

## Original Article

# Pregnant phenotype in aquaporin 8-deficient mice

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**Aim:** Aquaporin 8 (AQP8) is expressed within the female reproductive system but its physiological function remains to be elucidated. This study investigates the role of AQP8 during pregnancy using AQP8-knockout (AQP8-KO) mice.

**Methods:** Homozygous AQP8-KO mice were mated, and the conception rate was recorded. AQP8-KO pregnant mice or their offspring were divided into 5 subgroups according to fetal gestational day (7, 13, 16, 18 GD) and newborn. Wild type C57 pregnant mice served as the control group. The number of pregnant mice, total embryos and atrophic embryos, as well as fetal weight, placental weight and placental area were recorded for each subgroup. The amount of amniotic fluid in each sac at 13, 16, and 18 GD was calculated. Statistical significance was determined by analysis of variance of factorial design and chi-square tests.

**Results:** Conception rates did not differ significantly between AQP8-KO and wild type mice. AQP8-KO pregnant mice had a significantly higher number of embryos compared to wild type controls. Fetal/neonatal weight was also significantly greater in the AQP8-KO group compared to age-matched wild type controls. The amount of amniotic fluid was greater in AQP8-KO pregnant mice than wild type controls, although the FM/AFA (fetal weight/amniotic fluid amount) did not differ. While AQP8-KO placental weight was significantly larger than wild type controls, there was no evidence of placental pathology in either group.

**Conclusion:** The results suggest that AQP8 deficiency plays an important role in pregnancy outcome.

**Keywords:** AQP8 gene; knockout mice; pregnancy outcome; female reproductive system

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## Introduction

Aquaporins (AQPs) constitute a growing family of water-specific membrane channel proteins that transport water across cell membranes<sup>[1,2]</sup>. There are currently 13 known mammalian aquaporins (AQP0-12); a subset of these molecules are capable of increasing the permeability of small molecules such as glycerol (AQP3, 7, 9) and urea (AQP3, 7, 8, 9). AQP8 is reportedly permeable to ammonia<sup>[3]</sup>. The majority of aquaporins are constitutively expressed in the plasma membrane (AQP0, 1, 3, 4, 7, 8, 9, 10), while others are almost exclusively restricted to intracellular membranes (AQP6, 11, 12)<sup>[4]</sup>.

AQP8 complementary DNAs (cDNAs) were cloned from rat and mouse<sup>[5-7]</sup> in 1997 and human in 1998<sup>[8]</sup>. When expressed in *Xenopus* oocytes, mouse, rat or human aquaporin 8 genes increase the water permeability of oocytes by 10 to 20 fold. In addition, the cytoplasmic localization of AQP8 may also hint at its involvement in intracellular osmoregulation<sup>[9]</sup>.

Subsequent studies<sup>[10-16]</sup> have shown that AQP8 has a wide tissue distribution in mammals, with expression noted in the testis, kidney, heart, gastrointestinal tract, airway, salivary gland, pancreas and placenta. In the reproductive system, AQP8 is strongly expressed in the testis and sperm in the male and in the ovary, oviduct, uterus, placenta, amnion, chorion and cervix in the female<sup>[16-25]</sup>. While an earlier study<sup>[26]</sup> of AQP8 null mice reported no significant differences in comparison to wild type controls with the exceptions of an increase in testicular volume and a reduction in water permeability within the testes, we observed that AQP8 deficiency increased the number of mature follicles and the resulting fertility of female mice<sup>[27]</sup>. The present study aims to survey the role of AQP8 during pregnancy in AQP8-KO mice.

## Materials and methods

### Mice

AQP8 null mice were generated by targeted gene disruption as described in a previous study<sup>[26]</sup>. Wild type and AQP8-KO mice in a C57 genetic background were used at age 6–8 weeks. All animals were maintained in accordance with Institutional Guidelines for Care and Use of Laboratory Animals. Mice were housed under standard lighting (12-h light/dark cycle)

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and temperature ( $23\pm 1$  °C) conditions, with free access to a nutritionally balanced diet and deionized water. Protocols for mouse experiments were approved by the Committee on Animal Research of Jilin University Bethune Second Hospital.

Homozygous AQP8-KO mice and wild type mice were mated. Gestational day (GD) 1 was assigned as the day a copulation plug was observed. Pregnant mice that delivered pups did so at term (19–21 GD); thus, there were no premature or post-term deliveries. Embryos or offspring from AQP8-KO pregnant mice and wild type C57 pregnant mice (control group) were divided into 5 subgroups according to gestational age (7 GD, 13 GD, 16 GD, 18 GD, and newborn).

#### Gestational age-dependent embryo quantification

In total, 225 sacs from 31 AQP8-KO pregnant mice and 212 sacs from 33 wild type pregnant mice were used for embryo quantification studies (including all subgroups).

Mice that had shown copulatory plugs were anesthetized, and a Cesarean section was performed. Pregnant or non-pregnant status was recorded. The number of embryos per female was recorded at 7 GD, 13 GD, 16 GD, and 18 GD. Evidence of macroscopic atrophies were counted and recorded. The number of newborns delivered from 13 pregnant females was also recorded.

#### Fetal, placental and amniotic fluid measurements

AQP8-KO and wild type C57 pregnant mice were anesthetized, and Cesarean sections were performed at 13 GD, 16 GD, and 18 GD. Parameters, including fetal weight, placental weight and placental area, were measured in each subgroup. A total of 260 sacs were measured in this study.

The entire gestational sac was weighed before rupture of the amniotic membrane. After rupture of the amniotic membrane and absorption of the amniotic fluid, the fetus, placenta and fetal membranes were weighed. The placental area was calculated by measuring the placental diameter. The weight of amniotic fluid was estimated as the difference in weight pre- and post-rupture.

#### Statistical analysis

Differences in conception rate were assessed by a chi-square test. Analysis of variance (ANOVA) of factorial design was used to determine differences in embryo number, fetal weight, placental area and weight, weight of amniotic fluid and the ratio of fetal weight (FW)/amniotic fluid weight (AFA) between AQP8-KO and wild type groups. Parameters with significant interactions were assessed by LSD (Least Significant Difference) *post hoc* tests. Statistical significance was set at  $P < 0.05$ .

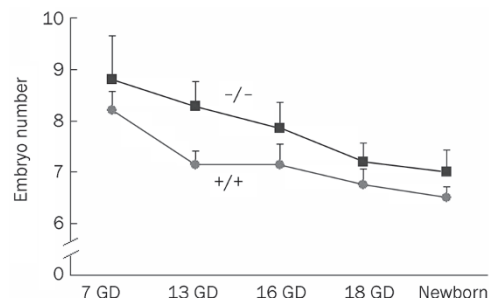
## Results

#### Embryo number

Fifty-three mated AQP8-KO females successfully conceived 40 mice (75.47%), and 54 mated wild type females conceived 35 mice (64.81%) ( $P = 0.321$ ).

As pregnancy progressed, the number of embryos declined

in both AQP8-KO and wild type groups ( $P = 0.004$ ). However, the total number of embryos per female was significantly greater among the AQP8-KO group for all gestational days compared to the wild type group ( $P = 0.018$ ), without interactions between the AQP8 gene and gestational age ( $P = 0.93$ ). Gestational age-dependent results are shown in Figure 1.



**Figure 1.** The mean number of embryos/pups at different gestational ages. The number of embryos per female decreased both in the AQP8-KO and wild type group as pregnancy progressed. The total number of embryos/pups of AQP8-KO mice during gestation was higher than wild type ( $P = 0.018$ ) although statistical significance was not achieved ( $P_{7\text{ GD}} = 0.54$ ,  $P_{13\text{ GD}} = 0.056$ ,  $P_{16\text{ GD}} = 0.293$ ,  $P_{18\text{ GD}} = 0.383$ ,  $P_{\text{Newborn}} = 0.354$ ). The number of pregnant mice examined at each checkpoint were 5, 7, 7, 5, 7 in AQP8-KO ( $n_1 = 31$ ) and 5, 7, 7, 8, 6 in wild type ( $n_2 = 33$ ) as pregnancy progressed. -/-: AQP8-KO; +/+ : Wild type.

While the number of macroscopic atrophic embryos was recorded for each gestational age investigated, no differences were observed among groups or during pregnancy.

#### Fetal/neonatal weight (mg)

Fetal weight varied as a result of both the presence/absence of the AQP8 gene and gestational age ( $P < 0.001$ ). Fetal weight progressively increased with gestational age ( $P < 0.001$ ) and was greater in AQP8-KO mice when compared to age-matched wild type controls ( $P < 0.001$ ) for gestational days 16, 18, and newborn (Table 1;  $P_{16\text{ GD}} = 0.001$ ,  $P_{18\text{ GD}} < 0.001$ ,  $P_{\text{New born}} < 0.001$ ) but not 13 ( $P_{13\text{ GD}} = 0.072$ ).

#### Amniotic fluid

##### Amniotic fluid amount

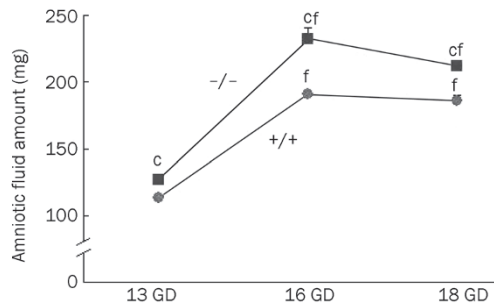
The weight of calculated amniotic fluid increased progressively during pregnancy for both AQP8-KO and wild type groups ( $P < 0.001$ ), although the increase in AQP8-KO mice was greater than that of wild type for each gestational age investigated ( $P < 0.001$ ). There were also significant interactions between the AQP8 gene and gestational age ( $P < 0.001$ ). As seen in Figure 2, the amount of amniotic fluid in AQP8-KO pregnant mice was elevated above wild type at each gestational age ( $P_{13\text{ GD}} < 0.001$ ,  $P_{16\text{ GD}} < 0.001$ ,  $P_{18\text{ GD}} < 0.001$ ).

##### Fetal weight (FW)/amniotic fluid amount (AFA)

There was no interaction between the AQP8 gene and ges-

**Table 1.** Fetal weight (mg) of AQP8-KO and wild type mice at 13, 16, 18 GD and newborn. Values are reported as Mean±SEM.

Mice type	Gestational age			
	13 GD (n=49, 33)	16 GD (n=47, 45)	18 GD (n=42, 44)	Newborn (n=43, 49)
AQP8-KO (n=181)	68.500±1.59	395.750±4.80	838.674±9.08	964.6±13.3
C57 (n=171)	65.027±1.05	368.065±6.80	777.984±7.60	885.2±10.7
P	0.072	0.001	<0.001	<0.001

**Figure 2.** The amniotic fluid amount (AFA) differed both between groups and during gestation. AFA in AQP8-KO mice was significantly greater than wild type throughout pregnancy ( $P_{13\text{ GD}} < 0.001$ ,  $P_{16\text{ GD}} < 0.001$ ,  $P_{18\text{ GD}} < 0.001$ ). The number (n) of embryos examined at each checkpoint was in Table 2. -/-: AQP8-KO; +/+ : wild type. <sup>c</sup> $P < 0.01$  vs wild type. <sup>f</sup> $P < 0.01$  vs 13 GD.

tational age ( $P=0.35$ ), and no difference in FM/AFA was observed between the two groups ( $P=0.157$ ). As pregnancy progressed, FM/AFA did increase ( $P < 0.001$ ).

## Placenta

### Placental area

Calculated placental area did not differ between AQP8-KO pregnant and wild type mice ( $P=0.862$ ). Placental area visibly increased from 13 GD to 18 GD ( $P < 0.001$ ). However, an interaction between the AQP8 gene and gestational age for placental area was not observed ( $P=0.815$ ).

### Placental weight

Placental weight was dependent on both the AQP8 gene and gestational age ( $P < 0.001$ ). The placenta grew heavier as pregnancy progressed ( $P < 0.001$ ). As summarized in Table 2, the placental weight of AQP8-KO mice was greater in

**Table 2.** Placenta weight (mg) of AQP8-KO and wild type mice at 13, 16 and 18 GD. Values are reported as Mean±SEM.

Mice type	Gestational age		
	13 GD (n=49, 33)	16 GD (n=47, 45)	18 GD (n=42, 44)
AQP8-KO (n=138)	60.275±1.15	101.317±1.67	101.949±1.55
C57 (n=122)	58.568±1.77	87.357±0.99	84.138±1.20
P	0.401	<0.001	<0.001

comparison to age-matched wild type controls, specifically at 16 GD and 18 GD ( $P_{13\text{ GD}} = 0.401$ ,  $P_{16\text{ GD}} < 0.001$ ,  $P_{18\text{ GD}} < 0.001$ ).

## Pathology of the placenta

Placental hematoxylin and eosin staining revealed no differences in structure between AQP8-KO and wild type placentas and no signs of pathology in either genetic background. Placentas of both groups were composed of three trophoblast cell layers: trophoblast giant cells, spongiotrophoblasts and labyrinthine trophoblasts.

## Discussion

Findings reported in the present study suggest that AQP8 deficiency plays an important role in pregnancy outcome. Comparison of the pregnant phenotype of AQP8-deficient mice with that of wild type controls revealed elevations in embryo number, fetus/newborn weight (16 GD, 18 GD, and newborn), and the amount of amniotic fluid at each gestational age.

While a broad tissue distribution of AQP8 has been reported in rat, mouse and human, strong expression has been reported within the female reproductive system, in particular in the placentas and fetal membranes of sheep, human and mouse and in the cervix of the mouse<sup>[16–19]</sup>. The reproductive impact of AQP8 deficiency has been addressed previously. Yang *et al*<sup>[26]</sup> reported that the weight and size of the testes in AQP8-KO mice were remarkably elevated, and a recent study<sup>[27]</sup> from our laboratory has shown elevated fertility in female AQP8-KO mice. Consistent with these studies, data presented here suggested an AQP8-mediated effect on female fertility. A nonsignificant increase in conception rate for AQP8-KO mice (75.47%) was observed compared to wild type mice (64.81%). Furthermore, while embryo number progressively decreased with advancing gestational age in both groups, embryo number was greater in the AQP8-KO group compared to wild type, with a comparable incidence of atrophic embryos. These results may be associated with an increase in follicular maturation and ovulation, as an increase in corpus luteum number has previously been reported in mature AQP8-KO ovaries<sup>[27]</sup>.

Importantly, previous studies<sup>[26, 28]</sup> on AQP8-KO mice have shown that the number, survival, and growth of offspring, as well as urinary concentrating ability, salivary gland fluid secretion ability, pancreatic function and organ weight, do not differ in comparison to wild type mice, with the notable exception of increased testicular weight in AQP8 null mice. Further, osmotically driven water transport, active fluid absorption,

and cholera toxin-driven fluid secretion are unimpaired in AQP8 null mice, with the exception of mild hypertriglyceridemia in null mice in closed intestinal loop measurements<sup>[26]</sup>. However, data presented here indicate that fetal weight increased, together with an enlarged and structurally normal placenta in AQP8 null mice.

The placenta is a special organ, constructed and utilized only during pregnancy. Nearly all material exchanges between mother and fetus take place via the placenta, including those involving nutrients and waste. Our results suggest that AQP8 plays a direct or indirect role in fetal and placental growth, as the fetuses of AQP8-KO mice were significantly heavier than those of wild type controls.

Mann *et al*<sup>[29]</sup> reported that AQP1-KO pregnant mice had a greater volume of amniotic fluid than wild type and heterozygous groups; they speculated that idiopathic polyhydramnios might contribute to amniotic fluid volume regulation and that the AQP1 knockout mice provided a polyhydramnios animal model. In our research, although the amniotic fluid amount also increased in AQP8-KO pregnant mice, the increase in fluid was related to fetal weight, which was confirmed by the consistent and comparable FW/AFA ratios between the two groups.

Ye *et al*<sup>[30]</sup> detected that the embryos from LPA<sub>3</sub>-deficient (lysophosphatidic acid, LPA) uteri were consistently smaller than those from wild-type/heterozygote controls on embryonic days 10.5 and 18.5; however, newborns from LPA<sub>3</sub>-deficient females were heavier. Prolonged pregnancy and/or smaller litter size were presumed to induce the above results. Regarding our research, the number of embryos per female was elevated in AQP8-KO mice, as were fetal/neonatal weights, without post-term delivery. The rise in number of embryos per female may be associated with an increase in follicular maturation and ovulation in AQP8-KO mice<sup>[27]</sup>, and the increase in amniotic fluid and larger placentas may provide a superior environment in utero as well as more nutrients to the fetuses to allow higher fetal/neonatal weights.

Our previous study<sup>[27]</sup> suggested that AQP8-KO mice displayed a greater number of mature follicles via reduced granulosa cell apoptosis, thus increasing the fertility of female mice. However, it remains unclear whether the findings reported here, namely, elevation in fetal/neonatal weight, amount of amniotic fluid and placental weight, are related to reduced apoptosis of placental cells. Further studies are needed to elucidate the molecular mechanism and cellular events occurring in the AQP8-KO placenta.

In conclusion, our collective results provide evidence that AQP8 deficiency plays an important role in pregnancy outcome.

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### Author contribution

Hui-shu LIU and Tong-hui MA designed research; Zheng-fang XIONG, Xiao-yan SHA, and Zheng ZHENG performed the research; Hui-shu LIU and Tong-hui MA contributed new analytical tools and reagents; Xiao-yan SHA analyzed the data; Xiao-yan SHA, Zheng-fang XIONG, and Hui-hu LIU wrote the paper.

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