Moderate maternal folic acid supplementation ameliorates adverse embryonic and epigenetic outcomes associated with assisted reproduction in a mouse model

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Submitted on September 24, 2018; resubmitted on January 14, 2019

STUDY QUESTION: Could clinically-relevant moderate and/or high dose maternal folic acid supplementation prevent aberrant developmental and epigenetic outcomes associated with assisted reproductive technologies (ART)?

SUMMARY ANSWER: Our results demonstrate dose-dependent and sex-specific effects of folic acid supplementation in ART and provide evidence that moderate dose supplements may be optimal for both sexes.

WHAT IS KNOWN ALREADY: Children conceived using ART are at an increased risk for growth and genomic imprinting disorders, often associated with DNA methylation defects. Folic acid supplementation is recommended during pregnancy to prevent adverse offspring outcomes; however, the effects of folic acid supplementation in ART remain unclear.

STUDY DESIGN, SIZE, DURATION: Outbred female mice were fed three folic acid-supplemented diets, control (rodent daily recommended intake or DRI; CD), moderate (4-fold DRI; 4FASD) or high (10-fold DRI; 10FASD) dose, for six weeks prior to ART and throughout gestation. Mouse ART involved a combination of superovulation, *in vitro* fertilisation, embryo culture and embryo transfer.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Midgestation embryos and placentas ($n = 74$ – 99 /group) were collected; embryos were assessed for developmental delay and gross morphological abnormalities and embryos and placentas were examined for epigenetic defects. We assessed methylation at four imprinted genes (*Snrpn*, *Kcnqlot1*, *Peg1* and *H19*) in matched midgestation embryos and placentas ($n = 31$ – 32 /group) using bisulfite pyrosequencing. In addition, we examined genome-wide DNA methylation patterns in placentas ($n = 6$ normal placentas per sex/group) and embryos ($n = 6$ normal female embryos/group; $n = 3$ delayed female embryos/group) using reduced representation bisulfite sequencing (RRBS).

MAIN RESULTS AND THE ROLE OF CHANCE: Moderate, but not high dose supplementation, was associated with a decrease in the proportion of developmentally delayed embryos. Although moderate dose folic acid supplementation reduced DNA methylation variance at certain imprinted genes in embryonic and placental tissues, high dose supplementation exacerbated the negative effects of ART at imprinted loci. Furthermore, folic acid supplements resolved female-biased aberrant imprinted gene methylation. Supplementation was more effective at correcting ART-induced genome-wide methylation defects in male versus female placentas; however, folic acid supplementation also led to additional methylation perturbations which were more pronounced in males.

LARGE-SCALE DATA: The RRBS data from this study have been submitted to the NCBI Gene Expression Omnibus under the accession number GSE123143.

LIMITATIONS REASONS FOR CAUTION: Although the combination of mouse ART utilised in this study consisted of techniques commonly used in human fertility clinics, there may be species differences. Therefore, human studies, designed to determine the optimal levels of folic acid supplementation for ART pregnancies, and taking into account foetal sex, are warranted.

WIDER IMPLICATIONS OF THE FINDINGS: Taken together, our findings support moderation in the dose of folic acid supplements taken during ART.

STUDY FUNDING/COMPETING INTEREST(S): This work was funded by the Canadian Institutes of Health Research (FDN-148425). The authors declare no conflict of interest.

Key words: folic acid supplementation / assisted reproductive technologies / DNA methylation / genomic imprinting / embryo development

Introduction

Although assisted reproductive technologies (ART) have been successful in helping infertile couples have children, these procedures are associated with an increased incidence of adverse outcomes including birth defects (Wen et al., 2012; Hansen et al., 2013) and imprinting disorders (Lazaraviciute et al., 2014). In addition, DNA methylation defects have been reported in ART-conceived children at imprinted genes (Denomme and Mann, 2012) and throughout the genome (Katari et al., 2009; Melamed et al., 2015).

Assisted reproduction takes place during periods in which DNA methylation patterns are highly dynamic. For instance, some techniques such as superovulation are performed during oogenesis, when most sex-specific DNA methylation profiles are established (Denomme and Mann, 2012). Conversely, the timing of other ART procedures, including embryo culture, coincides with preimplantation development where DNA methylation erasure occurs across the genome except at imprinted loci and certain repeat regions (Smith and Meissner, 2013). Therefore, due to the coincidental timing of ART and these important epigenetic reprogramming events, ART may be inducing epigenetic instability and consequently compromising offspring health (Denomme and Mann, 2012).

Methylation reactions are highly dependent on the availability of methyl groups, which may be compromised with low folate intake or the reduced activity of enzymes involved in folate metabolism (Crider et al., 2012). In fact, low folate status is associated with negative pregnancy outcomes, most notably neural tube defects (NTD) (Wald et al., 1991; Czeizel and Dudás, 1992). To prevent such pregnancy complications, many countries fortify the food supply with folic acid (Berry et al., 2010). In addition, periconceptional folic acid supplementation (≥ 0.4 mg per day) is recommended for women of reproductive age (Wilson et al., 2015; Gomes et al., 2016).

The recommended dose of daily folic acid supplements depends on the risk associated with pregnancy, such that in Canada high doses (4 mg/day) are recommended for pregnancies at high risk for an NTD (Wilson et al., 2015). Although the data are inconsistent, there is some concern regarding the safety of high dose folic acid supplements in terms of offspring health. For instance, a daily folic acid intake >5 mg per day during pregnancy was associated with impaired psychomotor development during infancy (Valera-Gran et al., 2014). In addition, it was recently reported that the use of folic acid supplements containing >1 mg per day

was associated with adverse neurocognitive outcomes during early childhood (Valera-Gran et al., 2017). Deleterious effects of high dose folic acid supplementation on offspring outcomes have also been demonstrated in animal models. A folic acid dose 10-fold the recommended intake for rodents, approximately equivalent to the 4 mg/day human recommendation, was associated with embryonic loss, embryonic delay and birth defects at midgestation (Mikael et al., 2013) and compromised short-term memory in juvenile mice (Bahous et al., 2017).

Although the effects of folic acid supplementation on spontaneous pregnancies have been examined, little work has been done to identify the optimal folic acid dose for ART pregnancy outcomes. Determining the effects of various levels of folic acid on ART-conceived progeny is of importance since cross-sectional data suggests that infertile women are consuming more folic acid than fertile women (Murto et al., 2014, 2015). To our knowledge, this study is the first to examine the impact of clinically-relevant doses of folic acid on ART pregnancies using a mouse model. Our aims were to determine whether folic acid supplementation could prevent developmental defects and epigenetic aberrations associated with ART and to assess potential deleterious effects of higher dose supplements.

Materials and Methods

Ethical approval

All animal work was conducted with approval by the Animal Care Committee at the Research Institute of the McGill University Health Centre in Montreal, Quebec.

Animals, diets and rationale with respect to clinical practice

Mice were exposed to a 12-h light:12-h dark cycle and provided with food and water *ad libitum*. Female outbred CFI mice (Envigo) were fed one of three amino acid defined diets (Envigo Teklad): a folic acid control diet (CD, 2 mg/kg diet, TD.130565) containing the recommended level of folate for rodents (Reeves, 1997), a 4-fold folic acid-supplemented diet (4FASD, 8 mg/kg diet, TD.160058) or a 10-fold folic acid-supplemented diet (10FASD, 20 mg/kg diet, TD.160059). CFI donor and recipient females (3 weeks of age) were fed the diet for 6 weeks prior to ART and throughout gestation. All males were fed regular mouse chow diet (4 mg folic acid/kg diet, Envigo Teklad).

The mouse diet regimens were designed to emulate clinical practice. It is recommended that women consume a folic acid supplement from at least 3 months prior to conception to ensure they have achieved a folate status that will optimise NTD risk reduction. Since neural tube closure occurs early in pregnancy (21–28 days post-conception which is often before a woman knows she is pregnant), it is desirable to have a woman take the supplements ahead of conception. Based on our previous studies (Ly *et al.*, 2017; MacFarlane-personal communication), the diets used in our mouse study result in significant differences in circulating folate by 2–3 weeks and tissue folate by 5 weeks, ensuring that by 6 weeks of exposure (pre-conception) the females' folate status should reflect their dietary intake.

Measurement of plasma and red blood cell folate

Upon the sacrifice of CFI females, whole blood was collected by cardiac puncture and kept on ice. Plasma and red blood cell (RBC) sample ($n = 5$ –6 donors/ART group, $n = 4$ –5 recipients/ART) total folate concentrations were measured as previously described (Ly *et al.*, 2017).

ART protocol

The mouse ART protocol was adapted from the Jackson Laboratory method (Behringer *et al.*, 2013) and previously published work (Byers *et al.*, 2006; de Waal *et al.*, 2015). Briefly, CFI females were superovulated (Fortier *et al.*, 2008) and *in vitro* fertilisation was performed using spermatozoa from B6SJLFI/J males (Fig. 1A). Resulting zygotes were cultured in potassium simplex optimisation medium (1X) with 1/2 amino acids (KSOM^{1/2AA}; EMD Millipore) under mineral oil at 37°C in a humidified, reduced oxygen environment (5% CO₂, 5% O₂, 90% N₂) for 4 days. Ten blastocyst-stage embryos from a single CFI donor female were transferred non-surgically (ParaTechs NSET device) to a 2.5-days post-coitum (dpc) pseudo-pregnant CFI recipient female. The day of embryo transfer was denoted as E3.5 and midgestation embryos and placentas were collected 8 days later.

Natural mating protocol

CFI females were fed CD for 6 weeks then naturally mated with B6SJLFI/J males (NAT-CD). Diets were continued throughout gestation. Embryonic day (E) 0.5 corresponded to the day in which a vaginal plug was observed. Naturally-conceived control embryos and placentas were collected 10.5 dpc.

Figure 1 Folic acid supplementation increases folate concentrations in blood. (A) Experimental design where female mice in the naturally-mating (NAT) or ART groups were fed: a control diet (CD), a 4-fold folic acid-supplemented diet (4FASD) or a 10-fold folic acid-supplemented diet (10FASD). Embryos and placentas were collected at midgestation for assessment. The folate concentrations in the red blood cells (RBC) (B) and plasma (C) of diet-exposed females were quantified. Means $\pm$ SEM are shown. Student's *t*-test compared NAT-CD and CD groups; ns signifies no statistically significant difference between groups. One-way ANOVA with Tukey's correction for multiple comparisons was used to compare ART groups; groups with a common letter do not differ significantly and statistical significance was set at $P < 0.05$.

Table 1 Effect of maternal folic acid supplementation on fertilisation rates and embryonic development in pregnancies achieved using ART

Diet group	No. of superovulated donors	Mean no. of oocytes per donor	Mean fertilisation rate (%) ^a	Mean <i>in vitro</i> development rate (%) ^b	No. of recipients ^c	No. of litters	No. of live midgestation embryos collected
CD	20	43 ± 2.9	89 ± 2.7	72 ± 4.0	27	27	99
4FASD	14	43 ± 3.8	84 ± 4.3	70 ± 4.9	18	16	74
10FASD	20	40 ± 2.6	90 ± 3.0	69 ± 3.6	26	24	92

Data are presented as absolute values or means ± SEM; One-way ANOVA with Tukey's correction for multiple comparisons was used to compare the mean no. of oocytes per donor, mean fertilisation rate and mean *in vitro* development rate between experimental groups.

^aFertilisation rate refers to the proportion of cultured oocytes which have been fertilised.

^b*In vitro* development rate refers to the proportion of fertilised embryos which have developed into blastocysts.

^cTen blastocysts were non-surgically transferred to each recipient female.

Examination of embryos and placentas

The number of implantation sites, resorption sites and live embryos were quantified upon the examination of uteri. Pre- and post-implantation loss were determined and the embryos examined for developmental delay and the presence of malformations as previously described (Whidden et al., 2016). Embryos delayed by 2 days or more (i.e. staged E9.5 or less) were denoted as developmentally delayed. Embryos and placentas were snap frozen on dry ice upon collection and stored at −80°C until further use. Yolk sacs were collected and stored at −20°C for embryo sex determination as previously described (Whidden et al., 2016).

Tissue DNA isolation

Genomic DNA was isolated from whole yolk sacs by sodium hydroxide hydrolysis. Frozen tissues (embryos or placentas) were homogenised by mortar and pestle after which genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) or QIAamp DNA Micro kit (Qiagen) for smaller tissues (delayed embryos) as per the manufacturer's protocol.

Pyrosequencing

DNA methylation was quantified by bisulfite pyrosequencing at four imprinting control regions (ICR: *Kcnq1ot1*, *Snrpn*, *Peg1* and *H19*) as previously described (Whidden et al., 2016) and at several regions targeted for reduced representation bisulfite sequencing (RRBS) validation (for primer sequences, see Supplementary Table S1) as previously described (Whidden et al., 2016).

Reduced representation bisulfite sequencing

RRBS libraries were prepared as previously described (McGraw et al., 2015) with a minor modification: quantitative PCR (qPCR) was used instead of regular PCR to perform cycle optimisation and large-scale PCR. Briefly, after two rounds of bisulfite conversion using the EpiTect Bisulfite kit (Qiagen) as per the manufacturer's protocol, qPCR was performed and the amplification curve was used as a reference for cycle optimisation. The qPCR reaction involved the addition of 1 µl SYBR Green Nucleic Acid Stain 5X (Invitrogen) to the regular PCR reaction mix. Large-scale qPCR was performed with the optimised cycle number using the LightCycler 96 System (Roche).

RRBS libraries were sequenced at the McGill University and Genome Quebec Innovation Centre (Montreal, QC) and bioinformatics was carried out as previously described (McGraw et al., 2015). On average, there were 15–20 million sequencing reads, covering 1.8–2.0 million CpGs per sample, with the majority (70% or more) covered >10X. Differentially

methyated tiles (DMTs) were identified using the MethylKit software (version 0.5.3), utilising the Benjamini–Hochberg false discovery-based method for *P*-value correction (significance set at $q \leq 0.01$). RRBS was based on 100 bp tiling with the following inclusion criteria: ≥2 CpGs per tile, ≥10-fold coverage of each tile per sample and DNA methylation differences ≥10% between groups. Gene ontology analysis was performed using ToppGene Suite (Chen et al., 2009, <https://toppgene.cchmc.org/>) to identify the biological processes or cellular components enriched among genic DMTs. RRBS results were used to examine DNA methylation at LINE-1 repetitive elements. We identified loci in common between all four experimental groups that were annotated as LINE-1 elements for both embryonic and placental tissues. Importantly, no minimum methylation threshold was used for this analysis.

Statistical analyses

Graphs were made and statistical analyses of data were performed using GraphPad Prism Version 6.0e (GraphPad Software). Statistical significance was set at $P < 0.05$ for all analyses. Absolute values were compared by Fisher's exact test or Chi-square with Yates' correction as indicated. Methylation variance at each ICR for each diet group was calculated by averaging all CpG variances. The effect of ART was established by comparing the means of the NAT-CD and CD groups using the two-tailed, unpaired student's *t*-test. The effect of folic acid supplementation in ART pregnancies was assessed by comparing the means of the CD, 4FASD and 10FASD groups using the one-way ANOVA with Tukey's correction for multiple comparisons. Figs 4E,F and Supplementary Fig. S3E,F graphs were generated using Rstudio. NAT-CD group data were used to establish a 'normal methylation range' in embryonic and placental tissues for each imprinted locus. The lower limit equals the minimum methylation value −2 SD. The upper limit equals the maximum methylation value +2 SD. Samples were deemed abnormally methylated at an ICR if their mean placental and/or embryonic methylation values did not fall within the ICR's 'normal methylation range'.

Results

Increased folate concentrations in blood after folic acid supplementation

Body weights of mothers were not affected by the maternal diets (Supplementary Fig. S1). RBC folate reflects long-term folate status as it is an indicator of intracellular folate storage; conversely, plasma

Figure 2 Folic acid supplementation does not affect the rate of embryonic loss in assisted reproduction. The effect of folic acid supplementation in assisted reproduction on rates of preimplantation loss (A), post-implantation loss (B) and embryo viability (C) ($n = 8$ for NAT-CD group; $n = 18$ – 27 per ART group). Means $\pm$ SEM are shown. Student's t -test compared NAT-CD and CD groups; $**P < 0.01$, $****P < 0.0001$. One-way ANOVA with Tukey's correction for multiple comparisons was used to compare ART groups; groups with a common letter do not significantly differ and statistical significance was set at $P < 0.05$.

Figure 3 Effects of folic acid supplementation on the rate of embryonic developmental delay and gross abnormalities. (A) The proportion of developmentally delayed embryos for each group. (B) The proportion of embryos exhibiting gross abnormalities per group. (C) Breakdown of embryos with various types of morphological anomalies. Absolute values and percentages are shown. $*P < 0.05$ by Fisher's exact test.

folate reflects circulating folate concentrations. No differences were observed in RBC or plasma folate concentrations between NAT-CD and CD groups (Fig. 1B and C), indicating that ART does not significantly affect folate status. Relative to the CD, exposure to the two folic acid-supplemented diets was associated with significant increases in RBC and plasma folate concentrations (Fig. 1B and C).

Dose-dependent effect of folic acid supplementation on reproductive outcomes

In the ART groups (Fig. 1A), folic acid supplementation did not affect the response to superovulation as the number of ovulated oocytes was similar between diet groups (Table 1). Likewise, no significant

Figure 4 Dose-dependent and sex-specific effects of folic acid supplementation in ART on DNA methylation at imprinting control regions (ICRs). Bisulfite pyrosequencing ($n = 31\text{--}32/\text{group/tissue}$) was used to assess DNA methylation in placentas at *Kcnq1ot1* (A) and *H19* (B) and in embryos at *Kcnq1ot1* (C) and *H19* (D). Embryos and placentas were matched. Each dot/circle corresponds to an individual placenta or embryo. Variance is shown in the upper right corner of each panel. Means $\pm$ SEM are shown. Student's *t*-test compared NAT-CD and CD groups; ***P*

differences in fertilisation rates and *in vitro* development rates were observed between diet groups (Table 1).

Embryonic loss and development ($n = 74\text{--}99$ embryos/diet group) were assessed at midgestation (Supplementary Tables SII–SV for detailed litter characteristics). Embryo crown-rump length was unaffected by maternal folic acid diets (Supplementary Fig. S2). ART was associated with an increase in embryonic mortality, as demonstrated by the significant increase in pre- and post-implantation losses in the CD group compared to the NAT-CD group (Fig. 2A and B). Folic acid supplementation did not affect the rate of embryonic loss (Fig. 2A and B). The ART-induced decrease in embryo viability was unaffected by folic acid supplementation (Fig. 2C).

ART resulted in a significant increase in developmental delay affecting 24.5% of embryos from the CD group versus 11.2% for the NAT-CD group ($P = 0.0116$; Fig. 3A). The 4FASD resolved this adverse ART outcome as demonstrated by the significantly lower proportion of delayed embryos (11.0%) compared with the CD group ($P = 0.0294$; Fig. 3A). Interestingly, 26.1% of live embryos were delayed in the 10FASD group (Fig. 3A).

Neither ART nor folic acid supplementation (4FASD and 10FASD) significantly affected the overall rate of embryonic abnormalities (Fig. 3B) or the specific types of morphological abnormalities (Fig. 3C).

Dose-dependent and sex-specific effects of folic acid supplementation on DNA methylation at imprinting control regions

Imprinted genes are critical for growth, development, neurological processes and placental function (Barlow and Bartolomei, 2014). Therefore, we performed bisulfite pyrosequencing on a representative subset of matched embryos and placentas ($n = 31/\text{group}/\text{tissue}$; Subset details found in Supplementary Table SVI) to examine DNA methylation at imprinting control regions (ICRs) of interest: three maternally-methylated ICRs (*Kcnq1ot1*, *Snrpn* and *Peg1*) and one paternally-methylated ICR (*H19*). Developmentally normal embryos and their corresponding placentas from the NAT-CD group were also examined to determine the normal DNA methylation levels ($n = 16$ per sex).

In placentas, ART was associated with decreased mean methylation and increased methylation variance at all four imprinted loci, as demonstrated by the significant differences between NAT-CD and CD groups (Fig. 4A and B, Supplementary Fig. S3A and B). In embryos, ART similarly led to increased variance at all four imprinted loci (Fig. 4C and D, Supplementary Fig. S3C and D), however lower mean methylation was observed only at the *Peg1* and *H19* ICRs.

Next we assessed whether folic acid supplementation could modify these ART-induced imprinting defects (Fig. 4, Supplementary Fig S3). Neither level of folic acid supplementation affected mean methylation

at the four ICRs for placenta. However, in embryos, the 10FASD resulted in lower methylation levels for *Snrpn*. In both placentas and embryos the 4FASD decreased methylation variance at the *Kcnq1ot1* and *H19* ICRs compared to the CD group (Fig. 4A–D). Methylation variance for *Snrpn* was increased by 10FASD in the placenta and decreased by 4FASD in the embryo. *Peg1* methylation variance was not affected in a major way by either dose of folic acid (Supplementary Fig. S3A–D).

To assess for potential sex differences, matched embryo and placenta ICR methylation profiles were compared between males and females (Fig. 4E and F, Supplementary Fig. S3E and F). We established a normal methylation range for each ICR using methylation data from the NAT-CD group. All samples within both the normal placental and embryonic methylation ranges (i.e. within the rectangles depicted on each graph) were deemed 'normally methylated', while those outside these limits were denoted as 'abnormally methylated'. ART was associated with significant female-biased methylation defects at *Kcnq1ot1*, *Snrpn* and *H19* ICRs, as evidenced by the higher proportion of females exhibiting abnormal methylation relative to males within the CD group (Fig. 4E and F, Supplementary Fig. S3E). Interestingly, this female-bias for acquiring abnormal methylation was no longer observed at the *Kcnq1ot1* (Fig. 4E) and *Snrpn* (Supplementary Fig. S3E) ICRs in embryos exposed to either level of folic acid supplementation or at the *H19* ICR (Fig. 4F) with exposure to the 10FASD.

Effect of assisted reproduction on genome-wide DNA methylation in embryos and placentas

To determine whether ART-induced methylation defects extended beyond imprinted genes, we assessed genome-wide DNA methylation using RRBS. DNA methylation changes from selected DMTs were validated by bisulfite pyrosequencing. Hypomethylation and hypermethylation induced by ART and folic acid supplementation, respectively, validated well (Supplementary Table SVII).

The CD group was compared with the NAT-CD group which allowed us to assess the effect of ART on genome-wide methylation. In placental tissue corresponding to normal embryos, ART-induced 12206 and 10102 DMTs in male and female placentas, respectively. For both sexes, >90% of DMTs demonstrated DNA hypomethylation (Fig. 5A). Among hypomethylated DMTs, females exhibited larger magnitude methylation changes compared with males (Supplementary Fig. S4A). Gene ontology analysis was performed to assess whether ART affected DNA methylation at regions of biological significance. The eight most significantly enriched pathways among hypomethylated genic DMTs for both males and females involved neurodevelopment and cellular morphology, with the strongest enrichment observed in females compared with males (Supplementary Fig. S5A). For hypermethylated genic DMTs,

< 0.01 , *** $P < 0.001$, **** $P < 0.0001$ and ns signifies no statistically significant difference. One-way ANOVA with Tukey's correction for multiple comparisons was used to compare ART groups; groups with different letters significantly differ and statistical significance was set at $P < 0.05$. Compilation of DNA methylation profiles for matched embryos and placentas across *Kcnq1ot1* (4 CpG sites) (E), and *H19* (6 CpG sites) (F) ICRs ($n = 31\text{--}32/\text{group}$). A dot denotes the average methylation across the ICR for one sample. All samples within the rectangle exhibit normal methylation at the particular ICR. Filled circles represent males and hollow circles represent females. Means and group density curves are shown. The sex distributions among abnormally and normally methylated samples were compared by Fisher's exact test within each group. Significant P -values are shown in bold in the tables ($P < 0.05$).

Figure 5 Genome-wide DNA methylation defects induced by ART and corrected by folic acid supplementation. Genome-wide DNA methylation in placentas corresponding to normal embryos ($n = 6$ per sex/group) was compared between the CD group and the NAT-CD group. We identified differentially methylated tiles (DMTs) caused by ART (A). Genome-wide DNA methylation was also assessed in normal ($n = 6$ /group) and delayed ($n = 3$ /group) female embryos to compare the CD group to the NAT-CD group, allowing for the identification of DMTs caused by ART (B). Genome-wide DNA methylation in placentas corresponding to normal embryos ($n = 6$ per sex/group) was assessed for the folic acid supplementation groups and compared with the CD group. We identified ART-specific DMTs corrected by folic acid supplementation and additional diet-specific DMTs (C). Similarly, genome-wide DNA methylation in normal ($n = 6$ /group) and delayed ($n = 3$ /group) female embryos was assessed for the folic acid supplementation groups and compared with the CD group. The number of ART-specific DMTs corrected by folic acid supplementation and the additional diet-specific DMTs are shown (D).

enrichment for biological processes was exclusively observed for female placentas (Supplementary Fig. S5A). These biological pathways were mainly involved in embryonic development and morphogenesis. We also determined whether certain regions susceptible to ART were common to males and females. We identified 1533 hypomethylated and 18 hypermethylated DMTs shared between both sexes (Supplementary Fig. S5B); such shared genic DMTs were enriched for biological pathways related to neurodevelopment.

Since female placentas were the most affected by ART, we examined genome-wide DNA methylation in the corresponding normal female embryos. ART led to far fewer DMTs in the embryo (1517 DMTs) compared to the placenta; however, similarly to the placenta, the majority of DMTs were hypomethylated (Fig. 5B). Upon examining delayed female embryos, strikingly more DNA methylation changes were observed (10520 DMTs), again with a preponderance towards hypomethylation (Fig. 5B). A larger proportion of DMTs in delayed

embryos exhibited magnitude of methylation changes >15%, compared with normal embryos (Supplementary Fig. S4B).

Modifying effect of folic acid supplementation in assisted reproduction on genome-wide DNA methylation in embryos and placentas

Next we examined whether the genome-wide DNA methylation perturbations associated with ART could be resolved by folic acid supplementation. Each of the folic acid supplementation groups (4FASD and 10FASD) was compared to the CD group. We intersected these DMTs with the list of DMTs caused by ART. A tile was deemed to have undergone correction if the methylation change at the DMT went in one direction following ART alone and in the opposite direction following a combination of ART and folic acid supplementation.

In male placentas, 14.5% of DNA methylation defects induced by ART were corrected by exposure to the 10FASD (1768 DMTs) compared with 6.7% for the 4FASD (818 DMTs) (Fig. 5C). In contrast, both supplementation regimens were associated with a similar level of correction in female placentas, as 8.7% (875 DMTs) and 7.1% (719 DMTs) of ART-induced methylation perturbations were corrected by the 4FASD and 10FASD, respectively (Fig. 5C). Folic acid supplementation also demonstrated diet-specific effects, whereby additional DNA methylation defects (unrelated to ART) were more pronounced in male placentas than in female placentas (Fig. 5C). The 10FASD demonstrated more additional diet-specific DMTs compared with the 4FASD for both sexes (Fig. 5C). Among the corrected ART-specific DMTs, we considered tiles exhibiting a rectification of 80–120% in DNA methylation as having undergone effective correction while all others were either under-corrected (<80% corrected) or over-corrected (>120%). For both folic acid-supplemented diets, we observed a greater proportion of effectively corrected ART-specific DMTs in male placentas compared to female placentas (Supplementary Fig. S5C); furthermore, males exhibited more over-correction whereas females demonstrated more under-correction. Pathway analysis revealed that the corrected ART-specific genic DMTs were only enriched for important cellular components in male placentas (Supplementary Fig. S5C). For the additional diet-specific DMTs, biological pathways were also more significantly enriched in males compared with females (Supplementary Fig. S5D).

A similar analysis was performed on female embryos to determine whether folic acid supplementation could rectify DNA methylation defects associated with ART. In normal female embryos, folic acid supplementation with the 4FASD and 10FASD led to 771 and 833 DMTs, respectively, the majority of which were hypermethylated (Supplementary Fig. S5E). Delayed embryos exhibited 6450 and 5766 DMTs when exposed to the 4FASD and 10FASD, respectively. Following both moderate or high dose folic acid supplementation, a greater proportion of ART-induced DNA methylation defects were corrected in delayed embryos (~25%) relative to normal embryos (~18%) (Fig. 5D). Nonetheless, there were also more additional diet-specific DMTs in delayed embryos (Fig. 5D).

The quantification of DNA methylation at long interspersed nuclear elements (LINEs) is often used as a surrogate for global DNA methylation. Using the RRBS data, we examined DNA methylation at LINE-I elements in placentas and embryos as an additional measure of the impact of ART and/or folic acid supplementation. We identified 9020 and 14011 tiles located in LINE-I elements in common between all four experimental groups in the embryo and placenta, respectively.

Similar to the genome-wide results, and most notable for the placenta, ART resulted in significantly decreased LINE-I methylation that was partially ameliorated by the 4FASD (Supplementary Fig. S6).

Discussion

Given that there is little to no empirical evidence suggesting that high dose folic acid supplementation benefits women undergoing ART, it is important to identify the optimal dose of folic acid for successful ART interventions. The aim of this preclinical animal study was to assess whether folic acid supplementation could prevent adverse outcomes associated with ART. Using a mouse model, we demonstrated that moderate dose folic acid supplementation in ART is beneficial, whereas high dose folic acid showed evidence of deleterious outcomes. The results are important as they suggest that ART pregnancies may benefit from some supplementation but there is such a thing as too much folic acid.

Consumption of the folic acid-supplemented diets led to elevated plasma and RBC folate levels in female mice as reported in previous studies (Swayne *et al.*, 2012; Ly *et al.*, 2017). Maternal plasma folate reflects the form transferred to the embryo by the placenta, and showed a clear dose-dependent increase between the three diet groups.

The effects of ART on offspring and epigenetic patterning have been the subject of many studies (e.g. Fortier *et al.*, 2008; Chen *et al.*, 2015; de Waal *et al.*, 2015). Here, we show that ART is associated with increased embryonic loss and embryonic developmental delay. ART-induced ICR hypomethylation may contribute to these adverse developmental outcomes, as many of the examined imprinted loci have been implicated in growth/development. However, a broad effect on epigenetic regulation is also a probable cause as DNA hypomethylation was prominent throughout the genome suggesting that epigenetic abnormalities associated with ART extend beyond effects on imprinted regions alone. This is further supported by the fact that normal female embryos demonstrated subtle genome-wide DNA methylation changes following ART or folic acid supplementation whereas delayed embryos exhibited more severe epigenetic perturbations.

Folic acid supplementation demonstrated a dose-dependent mitigating effect for several ART-dependent endpoints. Moderate dose folic acid supplementation in ART was associated with beneficial effects including decreased embryonic developmental delay, reduced DNA methylation variance at certain ICRs and slightly increased global DNA methylation in embryonic and placental tissues compared with the CD and/or 10FASD. In contrast, high dose folic acid supplementation led to deleterious effects as it further increased ICR methylation variance in embryos/placentas, and further decreased mean methylation at certain ICRs in embryos in comparison to the CD and/or 4FASD. Our results suggest that negative ART outcomes may be related to inadequate methyl donor availability and are rectified upon moderate folic acid supplementation but that too high a dose may be detrimental.

Effects of ART and folic acid supplements were also evident for the repetitive element LINE-I. LINEs are good surrogate markers for global methylation due to their high levels of methylation and their presence at high copy numbers throughout the genome. Our results are in agreement with previous findings from human studies demonstrating that LINE-I methylation is sensitive to ART with hypomethylation reported in placenta (Choux *et al.*, 2017; Ghosh *et al.*, 2017). As in

our study, the absolute differences in methylation between groups in the two recent human ART studies were small, in the range of a few percent. Thus, the partial, small magnitude, but significant correction of LINE-1 methylation levels by the folic acid supplements, in line with the partial correction of the genome-wide DNA methylation we found in our mouse study, may be physiologically relevant and worth examining further in future studies.

High dose folic acid supplementation has also been associated with adverse outcomes in spontaneous pregnancies in mice (Pickell et al., 2011; Mikael et al., 2013). Individuals with high folic acid intake have elevated levels of circulating unmetabolised folic acid (Kelly et al., 1997; Bailey et al., 2010), which is concerning as it may contribute to the downregulation of critical folate pathway enzymes. Through this mechanism, we suspect that maternal exposure to the 10FASD in ART pregnancies in the current study indirectly compromised the provision of methyl donors thus causing detrimental outcomes. Similar to the DNA hypomethylation found in our current study, in a recent human study, an association between high maternal folate status and lower DNA methylation in cord blood of newborns was reported (Joubert et al., 2016).

There was evidence of sex-specific effects of ART on imprinted genes. ART alone resulted in a greater proportion of females than males showing abnormal methylation of three out of four of the imprinted genes examined. Similarly, in a recent human study comparing placentas from control versus ART pregnancies, where ART involved blastocyst transfer, hypomethylation of imprinted genes was more likely to occur in females than males (Choufani et al., 2018). Superovulation (Whidden et al., 2016) and prolonged embryo culture (Mann et al., 2004), coinciding with key epigenetic reprogramming events, are factors that have been implicated in perturbing early epigenetic patterning. DNA methylation is required for X chromosome events in female preimplantation embryos, which in turn may make them more vulnerable to conditions that interfere with enzymes or mechanisms involved in methylating DNA, leading to hypomethylation (Bestor, 2003; Whidden et al., 2016).

Sex-specific effects were also observed following the use of folic acid supplementation. For both the 4FASD and 10FASD groups, the predominance of females versus males with abnormal methylation of *Kcnq1ot1* and *Snrpn* was no longer seen. We suggest that this observation may be related to preimplantation female embryos' increased need for methyl groups as discussed above. While limited to the small number of samples studied, the genome-wide methylation data also showed evidence of sex-specific responses to folic acid. For instance, there was more under-correction of ART-specific DMTs in females than males and more induction of additional diet-specific DMTs in males than in females. Whether such sex-specific epigenetic responses have functional consequences for offspring will need further study. To our knowledge, no human studies have examined sex-specific differences in epigenetic patterning associated with folic acid supplementation.

Our results also demonstrate that ART affects the epigenome more drastically in placentas than embryos, a finding described previously (Mann et al., 2004; de Waal et al., 2015). The higher susceptibility of the placenta versus the embryo to ART-induced epigenetic defects may reflect the fact that the embryo is more protected from nutrient fluctuations and/or that the placenta is more resilient and can survive with higher levels of epigenetic variability (Mann et al., 2004).

In summary, we demonstrate that ART is associated with adverse developmental and epigenetic outcomes in the next generation, some of which can be mitigated by moderate folic acid supplementation. However, above a certain threshold, folic acid supplementation is no longer beneficial but instead becomes deleterious to ART-conceived embryos. This is of clinical importance since women undergoing infertility treatments are more likely to take folic acid supplements than fertile women (Murto et al., 2014, 2015). We also suggest that, in a mouse model, the impact of folic acid supplementation may be sex-dependent, such that female embryos appear to benefit more than males, with males being more susceptible to non-specific folic acid effects. Taken together, our findings support moderation in the dose of folic acid supplements taken during ART.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Acknowledgements

We would like to thank Dr Marisa Bartolomei and Ms Mitra Cowan for their advice in establishing our ART protocol. We thank Dr Loydie Jerome-Majewska for assessment of embryonic developmental delay and morphological abnormalities. We thank Dr Francesco Marchetti and Dr Anne Marie Gannon at Health Canada for critically reviewing our manuscript. We are grateful to the team at the McGill University and Genome Quebec Innovation Centre for performing the sequencing required for our RRBS experiments.

Authors' roles

S.R., J.M. and J.M.T. designed the study. S.R., J.M., G.K., C.A. and N.A.B. performed the experiments. S.R., D.C., J.M. and C.A. analyzed the data. S.R. interpreted the data and wrote the manuscript. A.J.M. provided advice regarding study design. J.M.T. and A.J.M. revised the manuscript.

Funding

This work was supported by a grant from the Canadian Institutes of Health Research (CIHR) to J.M.T. (FDN-148425). This research was enabled in part by support provided by Calcul Québec (www.calculquebec.ca) and Compute Canada (www.computeCanada.ca). S.R. was supported by the E. Belanger Fellowship from the McGill Faculty of Medicine, a Studentship from the McGill Centre for Research in Reproduction and Development and a RI-MUHC-Desjardins Studentship in Child Health Research. G.K. was supported by a Ferring Foundation Fellowship.

Conflict of interest

The authors declare no conflict of interest.

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