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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.

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Keywords

Choline; Placenta; Inflammation; Angiogenesis; Fetal Sex

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 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.

MATERIALS AND METHODS

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-hour 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 1.4g 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 2.8g choline chloride/kg diet (Dyets no. 103346; Dyets, Bethlehem, PA), or a 4X choline diet containing 5.6g 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).

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 minutes, 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).

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].

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 minutes, 42°C for 40 minutes and 95°C for 5 minutes. 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 minutes, followed by 40 cycles with 15 sec at 95°C, 30 sec at 63°C, and 30 sec 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 by the expression level of the housekeeping gene 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 under different choline intake levels [17].

Quantification of placental protein abundance

To evaluate the protein abundance of IL-1β, TNF-a and NF-κB in the placenta, frozen placental samples were homogenized in ten volumes of buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.5% IGEPAL CA-630 (Santa Cruz Biotechnology)] containing protease inhibitor cocktails (Sigma-Aldrich). The homogenates were centrifuged at 13,200 rpm for 25 minutes at 4°C. The total protein concentration in the supernatant was quantified by the Bradford assay (Thermo Scientific Pierce). Protein was loaded onto SDS-PAGE gel, subjected to electrophoresis, and then transferred onto Immobilon FL PVDF membranes (EMD Millipore). Membranes were blocked in blocking buffer (LI-COR). The membranes were then incubated overnight with primary antibodies for IL-1β (1:200; Santa Cruz Biotechnology), TNF-a, NF-κB or β-actin (1:200, 1:1000 and 1:5000, respectively; Cell Signaling Technology), after which secondary antibodies (IRDye 800CW goat anti-rabbit and IRDye 680RD goat anti-mouse (LI-COR), 1:10,000) were added to the membranes. Protein bands were visualized and quantified by the Odyssey imaging system (LI-COR). Data are expressed as the ratio of the intensity of targeted protein to the intensity of β-actin and compared between samples.

Measurement of circulating angiogenic factors in maternal serum

Circulating concentrations of sFLT1 and sENG in the maternal serum were measured using commercial ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Assessment of placental apoptosis

The placental tissues were fixed in 10% formalin, paraffin embedded and sectioned at 10μm. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was conducted using a commercial kit (Millipore, Billerica, MA) to assess placental apoptosis. The total number of cells and the number of TUNEL-positive cells in the placenta were quantified by the Aperio ImageScope software to determine the percentage of TUNEL-positive cells.

Assessment of maternal spiral artery area and placental labyrinth vasculature

Some formalin-fixed sections were subjected to immunohistochemistry as described previously [26]. To identify maternal spiral arteries for area evaluation, the placental sections were incubated with a smooth muscle actin (SMA) antibody (1:50, DakoCytomatin, Glostrup, Denmark), followed by incubation with a secondary antibody. All stained sections were imaged on an Aperio Scanscope (Vista, CA). The maternal spiral arteries were defined manually, and their areas were quantified using the Aperio ImageScope software. Data on the spiral artery area are presented as a ratio of the luminal area to the total vessel area.

To evaluate the vascular structure in the placental labyrinth, the placental sections were incubated with isolectin (1:100, Vector Laboratories, Burlingame, CA), which is a marker of the endothelial cells and has been used to stain the vasculature in other mouse tissues [27, 28], and then counterstained with hematoxylin. The placental labyrinth compartment was defined manually, and the intensity of the isolectin staining was determined using the Aperio ImageScope software. Data are expressed as the staining intensity per unit area of placental labyrinth.

Statistical analysis

Fetal measurements and the placental transcript and protein data were analyzed separately for each gestational day and fetal sex using a mixed linear model. Because some fetuses were fixed in formalin together with their placentas, fetal DNA was degraded and was not available for sex genotyping. Therefore, histology data were analyzed without stratifying by fetal sex. All mixed linear models included choline treatment as an independent fixed effect and maternal identification as an independent random effect. Litter size was included in the model as a covariate when it achieved $P \leq 0.05$. For the maternal measurements, data were analyzed separately for each gestational day using one-way ANOVA. The model included choline treatment as an independent fixed effect, and litter size as a covariate when it had a $P \leq 0.05$. Correlations between the choline metabolites in maternal liver and placental inflammatory or angiogenic markers at E18.5 were assessed using Pearson's correlation analysis (with log-transformed variables as needed). Bonferroni correction was used to adjust for multiple comparisons. Data are presented as means ± SEM. SPSS software, Version 23 (SPSS Inc, Chicago, IL) was used to perform the statistical analysis and

differences were considered statistically significant when $P_{adjusted} \leq 0.05$. Given that we hypothesized (*a priori*) that supplementing the maternal diet with extra choline would influence the outcome variables, unadjusted *P*-values ($P_{unadjusted}$) are also presented for variables whose significance was lost after adjusting for multiple testing.

RESULTS

Concentrations of choline (and its metabolites) in the maternal liver

Maternal liver concentration of choline was higher in response to MCS, but only the difference between 1X and 4X choline groups remained significant after adjusting for multiple testing (4X vs 1X choline: $P_{adjusted} \leq 0.001$; 4X vs 2X choline: $P_{unadjusted} = 0.02$, $P_{adjusted} = 0.06$; 2X vs 1X choline: $P_{unadjusted} = 0.032$, $P_{adjusted} = 0.09$; Figure 1A). Maternal liver concentrations of betaine, DMG and TMAO were higher in response to 2X and 4X choline ($P_{adjusted} < 0.05$ vs 1X choline; Figure 1B-1D).

Placental inflammation

In the female placentas, MCS influenced the transcript abundance of *Il1b* with the 4X choline group having lower abundance at E12.5 ($P_{adjusted} = 0.045$ vs 1X choline) and higher abundance at E15.5 ($P_{adjusted} = 0.031$ vs 1X choline; $P_{adjusted} = 0.006$ vs 2X choline). A lower *Il1b* transcript abundance in response to 4X choline was also detected at E18.5 ($P_{unadjusted} = 0.022$ vs 1X choline), but this difference was lost after adjusting for multiple testing ($P_{adjusted} = 0.067$ vs 1X choline) (Figure 2A). Protein concentrations of IL-1β exhibited expression patterns that mirrored those of mRNA abundance at E12.5 (4X vs 1X choline: $P_{unadjusted} = 0.039$, $P_{adjusted} = 0.11$), E15.5 (4X vs 2X choline: $P_{unadjusted} = 0.041$, $P_{adjusted} = 0.12$), and E18.5 (4X vs 1X choline: $P_{unadjusted} = 0.022$, $P_{adjusted} = 0.065$) (Figure 2B-C).

Nfkb1 transcript abundance in the female placentas was higher at E18.5 in the 4X choline group ($P_{adjusted} = 0.014$ vs 1X choline). Protein concentration of NF-κB exhibited an expression pattern similar to mRNA abundance but did not achieve statistical significance (4X vs 1X choline: $P_{unadjusted} = 0.059$, $P_{adjusted} = 0.177$) (Figure 3A-B). MCS had no detectable effects on the transcript abundance of *Tnf, Il6* and *Il10* ($P \geq 0.12$) in the female placentas at any time points. Correlation analyses indicated a modest but significant negative correlation ($r = -0.54$, $P = 0.02$) of *Il1b* abundance in the E18.5 placentas with TMAO concentration in the maternal liver. The placental *Nfkb1* transcript abundance at E18.5 was also positively associated with the concentrations of choline ($r = 0.7$, $P = 0.001$), betaine ($r = 0.65$, $P = 0.004$) and DMG ($r = 0.48$, $P = 0.044$) in the maternal liver.

In the male placentas, 4X choline decreased the transcript abundance of *Il1b* at E18.5 ($P_{adjusted} = 0.035$ vs 1X choline) (Figure 4A). The protein abundance of the precursor form of IL-1β was also reduced in the 4X choline group ($P_{adjusted} = 0.01$ vs 1X choline). Similarly, a reduction in the mature form of IL-1β was detected in the 4X choline group ($P_{unadjusted} = 0.035$ vs 1X choline) but statistical significance was lost after adjusting for multiple testing ($P_{adjusted} = 0.1$ vs 1X choline; Figure 4B).

The male placentas in the 2X and 4X choline groups also had lower ($P_{adjusted}$ = 0.008 and 0.033 vs 1X choline, respectively) transcript abundance of *Tnf* at E10.5. Similarly, the protein concentration of TNF-a was lower in the 2X and 4X choline groups at E10.5 ($P_{unadjusted}$ = 0.05 and 0.02 vs 1X choline, respectively) but statistical significance was lost after adjusting for multiple testing ($P_{adjusted}$ = 0.15 and 0.06 vs 1X choline, respectively) (Figure 4C-D). MCS had no effects ($P \geq 0.1$) on the transcript abundance of *Il6, Il10* and *Nfkb1* or the protein concentration of NF-κB in the male placentas.

Placental angiogenic markers

In the female placentas, a higher expression of *Vegfa* was observed at E15.5 ($P_{adjusted}$ = 0.034 vs 2X choline) and E18.5 ($P_{adjusted}$ = 0.026 vs 1X choline) in response to 4X choline (Figure 5). Correlation analyses showed significant modest correlations between *Vegfa* abundance in the E18.5 placentas and all four choline metabolites in the maternal liver (choline: r = 0.57, P = 0.014; betaine: r = 0.48, P = 0.045; DMG: r = 0.51, P = 0.032; TMAO: r = 0.58, P = 0.011). MCS had no detectable effects on the transcript abundance of *Pgf, sFlt1*, *Mmp14* and *Eng* ($P \geq 0.1$).

In the male placentas, *sFlt1* transcript abundance tended to be lower in response to 2X and 4X choline ($P_{unadjusted}$ = 0.07 vs 1X choline) at E18.5; however, this tendency was not detected after adjusting for multiple testing ($P_{adjusted}$ = 0.22 vs 1X choline). Other angiogenic factors in the male placentas remained unchanged ($P \geq 0.1$) in response to MCS.

Maternal circulating concentration of sFLT1 and sENG

4X choline decreased sFLT1 concentration in the maternal serum at E18.5 ($P_{unadjusted}$ = 0.05 vs 1X choline), but this difference was lost after adjusting for multiple testing ($P_{adjusted}$ = 0.15 vs 1X choline). MCS did not affect the concentration of sENG ($P \geq 0.5$) in the maternal serum.

Placental apoptosis

Fewer TUNEL-positive cells were detected in the placentas of the 2X ($P_{adjusted}$ = 0.04 vs 1X choline) and 4X choline ($P_{adjusted}$ = 0.011 vs 1X choline) groups at E10.5 (Figure 6A-B). No effects of MCS ($P \geq 0.18$) were detected on the apoptotic index in the placentas at any other time points (Figure 6A).

Placental vasculature

Placentas from the 2X and 4X choline groups exhibited a larger ($P_{adjusted} \leq 0.05$) maternal spiral artery luminal area across all four gestational time points as compared to the 1X choline group (Figure 6C-D). The isolectin staining intensity in the placental labyrinth did not differ in response to MCS at any of the gestational time points ($P \geq 0.13$).

Phenotypic measurements of the fetus and the placenta

Fetal weight and crown rump measurements were not affected ($P \geq 0.28$ and $P \geq 0.6$, respectively) by MCS. Maternal choline treatment also had no effects on placental weight ($P \geq 0.23$) or placental efficiency (the ratio of fetal weight to placental weight; $P \geq 0.19$) (Supplemental table 2).

DISCUSSION

Previous investigations from our group have shown that extra dietary choline during the third trimester of human pregnancy suppresses placental production of an anti-angiogenic factor sFLT1 [19] while choline inadequacy in a cell culture model leads to a molecular profile that impairs trophoblast function and *in vitro* angiogenesis [18]. In the current study, we show effects of MCS on placental markers of inflammation, angiogenesis and apoptosis, all of which can influence placental vascular development. We also demonstrate that most of these choline-induced effects manifest in a fetal sex- and gestational day-dependent manner. Finally, we present preliminary *in vivo* evidence suggesting that a higher maternal choline intake during murine pregnancy improves remodeling of the maternal spiral arteries, a finding that merits additional investigation in the future.

MCS alters the placental abundance of inflammatory and angiogenic markers in a fetal sex- and gestational day-dependent manner

Choline is an essential nutrient known to have an important role in fetal development [15]. In rodent studies, offspring from dams who received 4X choline (as compared to 1X choline) during pregnancy have improved cognitive function and attenuated age-related memory decline [20]. These neuroprotective consequences of extra maternal choline have been associated with inflammatory and angiogenic processes in the nervous system [17, 29]. We extend these findings to the mouse placenta whereby maternal choline supply modulated these same biological processes but in a manner that was dependent on fetal sex and gestational time point. Because aberrant expression of the inflammatory and angiogenic markers is associated with placental dysfunction, the choline-induced changes of these markers shown in the present study may have important clinical implications on pregnancy outcomes.

One striking difference between male and female placentas in response to MCS was the expression of the pro-inflammatory cytokine *Il1b*. In the female placentas, 4X (versus 1X) choline induced a 40% reduction at E12.5, a 43% increase at E15.5, and a 30% reduction at E18.5. Although statistical significance was not achieved after adjusting for multiple testing, IL-1β protein abundance exhibited an expression pattern that paralleled those of the transcript. In contrast, IL-1β expression remained largely unchanged in the male placentas until E18.5, when 4X choline yielded a 26% reduction in *Il1b* transcript abundance and a 55% reduction in IL-1β protein abundance as compared to 1X choline. As some immune responses are shown to be more active and stronger in females compared to males [30], we speculate that the less pronounced effects of MCS on the expression of IL-1β in the male placentas may relate to the sex-specific differences in immune regulation. Notably, however, the sex-specific immune response to maternal choline may also be cytokine dependent, as suggested by the downregulation of *Tnf* expression in the male placentas at E10.5, but not in the female placentas, in response to 4X choline supplementation.

The observed choline-induced downregulation of placental pro-inflammatory cytokines at several gestational time points may be beneficial to placental development. Excessive production of placental TNF-a and IL-1β have been shown to impair vascular remodeling [31] and increase the risk of adverse pregnancy outcomes in both animals [5] and humans [9,

32, 33]. Consistent with these data, pharmacological targeting of these pro-inflammatory cytokines in animal models reverses some of the placental vascular abnormalities and improves pregnancy outcomes [34, 35]. Therefore, supplementing the maternal diet with extra choline may be a nutritional strategy for lowering the risk of developing pregnancy disorders characterized by an intensified placental pro-inflammatory response.

As our prior investigation in extravillous human trophoblasts [18] found that cells cultured in a medium deficient in choline have an increased abundance of NF-κB, we expected that placental *Nfkb1* expression would be downregulated in response to MCS in the present study. Contrary to our hypothesis, the transcript abundance of *Nfkb1* at E18.5 was 28% higher in the 4X choline group, and this change was detected only in the female placentas. Although statistical significance was not achieved, the protein abundance in these placentas also showed a 30% increase, which was comparable to the change detected at the transcript level. The reason for this sex-specific difference and choline-induced upregulation of placental *Nfκb1* is unclear. However, consistent with the greater investment of female placentas in the maintenance of pregnancy [36], we hypothesize this induction of a pro-inflammatory state during late gestation may facilitate nutrient transport to the rapidly growing fetus [37].

MCS also affected the transcript abundance of angiogenic proteins in the female placentas, as evidenced by an approximately 30% upregulation of the pro-angiogenic factor *Vegfa* in late gestation. VEGF promotes endothelial cell proliferation and new blood vessel formation, and stimulates relaxation of the vascular system by increasing the production of nitric oxide [4]. Notably, reduced expression of VEGF is observed in placentas from preeclamptic women as compared to placentas from normotensive women [38], and adenoviral-mediated delivery of Vegf in a mouse model of preeclampsia resolves the maternal preeclamptic phenotype [23]. Taken together, these data suggest that the choline-induced increase of *Vegfa* may beneficially influence placental angiogenic balance, vascular development and pregnancy outcome.

Although the objectives of the present study did not focus on exploring the mechanisms by which extra choline affects placental inflammatory and angiogenic processes, we suggest that some of these choline-induced effects are mediated by signaling pathways related to acetylcholine and protein kinase C (PKC). Choline is a precursor of acetylcholine and we have shown that MCS during pregnancy increased the placental concentration of acetylcholine and upregulated placental cholinergic receptor muscarinic 4 (*CHRM4)* expression [19, 22]. Others have shown that acetylcholine can signal through the alpha-7 nicotinic acetylcholine receptor, resulting in the recruitment of VEGF and blood vessel formation [39] as well as the reduction of pro-inflammatory cytokines [40]. Furthermore, biosynthesis of phosphatidylcholine from choline can prevent the accumulation of diacylglycerol and subsequent activation of PKC [15] which induces TNF-a production [41] and attenuates the actions of VEGF [42, 43]. In previous work, we demonstrated that the addition of a PKC inhibitor partially rescues aberrant *IL1B* expression induced by choline inadequacy in a cell culture model of extravillous human trophoblasts [18]. Because choline can be oxidized to generate the methyl donor betaine, it may be possible that an epigenetic mechanism is also involved in mediating these choline-induced effects.

The reasons for the sexual dimorphic placental response to MCS are also unclear, but it may relate to different rates of fetal development and different strategies to meet nutrient demands [44]. Regardless, these observations are consistent with the theory of fetal programming suggesting that female fetuses tend to generate a more adaptive response to environmental triggers (such as maternal diet) and invest more resources in developing their placentas [44, 45].

MCS decreases placental apoptosis in early gestation

Consistent with our prior investigation in extravillous human trophoblasts [18], we found that supplementing the maternal diet with 2X and 4X choline decreased placental apoptosis at E10.5 by 57% and 68%, respectively. This reduction may be beneficial because it could increase the survival of trophoblasts and endothelial cells thereby enhancing the development of the placental vasculature.

MCS increases the luminal area of the maternal spiral arteries

In the present study, we found that placentas from the 4X choline supplemented groups exhibited larger maternal spiral artery luminal areas than the 1X choline group. To the best of our knowledge, these data are the first *in vivo* evidence indicating extra maternal choline may improve remodeling of the maternal spiral arteries. Nonetheless, blood flow measurements are needed to determine if the choline-induced increase in luminal area leads to enhanced uteroplacental perfusion.

Conclusion

Supplementing the maternal diet of mice with extra choline influences placental inflammatory, angiogenic and apoptotic processes, with possible consequences on placental vascular development. Of note, most of these choline-induced effects occur in a fetal sex- and gestational day-dependent manner, highlighting the importance of these variables in studies that examine the effects of dietary manipulation on placental development. A higher maternal choline intake also increased the luminal area of the maternal spiral arteries, which may influence placental perfusion. Overall, our data provide additional support for increasing maternal choline intake during normal pregnancy as a nutritional strategy to improve placenta-related pregnancy outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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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Highlights:

- Choline alters the abundance of inflammatory, angiogenic and apoptotic markers in the mouse placenta.
- The placental responses to maternal choline supplementation are fetal sex- and gestational day-dependent.
- Maternal choline supplementation increases the maternal spiral artery luminal area, which may enhance placental perfusion.

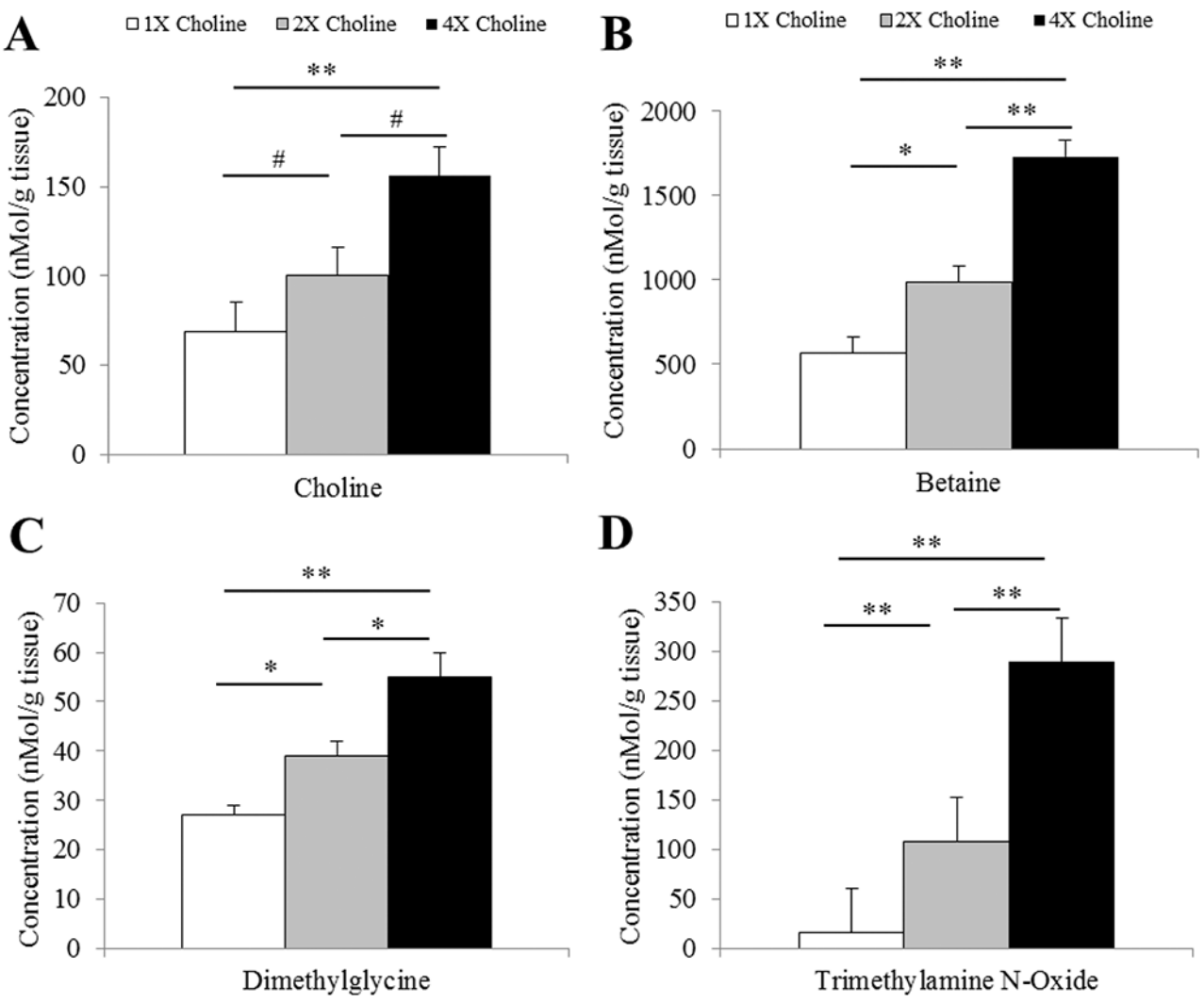

Figure 1.

Maternal hepatic concentrations of **A)** choline, **B)** betaine, **C)** dimethylglycine and **D)** trimethylamine *N*-oxide at E18.5 in response to three different choline treatments (1X, 2X and 4X). Data were analyzed using ANOVA followed by post-hoc Bonferroni corrections. Values are presented as mean ± SEM. $^{*}P \leq 0.05$, $^{**}P \leq 0.001$. $^{\#}$ $P_{unadjusted} \leq 0.05$, $P_{adjusted} > 0.05$.

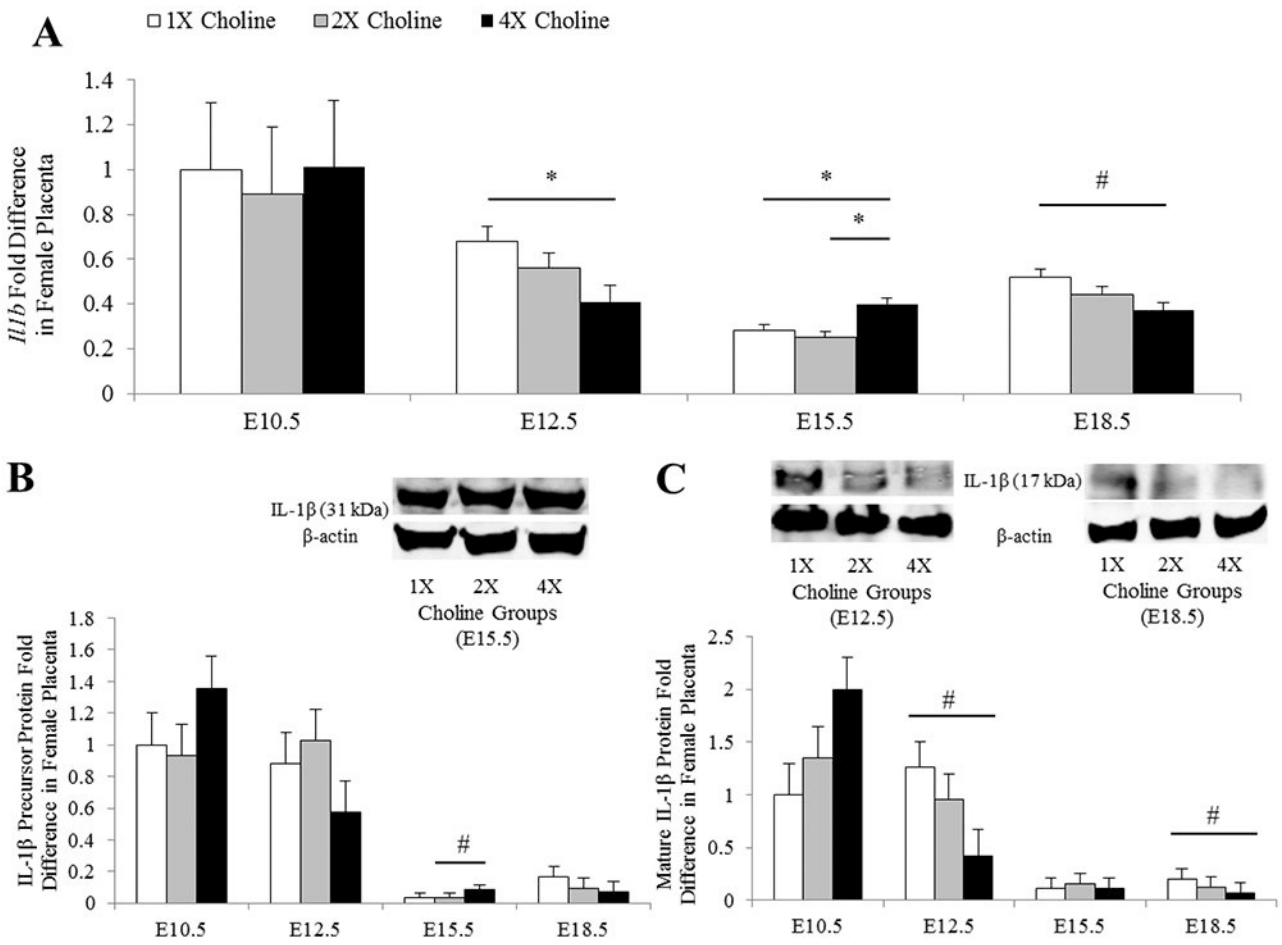

Figure 2.

A) Transcript and **B-C)** protein abundance of IL-1β in the female placentas obtained from dams receiving 1X, 2X or 4X choline treatments at E10.5, E12.5, E15.5 and E18.5. The transcript data are expressed as fold-change relative to the housekeeping gene *Tbp* and the protein data are expressed relative to β-actin. After normalization, the mean value of the control group at E10.5 was assigned a value of 1 and the mean values of the other groups were presented as a fraction of this value. Statistical analysis was done using the mixed linear model followed by post-hoc Bonferroni corrections. Values are given as mean ± SEM. $^{*}P \leq 0.05$. $^{\#}P_{unadjusted} \leq 0.05$, $P_{adjusted} > 0.05$.

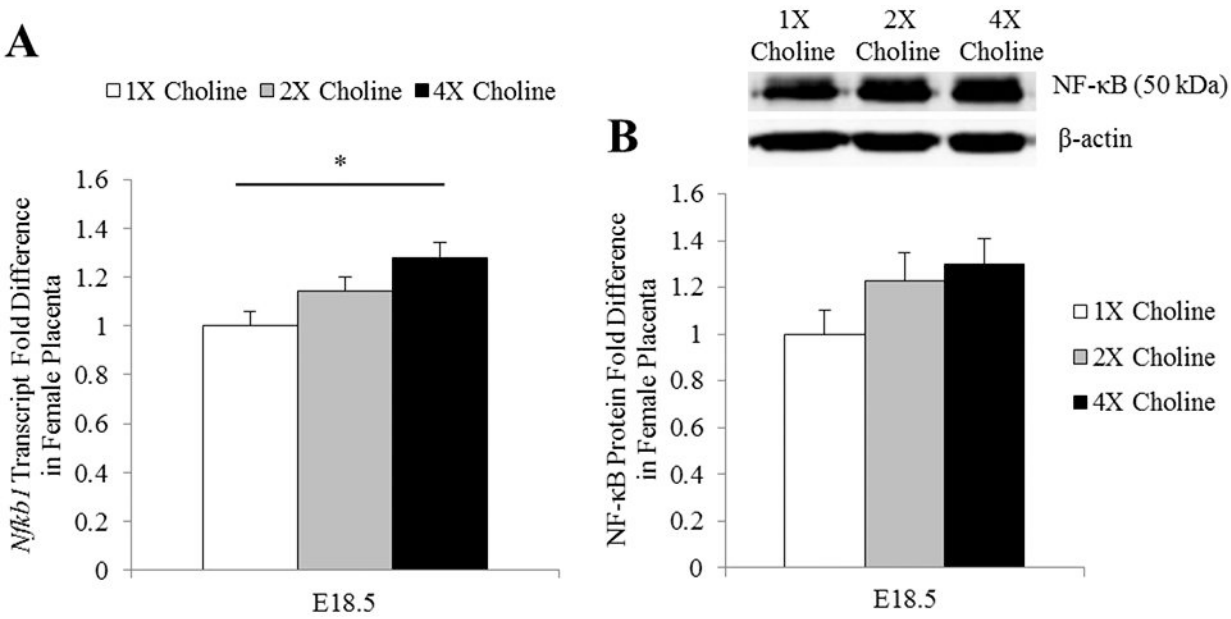

Figure 3.

A) Transcript and **B)** protein expression of NF-κB in the E18.5 female placentas obtained from dams receiving 1X, 2X or 4X choline treatments. The transcript data are expressed as fold-change relative to the housekeeping gene *Tbp* and the protein data are expressed relative to β-actin. After normalization, the mean value of the control group was assigned a value of 1 and the mean values of the treatment groups were presented as a fraction of this value. Statistical analysis was done using the mixed linear model followed by post-hoc Bonferroni corrections. Values are given as mean ± SEM. $*P \leq 0.05$.

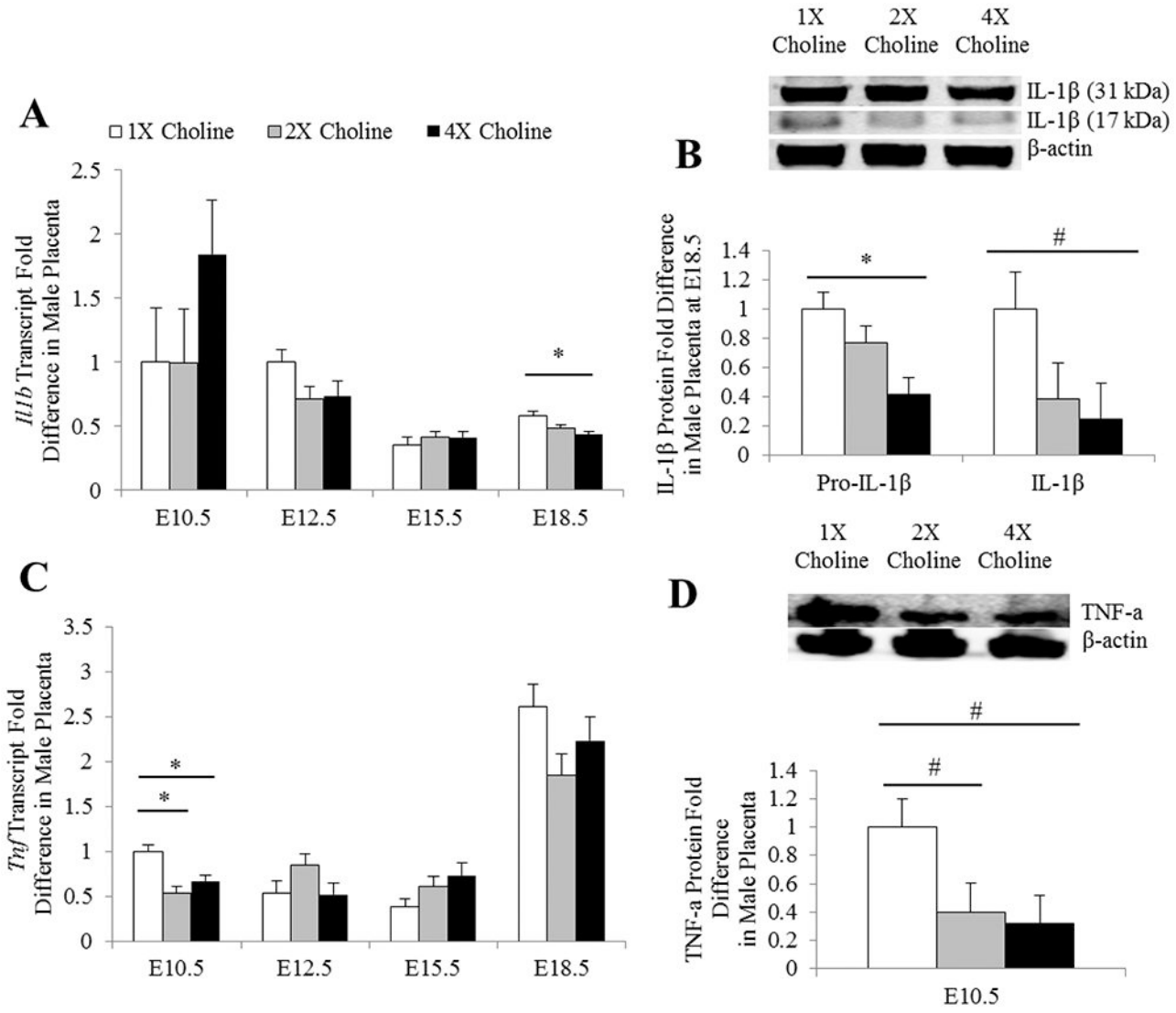

Figure 4.

mRNA and protein abundance of **A-B)** IL-1β and **C-D)** TNF-a in the male placentas obtained from dams receiving 1X, 2X or 4X choline treatments. The transcript data are expressed as fold-change relative to the housekeeping gene *Tbp* and the protein data are expressed relative to β-actin. After normalization, the mean value of the control group at E10.5 (for mRNA data) or the mean value of the control group (for protein data) was assigned a value of 1 and the mean values of the other groups were presented as a fraction of this value. Statistical analysis was done using the mixed linear model followed by post-hoc Bonferroni corrections. Values are given as mean ± SEM. $^{*}P \leq 0.05$. $^{\#}P_{unadjusted} \leq 0.05$, $P_{adjusted} > 0.05$.

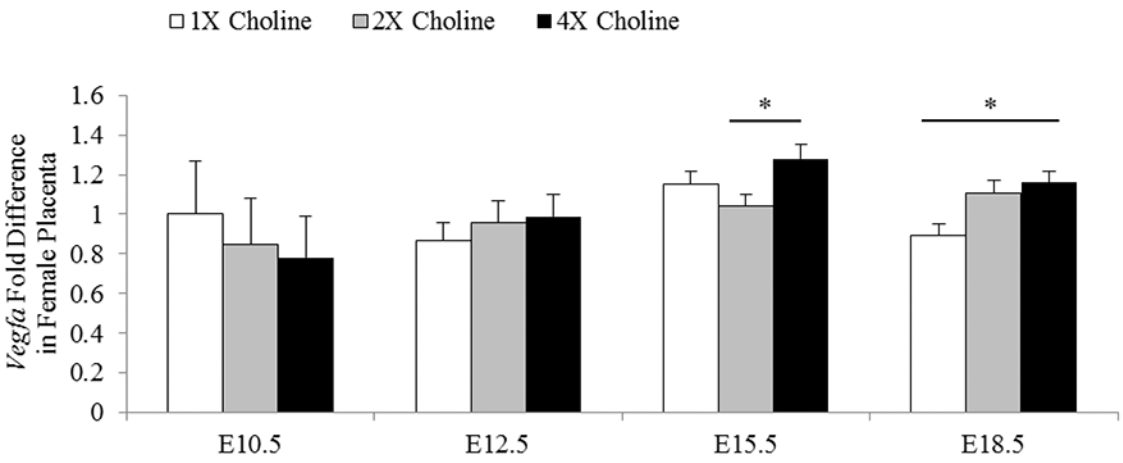

Figure 5.

mRNA abundance of *Vegfa* in the female placentas obtained from dams receiving 1X, 2X or 4X choline treatments at E10.5, E12.5, E15.5 and E18.5. Data are expressed as fold-change relative to the housekeeping gene *Tbp*. After normalization, the mean value of the control group at E10.5 was assigned a value of 1 and the mean values of the other groups were presented as a fraction of this value. Statistical analysis was done using the mixed linear model followed by post-hoc Bonferroni corrections. Values are given as mean ± SEM. $*P \leq 0.05$.

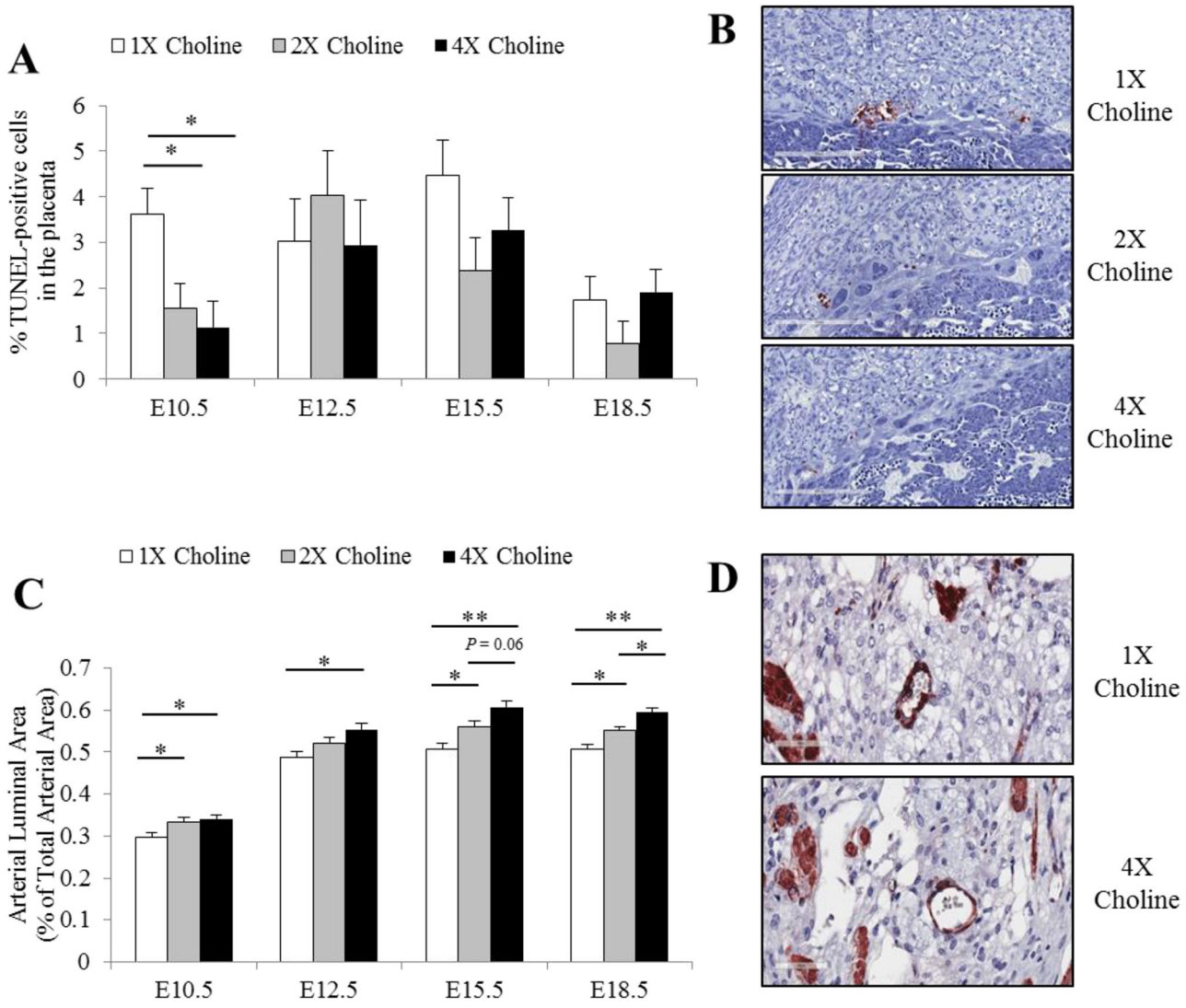

Figure 6.

A) The percentage of TUNEL-positive cells in the placenta from dams receiving 1X, 2X or 4X choline treatments at E10.5, E12.5, E15.5 and E18.5. **B)** Representative images of the apoptotic nuclei within the E10.5 placentas are shown. **C)** Arterial luminal area in the maternal decidua from dams receiving 1X, 2X or 4X choline treatments at E10.5, E12.5, E15.5 and E18.5. **D)** Representative images of the smooth muscle actin staining within the maternal decidua are shown. Data were analyzed using the mixed linear model followed by post-hoc Bonferroni corrections. Values are presented as mean ± SEM. *$P \leq 0.05$, **$P \leq 0.001$.