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Maternal choline supplementation during murine pregnancy modulates placental markers of inflammation, apoptosis and vascularization in a fetal sex-dependent manner

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ABSTRACT

Introduction: Normal placental vascular development is influenced by inflammatory, angiogenic and apoptotic processes, which may be modulated by choline through its role in membrane biosynthesis, cellular signaling and gene expression regulation. The current study examined the effect of maternal choline supplementation (MCS) on placental inflammatory, angiogenic and apoptotic processes during murine pregnancy.

Method: Pregnant dams were randomized to receive 1, 2 or 4 times (X) the normal choline content of rodent diets, and tissues were harvested on embryonic day (E) 10.5, 12.5, 15.5 or 18.5 for gene expression, protein abundance and immunohistochemical analyses.

Results: The choline-induced changes in the inflammatory and angiogenic markers were a function of fetal sex. Specifically, 4X (versus 1X) choline reduced the transcript ($P \leq 0.05$) and protein ($P \leq 0.06$) expression of TNF-a and IL-1β in the male placentas at E10.5 and E18.5, respectively. In the female placentas, 4X (versus 1X) choline modulated the transcript expression of *Il1b* in a biphasic pattern with reduced *Il1b* at E12.5 ($P = 0.045$) and E18.5 ($P = 0.067$) but increased *Il1b* at E15.5 ($P = 0.031$). MCS also induced an upregulation of *Vegfa* expression in the female placentas at E15.5 ($P = 0.034$; 4X versus 2X) and E18.5 ($P = 0.026$; 4X versus 1X). MCS decreased ($P = 0.011$; 4X versus 1X) placental apoptosis at E10.5. Additionally, the luminal area of the maternal spiral arteries was larger ($P \leq 0.05$; 4X versus 1X) in response to extra choline throughout gestation.

Discussion: MCS during murine pregnancy has fetal sex-specific effects on placental inflammation and angiogenesis, with possible consequences on placental vascular development.

1. Introduction

The placenta is the organ of pregnancy that mediates nutrient and oxygen supply to the developing fetus, and is therefore a critical determinant of fetal growth and development. Efficient placental transport requires proper remodeling of the maternal uterine spiral arteries and the development of a vascular network within the chorionic villi (in human placenta) or labyrinth (in mouse placenta) [1,2]. When placental vascularization is compromised, the placenta is unable to provide sufficient nutrients and oxygen to the developing fetus, which increases the risk of fetal growth restriction and abnormal birth weight [1].

Normal placental vascular development is influenced by the balance of pro- and anti-angiogenic factors. Pro-angiogenic factors

Abbreviations: Vegfa, vascular endothelial growth factor; Pgf, placental growth factor; sFLT1, soluble fms-like tyrosine kinase-1; sENG, soluble endoglin; Tnf, tumor necrosis factor alpha; Il6, interleukin 6; Il1b, interleukin 1 beta; Il10, interleukin 10; MCS, maternal choline supplementation; NSA, non-Swiss Albino; DMG, dimethylglycine; TMAO, trimethylamine *N*-oxide; Nfkb1, nuclear factor of kappa light polypeptide gene enhancer in B-cells; Eng, endoglin; Mmp14, matrix metalloproteinase 14; Tbp, TATA box binding protein; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; SMA, smooth muscle actin.

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such as vascular endothelial growth factor (VEGF) and placental growth factor (PGF) play a regulatory role in the growth and proliferation of endothelial cells, angiogenesis and vasodilation while anti-angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFLT1) and soluble endoglin (sENG) interfere with normal pro-angiogenic signaling, disrupt endothelial tube formation and damage the placental vasculature [3,4]. The inflammatory milieu also plays a role in placental vascular development. Heightened levels of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-a) and interleukin 6 (IL-6) have been shown to cause endothelial cell dysfunction, reduce vascular relaxation, inhibit trophoblast invasion into the maternal decidua and adversely affect placental vascularization [4–6].

Abnormal angiogenesis and inflammation may be causal in pregnancy disorders such as preeclampsia. Aberrant expression of these proteins and others including interleukin 1 beta (IL-1β) and interleukin 10 (IL-10) is detected among women with placental dysfunction [7–11]. Recent work also reveals that placental angiogenesis and inflammation may be a sexual dimorphic phenomenon, underscoring the importance of considering fetal sex when studying these placental markers [12–14].

Choline is an essential micronutrient required for membrane biosynthesis and cellular signaling, and plays a regulatory role in gene expression via epigenetic processes (e.g., DNA and histone methylation) [15]. Consequently, choline may modulate physiological processes such as inflammation, angiogenesis and apoptosis that are central to placental function and fetal development [15–17]. Notably, we have shown an effect of choline on these processes in a cell culture model of extravillous human trophoblast cells where increasing choline concentrations decreased the abundance of pro-inflammatory, anti-angiogenic and pro-apoptotic markers [18]. Similarly, we found that supplementing the maternal diet of healthy pregnant women with extra choline (930 vs. 480 mg/d) throughout the third trimester of pregnancy decreased placental production and circulating concentrations of sFLT1 [19]. However, apart from the choline-induced reduction in placental sFLT1 expression, it is unknown whether maternal choline supplementation (MCS) can influence inflammatory, angiogenic and apoptotic processes in an *in vivo* model of normal pregnancy. A better understanding of the functional role of choline in placental vascular development is also needed. Accordingly, we conducted a choline supplementation study in pregnant mice and examined biomarkers of placental inflammation, angiogenesis, and apoptosis at four gestational time points. We also conducted a preliminary histological investigation to examine the effect of MCS on vascular indicators within the maternal decidua and the feto-placental unit.

2. Materials and methods

2.1. Mice and diets

All animal protocols and procedures used in this study were approved by the Institutional Animal Care and Use Committees at Cornell University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Adult male and female non-Swiss Albino (NSA) mice were purchased from Harlan (Indianapolis, IN). The animals were housed in microisolator cages (Ancare) in an environmentally-controlled room (22–25 °C and 70% humidity) with a 12-h light-dark cycle. The mice in the breeding colonies were given *ad libitum* access to a commercially available rodent chow and water. After weaning at 3 weeks of age, both females and males were given *ad libitum* access to the AIN-93G purified rodent diet (Dyets no. 103345; Dyets, Bethlehem, PA) containing 1.4 g choline chloride/kg diet (1X choline diet). This dietary regimen was continued until five days prior to mating at which time female mice were randomized to the 1X choline diet, a 2X choline diet containing 2.8 g choline chloride/kg diet (Dyets no. 103346; Dyets, Bethlehem, PA), or a 4X choline diet containing 5.6 g choline chloride/kg diet (Dyets no. 103347; Dyets, Bethlehem, PA). These dosages were selected based on our studies conducted in third-trimester pregnant women showing a choline lowering effect on sFLT1 with 2X choline supplementation [19] and evidence from rodent studies reporting improvements in brain development in the adult offspring whose mothers were supplemented with 4X choline [20]. Day of conception was determined by the presence of a vaginal plug and was defined as gestational day (E) 0.5. The female mice continued to consume their assigned diet until they were euthanized at one of four gestational time points (i.e., E10.5, E12.5, E15.5 or E18.5; n = 6–8 dams/treatment group/time point).

2.2. Tissue collection and processing

Maternal blood was collected by cardiac puncture into microtainer collection tubes with clot activator and SST gel (Becton Dickinson, Franklin Lakes, NJ), and was allowed to clot at room temperature for one hour. The sample was then centrifuged at 14,000 rpm for 6 min, and the serum was collected and stored at −80 °C. Maternal liver was removed, immediately frozen in liquid nitrogen and stored at −80 °C. The gravid uterus was removed, the fetuses and placentas were then carefully dissected and weighed. One-third of the placental disks were fixed in 10% formalin for histology analysis, while the remaining placental disks were cut in half across the chorionic plate and placed in RNA*later* or immediately frozen in liquid nitrogen and stored at −80 °C. The fetuses were imaged to obtain crown rump measurements using the Image J Analysis Software (NIH). Fetal DNA was extracted and subjected to PCR using a commercial kit (Qiagen) for sex determination (Supplemental Table 1).

2.3. Measurement of choline metabolites in maternal liver

The concentrations of choline and its metabolic derivatives [betaine, dimethylglycine (DMG) and trimethylamine *N*-oxide (TMAO)] were measured in maternal liver obtained at the last study time point (i.e.: E18.5) by LC/MS according to the method of Holm et al. [21] with modifications based on our equipment [22].

2.4. Quantification of placental transcript abundance

Total RNA was extracted from the placental tissues fixed in RNA*later* by TRIzol reagent (Invitrogen). Reverse transcription was performed using ImProm-II Reverse Transcription System (Promega) with the following reaction conditions: 25 °C for 10 min, 42 °C for 40 min and 95 °C for 5 min. Quantitative PCR was performed using the SYBR Green system in Roche LightCycler480. All primers for the targeted genes (*Tnf, Il1b, Il6, Il10, Nfkb1, Vegfa, Pgf, sFlt1, Eng, Mmp14*) were designed using Primer-BLAST available on the NCBI website (Supplemental Table 1). These genes were selected because of their importance in placental development and association with adverse pregnancy outcomes [5,7–11,23,24] and their responsiveness to choline in prior investigations [18,19]. The reaction conditions were as follows: 95 °C for 5 min, followed by 40 cycles with 15 s at 95 °C, 30 s at 63 °C, and 30 s at 72 °C. To ensure the specificity of the PCR product, a dissociation stage was included at the end of the amplification cycles. Data are expressed by the $\Delta\Delta C_t$ method, in which the expression level of the gene of interest is normalized to the expression level of the housekeeping gene and presented as fold change before comparison between samples. TATA box binding protein, *Tbp*, was selected as the housekeeping gene because its expression is stable in placental tissue [25] and remains unchanged