we show markedly reduced sulfate levels in the serum of maternal *Nas1*^{-/-} mice and in fetal serum and amniotic fluid of their progeny. From mid-gestation, pregnant *Nas1*^{-/-} mice have enhanced fetal reabsorption, enlarged placental labyrinth and spongiotrophoblast layers and decreased fetal numbers. The progeny of maternal *Nas1*^{-/-} mice show increased body weight at birth, and female progeny have increased body weight in adulthood. Since all progeny from the reciprocal matings in this study were *Nas1*^{+/+}, and because NaS1 is not expressed in the placenta or fetus [7, 18], we propose that these abnormal features are due to reduced sulfate supply from maternal circulation to the fetoplacental unit as a consequence of maternal hyposulfataemia.

Previous human studies showed that maternal serum sulfate levels increased (by $\approx$ 2-fold) during pregnancy [9, 11, 12]. These

Fig. 4. Body weights of progeny from *Nas1*^{+/+} female x *Nas1*^{-/-} male mice (filled circles and dash line) and *Nas1*^{-/-} female x *Nas1*^{+/+} male mice (open squares and solid line). (A) Male and (B) female body weights were determined up to 16 weeks of age. Each point represents the mean $\pm$ SD derived from 6 to 37 measurements. *P<0.05 when compared to progeny of *Nas1*^{+/+} female mice.

findings led us to investigate circulating sulfate levels in pregnant *Nas1*^{-/-} mice and compare them with pregnant *Nas1*^{+/+} mice. Our data demonstrate that NaS1 is essential for maintaining high maternal circulating sulfate levels throughout pregnancy, and that maternal hyposulfataemia leads to markedly reduced fetal serum and amniotic fluid sulfate levels. This is an important finding because the placenta and fetus have a relatively low capacity to generate sulfate [13, 14], and rely on a sufficient supply of sulfate from the mother's circulation for sulfonation reactions to function [2]. Whilst the precise effect of hyposulfataemia on sulfonation in the placenta and fetus is at present unclear, our data demonstrate that loss of NaS1 causes reduced fecundity via maternal hyposulfataemia throughout pregnancy which leads to reduced fetal and amniotic fluid sulfate levels in *Nas1*^{-/-} mice.

Reduced litter sizes arising from *Nas1*^{-/-} female mice [15] led us to study the viability and growth of fetuses in pregnant *Nas1*^{-/-} and *Nas1*^{+/+} mice at three gestational ages that frame the period of chorioallantoic placental development: E7.5, prior to chorioallantoic attachment and any fetal losses in *Nas1*^{-/-} pregnancies; E12.5, when a functional placental labyrinth is present, which is the site of

fetal-maternal exchange [26]; and near the end of gestation at E18.5, when labyrinth development continues and rapid fetal growth is occurring [27]. In this study, we show similar numbers of viable fetuses in pregnant *Nas1^{-/-}* and *Nas1^{+/+}* mice at E7.5, indicating no change in fertility in *Nas1^{-/-}* mice *per se*. The reduced number of viable fetuses in pregnant *Nas1^{-/-}* mice at E12.5 and E18.5 were similar to the number of live births previously reported [15], suggesting that fetal loss occurs prior to day E12.5. This conclusion is consistent with the increased fetal reabsorption sites found in pregnant *Nas1^{-/-}* mice at E12.5 and E18.5, as well as our earlier observations of blood spotting and miscarriage in *Nas1^{-/-}* mice at 14 days of gestation [15]. Whilst fetal numbers were reduced by $\approx 50\%$ in *Nas1^{-/-}* mice at E12.5 and E18.5, we found similar weights of fetuses from *Nas1^{-/-}* and *Nas1^{+/+}* pregnant mice. A hallmark feature of normal rodent pregnancy is the inverse relationship between fetal number and weight [28, 29], which was not found in E12.5 and E18.5 fetuses from pregnant *Nas1^{-/-}* mice. This finding implies that the reduced number of surviving fetuses (≈ 4 per pregnancy) in pregnant *Nas1^{-/-}* mice have no growth advantage up to at least E18.5, when compared to fetuses (≈ 8 per pregnancy) from pregnant *Nas1^{+/+}* mice. This can be relevant to the enlarged labyrinth layer in pregnant *Nas1^{-/-}* mice, which may be compensating for a reduction in available sulfate. Enlargement of the labyrinth is proposed to be a physiological adaptation to maximize nutrient transfer in the placenta, as shown in a mouse model of over-expression of maternal IGFBP1 [30]. In addition, since impaired IGF1 expression can reduce fecundity and placental nutrient exchange [31], it is possible that low levels of IGF1 in *Nas1^{-/-}* mice [15] may be a contributing factor to the reduced fecundity and/or altered placental morphometry in pregnant *Nas1^{-/-}* mice. To our knowledge, this is the first study to show increased fetal reabsorption and altered placental morphology in a model of maternal hyposulfataemia.

Enlargement of the spongiotrophoblast layer in mouse placentae has been observed in several mouse models with decreased fetal survival, including over-expression of epidermal growth factor receptor [32], trisomy 15 syndrome [33] and hydroxysteroid 17 β dehydrogenase 2 null mice [34]. This is relevant to the enlarged spongiotrophoblast layer in placentae from pregnant *Nas1^{-/-}* mice, which have enhanced mid-gestational fetal loss. Spongiotrophoblasts produce numerous proteins with endocrine activity, including cytokines, prolactin-like hormones and lactogens [35], which are essential for the maintenance and progression of pregnancy and development of the fetus [36–38]. Whilst the precise mechanism underlying the placental phenotype in *Nas1^{-/-}* mice is at present not clear, our data demonstrate maternal hyposulfataemia accompanies an enlarged spongiotrophoblast layer.

Sulfotransferase (*Sult1e1*, *Hs3st1* and *Tpst1*) null mice exhibit mid-gestational fetal loss [16, 17, 39], indicating that sulfonation of estrogen, heparan sulfate and tyrosine, is critical for maintaining pregnancy. Our previous study demonstrated increased hepatic sulfotransferase and *Sat1* sulfate transporter mRNA levels in *Nas1^{-/-}* mice [23], suggesting that sulfotransferase and sulfate transporter mRNA expression is sensitive to changes in blood sulfate levels. In this study we investigated the mRNA expression of two sulfotransferases (*Sult1e1* and *Hs3st1*) and two sulfate transporters (*NaS2*

and *Slc26a2*), which are expressed in the placenta [16, 17, 24, 25]. However, our data show no change in *Sult1e1*, *Hs3st1*, *NaS2* and *Slc26a2* mRNA expression, indicating that these genes in the placenta are not sensitive to changes in maternal circulating sulfate levels.

The abnormal prenatal phenotypes of placental and fetal tissues from pregnant *Nas1^{-/-}* mice led us to investigate the postnatal growth of progeny from *Nas1^{-/-}* and *Nas1^{+/+}* female mice. Whilst the median fetal weight was similar in pregnant *Nas1^{-/-}* and *Nas1^{+/+}* mice at E18.5, we found significantly increased (by $\approx 7\%$) birth weights of male and female progeny from maternal *Nas1^{-/-}* mice when compared to the progeny of maternal *Nas1^{+/+}* mice, suggesting late prenatal accelerated growth in pregnant *Nas1^{-/-}* mice. Previous studies showed accelerated fetal growth from mid- to late-gestation [40] and a greater mass at weaning [41] in the progeny of rats fed on a low protein diet during pregnancy. Interestingly, the body mass of female progeny from maternal *Nas1^{-/-}* mice was increased (by 10%) from 5 weeks of age when compared to female progeny of maternal *Nas1^{+/+}* mice, which was not observed between male progeny from maternal *Nas1^{-/-}* and *Nas1^{+/+}* mice, indicating that male and female progeny may not have identical responses to maternal hyposulfataemia. Previous studies have shown that female post-natal growth is more responsive to intrauterine stressors when compared to males [42], suggesting that gender differences are associated with metabolic programming in the developing fetus.

Our earlier studies showed behavioural abnormalities in *Nas1^{-/-}* mice, including reduced object anxiety and locomotor activity [21]. In this study, we found no behavioural differences between adult progeny from maternal *Nas1^{-/-}* and *Nas1^{+/+}* mice in marble bury, elevated plus maze and open field tests, which are used to assess anxiety-like behaviours in rodents [43–45]. These findings indicate that exposure to maternal and fetal hyposulfataemia does not lead to anxiety-like symptoms in the adult progeny from *Nas1^{-/-}* female mice.

In summary, this study shows that NaS1 is essential for maintaining high circulating sulfate levels during pregnancy and that maternal hyposulfataemia leads to mid-gestational fetal loss, altered placental development and increased birth weights. These findings are relevant to diet, hormones and analgesics which regulate blood sulfate levels [46], prompting future assessment of pre- and post-natal development in humans with altered sulfate homeostasis.

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