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ORIGINAL ARTICLE

An exploratory study on the effect of choline and folate deficiency on levels of vascularization proteins and transcription factors in first trimester trophoblast HTR-8/SVneo cells

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Abstract

Aims: We studied the effect of choline and folate deficiencies on levels of predetermined placental proteins during early development.

Methods: We incubated HTR-8/SVneo cells under choline and folate deficiency conditions and measured levels of some placental proteins using ELISA methods.

Results: Concentrations of LRP2 protein in cell lysates were higher in cells incubated in choline and folate deficient media compared to the control media (mean [SD] = 2.95 [1.30] vs. 1.65 [0.27] ng/mg protein, $p = 0.004$). The levels of LRP2 protein in lysates of cells incubated in choline and folate deficient media were significantly higher than the concentrations in lysates of cells incubated in choline deficient but folate sufficient media (1.96 [0.28] ng/mg protein) or those incubated in choline sufficient but folate deficient media (1.77 [0.24] ng/mg protein) ($p < 0.05$ for both). The cellular levels of CDX2 protein were significantly higher in cells incubated in choline and folate deficient media compared to the control media (1.78 [0.60] vs. 0.99 [0.42] pg/mg protein, $p = 0.002$); and compared to CDX2 levels in cells incubated in choline deficient but folate sufficient media (0.87 [0.13] pg/mg protein, $p < 0.001$) or in choline sufficient but folate deficient media (0.96 [0.16] pg/mg protein, $p < 0.001$). The levels of sFLT-1 and IGF1 in culture media and that of EOMES in HTR-8/SVneo cell lysates remained unchanged under all deficiency conditions.

Discussion: LRP2 and CDX2 are likely to be molecular targets for early choline and folate deficiencies in human trophoblast cells. The results should be confirmed in animal models and in other models of placental cells.

KEYWORDS
choline deficiency, first trimester, placenta, pregnancy, vascularization

INTRODUCTION

The requirements of the nutrients choline and folate increase during pregnancy to support cell metabolism and proliferation.[1] Adequate maternal choline during early pregnancy is necessary for development of the fetus,[2] the retina,[3] and the brain[4–6] possibly by mechanisms related to DNA synthesis and methylation.[7] Choline (via betaine) and folate are key methyl donors in one-carbon metabolism. In addition, choline is a source of acetylcholine and phosphatidylcholine. Phosphatidylcholine is used for synthesis of cell membranes.

Choline is taken up by the placenta by saturable[8] and non-saturable[9] mechanisms. The placenta accumulates choline during early pregnancy and placental tissues contain higher choline levels than the liver of the mother or the liver of non-pregnant rats.[10] Maternal choline supplementation has been shown to influence fatty acids and glucose transporters in the placenta of pregnant mice.[11,12] In humans, higher maternal choline intake (×4-fold) reduced placental inflammation and apoptosis and enhanced placental vascular development.[13] Thus, choline deficiency during pregnancy could affect placental vascularization and functions.

Supplementation of 930 mg/d choline to third trimester pregnant women (vs. 480 mg/d) lowered plasma concentrations of soluble fms-like tyrosine kinase 1 (sFLT-1).[14] sFLT-1 is antiangiogenic factor that is released from the placenta leading to raised levels of this protein in plasma of women with preeclampsia.[15] The release of sFLT-1 is regulated by epidermal growth factor receptor (EGFR)[16] that is important for early embryogenesis.[17]

Placental low-density lipoprotein receptor-related protein 2 (LRP2, also called megalin) is a potential target of methyl donors insufficiency. LRP2 plays a role in endocytosis and trafficking of ligands.[18] The insulin-like growth factor (IGF-1) has growth promoting effects and its gene expression and protein levels were found to be higher in liver and muscles of pigs from methyl deficient mothers compared to offspring from mothers on a control diet.[19] Moreover, methyl donors insufficiency could affect transcription factors such as eomesodermin homolog (EOMES, also known as Tbr2) that is involved in trophoblast differentiation and gastrulation and neuronal division[20] or the caudal-related homeobox 2 (CDX2) that preferably binds to methyl-containing CpG sequences on DNA[21] and thereby regulates the transcription of intestinal epithelium genes.

Choline and folate deficiency in early pregnancy could cause inadequate trophoblastic invasion and placental endothelial dysfunction and could alter levels of placental proteins necessary for fetal development. We studied the effect of choline deficiency and simultaneous choline and folate deficiencies on levels of placental proteins in a model of human trophoblast cells (HTR-8/SVneo cells).

MATERIALS AND METHODS

HTR-8/SVneo cells were obtained from ECACC (Lot Nr. 70014079). This cell line is derived by transfecting the cells that grew out of chorionic villi explants of human first trimester placenta with the gene encoding for simian virus 40 large T antigen. The cells were cultured in advanced RPMI1640 (Gibco 12633, Life Technologies) in the presence of 2 mM glutamine, 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

We used a choline and folate deficient medium (a modified RPMI1640) that was completely free of the nutrients (Modified Gibco 12633, without choline, folic acid, and L-glutamine; REF ME 19630L1, Lot 11930501, Life Technologies).

For the control medium, we added choline bitartrate (obtained via P&G Health, Lot 14486/19, 161U176049) and calcium methylfolate (Metafolin, obtained via P&G Health, Lot 14898/19, LMCM067501) each at concentrations of 50 µmol/L in addition to 2 mmol/L glutamine and 1.25% FBS. The concentrations of L-glutamine and FBS were stable in all of the experiments. In the deficiency conditions, either choline or folate or both of them were not added to the medium. The selection of choline[22–24] and folate[25] concentrations in the medium was in line with earlier studies using different cell models and aimed at avoiding supraphysiological concentrations of the two nutrients. We verified concentrations of choline in all media using UPLC-MS/MS method that was described earlier.[26]

The HTR-8/SVneo cells were seeded at a density of 2.0×10^6 cells/plate in 100 mm petri dishes. Cells were cultured for 96 h in the control medium (with 50 µmol/L choline and folate) or the media that were deficient in choline and/or folate. Afterwards, the culture media were collected and stored at −80°C until analysis. The media were used to measure concentrations of sFLT-1, IGF-1, and sEGFR using commercially available ELISA reagents (Table S1). The remaining cells were washed using phosphate buffered saline and harvested by using a rubber policeman. After centrifugation, supernatant was discarded, and cells were lysed using cell extraction buffer (CellLytic M; Sigma Adrich). The cell lysates were used to measure concentrations of total proteins by using Pierce 660 nm Protein Assay (ThermoFisher). Concentrations of LRP2, EOMES, and CDX2 were measured in cell lysates by using commercially available ELISA methods (Table S1). All measurements in media and cell lysates were corrected for total protein levels in the cells.

Organic cations such as hemicholinium-3 (HC-3) have been shown to inhibit up to 75% of choline uptake into human term placenta.[27–29] In addition to using choline deficient media, we incubated the cells for 3 h with 5 µmol/L HC-3 to prevent choline uptake into the cells. In a subset of the experiments, we verified the choline concentrations in the cell pellets to ensure that the cells incubated in choline deficient media or with HC-3 contained lower choline compared to the control cells that were incubated at 50 µmol/L choline. Each experiment was repeated twice independently (i.e., on Day 1 and Day 2).

The ELISA methods were checked in a series of preliminary experiments to verify that the levels are within the measurement ranges of the ELISAs. The protein concentrations were measured in duplicate samples of medium or cell lysate and a mean value was calculated. The ranges of the measured concentrations of each protein marker (without adjustment for total cell proteins) are shown in (Table S1). Table S2 shows method performance shown as the mean and range of deviations of the duplicate measurements of the concentrations of the biomarkers in the same medium or cell lysate.

The concentrations of choline were verified in the media. We measured 19.2 µmol/L choline concentrations in the standard medium (Advanced RPMI1640; Life Technologies) that was used to propagate the cells. This medium contains 20 µmol/L choline according to the manufacturer. The control medium with sufficient choline contained approximately 60 µmol/L choline, and the choline deficient medium contained approximately 1.5 µmol/L (samples were measured in duplicates).

TABLE 1 Concentrations of markers in cell medium

Incubation conditions[a]	Culture well	sFLT1		IGF-1		sEGFR	
		Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Control + 50 μmol/L choline + 50 μmol/L folate	1	184.36	155.68	17.61	1.05	122.73	79.40
	2	87.91	138.00	6.89	11.72	60.19	89.58
	3	83.87	134.79	11.93	10.49	49.69	88.70
	4	121.81	*54.26*	4.30	5.02	73.12	*67.32*
	Within-day mean	***119.49***	***142.82***	***10.18***	***7.07***	***76.43***	***85.89***
	Mean (SD) of 2 days	***129.49***	**(*35.74*)**	***8.63***	**(*5.30*)**	***80.49***	**(*23.63*)**
50 μmol/L choline (0 μmol/L folate)	1	96.59	94.85	5.71	5.67	74.42	72.27
	2	105.94	110.25	9.88	4.06	73.05	69.69
	3	61.52	140.58	10.80	14.26	70.65	82.30
	4	*24.45*	168.57	*3.81*	8.74	*64.13*	93.21
	Within-day mean	***88.02***	***128.56***	***8.80***	***8.18***	***72.70***	***79.37***
	Mean (SD) of 2 days	***111.19***	**(*34.48*)**	***8.44***	**(*3.56*)**	***76.51***	**(*8.44*)**
50 μmol/L folate (0 μmol/L choline)	1	105.63	113.48	10.02	*3.10*	79.79	79.61
	2	157.30	122.17	7.92	7.00	78.46	57.33
	3	122.61	103.87	15.61	6.90	83.47	60.64
	4	109.76	90.03	7.24	8.10	77.57	68.75
	Within-day mean	**123.82**	**107.39**	**10.20**	**7.33**	**79.82**	**66.58**
	Mean (SD) of 2 days	**115.61**	**(19.86)**	**8.97**	**(3.12)**	**73.20**	**(9.76)**
Choline and folate deficient (0 μmol/L)	1	254.78	*391.81*	7.87	*19.64*	196.48	*254.56*
	2	392.81	125.81	*34.14*	17.06	224.81	75.42
	3	140.07	125.95	12.18	11.19	90.11	84.55
	4	59.02	123.86	6.32	2.59	68.26	77.98
	Within-day mean	***211.67***	***125.21***	***8.79***	***10.28***	***144.92***	***79.31***
	Mean (SD) of 2 days	***174.62***	**(*112.49*)**	***9.54***	**(*5.05*)**	***116.80***	**(*64.99*)**

Note: Concentrations are shown in pg per mg total proteins in the cultured cells. Concentrations were measured in cell medium of HTR-8/SVneo cells. Data are mean of duplicate measurements of the same well.

Abbreviations: IGF-1, insulin-like growth factor-1; sEGFR, soluble epidermal growth factor receptor; sFLT1, soluble fms-like tyrosine kinase 1.

The reagents used for the assays are described in Table S1. The between-group *p*-values (ANOVA) were as follow: For sFLT1, $p = 0.2146$; for IGF-1, $p = 0.9714$; for sEGFR, $p = 0.0862$.

[a]Choline was added as choline bitartrate, and folate was added as calcium methylfolate.

The experiments were conducted at BioTeSys GmbH Lab, Esslingen, Germany.

Statistical analyses were conducted using Prism 5 (GraphPad Software, Inc.). ANOVA and the Tukey's multiple comparison test post-hoc test were used to compare different incubation conditions. A p value < 0.05 was considered statistically significant.

RESULTS

Concentrations of sFLT-1 in culture media did not differ significantly when cells were incubated in choline and folate deficient media compared to choline and folate sufficient media (mean [SD] = 174.62 [112.49] vs. 129.49 [35.74] pg/mg protein; $p = 0.2146$) (Table 1). The concentration of sFLT-1 in the culture medium did not differ significantly between any of the four different incubation conditions (Tukey's multiple comparison test). The concentrations of IGF-1 in culture media did not differ significantly between cells incubated in choline and folate sufficient media versus those incubated in choline and folate deficient media (8.63 [5.30] vs. 9.54 [5.05] pg/mg protein; $p = 0.9714$). The concentrations of sEGFR in culture medium did not differ between cells incubated in choline and folate deficient media versus those incubated at choline and folate sufficient media (116.80 [64.99] vs. 80.49 [23.63] pg/mg protein; $p = 0.0862$). In a series of independent experiments, adding 5 μmol/L HC-3 (a choline uptake inhibitor) to the medium containing sufficient choline (50 μmol/L) resulted in significantly higher sEGFR concentrations compared to cell medium from cells incubated in the same choline sufficient media without HC-3 (Figure S1).

Concentrations of LRP2 protein in HTR-8/SVneo cell lysates were higher in cells incubated in choline and

TABLE 2 Concentrations of proteins measured in cell lysates and expressed in mg total protein

Incubation conditions[a]	Culture well	LRP2 in ng/mg protein		CDX2 pg/mg protein		EOMES ng/mg protein	
		Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Control + 50 μmol/L choline + 50 μmol/L folate	1	*3.70*	1.99	1.81	0.74	7.06	2.68
	2	1.39	1.62	0.98	0.57	4.10	3.20
	3	1.23	1.92	0.82	*1.06*	3.13	3.67
	4	1.62	1.77	1.28	0.75	4.40	*3.00*
	Within-day mean	***1.41***	***1.82***	***1.22***	***0.69***	***3.88***	***3.14***
	Mean (SD) of 2 days	***1.65***	**(*0.27*)**	***0.99***	**(*0.42*)**	***4.03***	**(*1.46*)**
50 μmol/L choline	1	1.70	1.85	1.15	0.95	*4.64*	3.50
	2	1.42	1.93	0.96	0.82	3.64	3.60
	3	1.79	2.20	0.87	0.90	3.78	4.22
	4	1.57	1.71	1.25	0.78	3.43	3.81
	Within-day mean	***1.62***	***1.92***	***1.06***	***0.86***	***3.62***	***3.78***
	Mean (SD) of 2 days	***1.77***	**(*0.24*)**	***0.96***	**(*0.16*)**	***3.71***	**(*0.26*)**
50 μmol/L folate	1	2.08	2.55	1.11	0.76	*3.95*	4.13
	2	2.01	1.70	0.98	0.87	4.84	3.43
	3	1.83	1.81	0.83	0.91	5.10	2.65
	4	1.66	2.05	0.79	0.68	4.76	3.48
	Within-day mean	***1.90***	***2.03***	***0.93***	***0.80***	***4.90***	***3.42***
	Mean (SD) of 2 days	***1.96***	**(*0.28*)**	***0.87***	**(*0.13*)**	***4.06***	**(*0.90*)**
Choline and folate deficient (0 μmol/L)	1	4.76	*8.15*	2.88	*5.90*	11.82	*11.79*
	2	4.90	2.01	2.18	1.93	12.16	4.14
	3	2.06	2.19	1.17	1.63	4.30	4.56
	4	2.18	2.57	1.26	1.40	4.47	4.37
	Within-day mean	***3.48***	***2.26***	***1.87***	***1.65***	***8.19***	***4.36***
	Mean (SD) of 2 days	***2.95***	**(*1.30*)**	***1.78***	**(*0.60*)**	***6.55***	**(*3.72*)**

Note: Concentrations were measured in HTR-8/SVneo cell lysates. Data are mean of duplicate measurements of the same well.
Abbreviations: CDX2, caudal-related homeobox 2; EOMES, eomesodermin homolog; LRP2, low-density lipoprotein receptor-related protein 2.
The reagents used for the assays are described in Table S1.
The between-group p values (ANOVA) were as follow:
For LRP2, p between groups = 0.004. The Tukey's multiple comparison test results: mean difference and 95% CI for 50 μM Choline + 50 μM folate versus choline and folate deficient = −1.304 (−2.279 to −0.3294). 50 μM choline (0 μM folate) versus choline and folate deficient −1.182 (−2.126 to −0.2377). 50 μM folate (0 μM choline) versus choline and folate deficient = −0.9916 (−1.936 to −0.04767).
For CDX2, p between groups = 0.0002. The Tukey's multiple comparison test results: mean difference and 95% CI 50 μM choline + 50 μM folate versus choline and folate deficient −0.7857 (−1.331 to −0.2401). 50 μM choline (0 μM folate) versus choline and folate deficient −0.8186 (−1.347 to −0.2902). 50 μM folate (0 μM choline) versus choline and folate deficient = −0.9123 (−1.441 to −0.3840).
For EOMES, p between groups = 0.0574.
[a]Choline was added as choline bitartrate, and folate was added as calcium methylfolate.

folate deficient media compared to the cells incubated in the control media (2.95 [1.30] vs. 1.65 [0.27] ng/mg protein, $p = 0.004$) (Table 2). The LRP2 levels in cell lysates in choline and folate deficient media (2.95 [1.30] ng/mg protein) were significantly higher than the concentrations in cell lysates incubated at choline deficient but folate sufficient media (1.96 [0.28] ng/mg protein) or those incubated at choline sufficient but folate deficient conditions (1.77 [0.24] ng/mg protein) compared to cells grown in the control media ($p < 0.05$).

The concentrations of EOMES in HTR-8/SVneo cell lysates did not differ significantly between cells incubated in choline and folate deficient media versus cells grown in choline and folate sufficient media (6.55 [3.72] ng/mg protein vs. 4.03 [1.46] ng/mg protein; $p = 0.0574$) (Table 2). The concentrations of CDX2 protein in cells incubated in choline and folate deficient media were significantly higher than levels measured in cells incubated in choline and folate sufficient media (1.78 [0.60] vs. 0.99 [0.42] pg/mg protein; $p = 0.002$); and compared to levels in cells incubated in choline deficient but folate sufficient media (0.87 [0.13] pg/mg protein; $p < 0.001$) or in folate deficient but choline sufficient media (0.96 [0.16] pg/mg protein; $p < 0.001$) (Table 2).

Adding 5 μmol/L HC-3 to the cell media containing 50 μmol/L choline had no effect on any other protein in the medium or in the cell lysate (data not shown).

DISCUSSION

We studied the effect of deficiency of choline, folate, or both of them in human trophoblast cells on candidate proteins related to placental vascularization or functions or early fetal development. HTR-8/SVneo cells were used as a model of first trimester placenta. We found that protein concentrations of LRP2 and CDX2 were upregulated in lysates of HTR-8/SVneo cells incubated in choline and folate deficient media compared to those grown in choline and folate sufficient media. In contrast, isolated choline or folate deficiency had no significant effect on LRP2 and CDX2 protein concentrations. The results suggest additive effects of choline and folate deficiencies on these placental proteins. Moreover, using a choline uptake inhibitor showed mostly similar results to those when using choline deficient media.

Choline and folate have unique and joint roles in cell metabolism. Both nutrients are methyl donors in one-carbon metabolism,[30–32] while choline has specific roles in synthesis of phospholipids and acetylcholine and folate has specific roles in synthesis of purine and thymidylate. Experimental dietary folate deficiency in rats caused depletion of liver choline derivatives, suggesting that more choline was used as methyl donor under conditions of folate deficiency.[30] In experimental models of dietary choline deficiency in newborn pigs, a combination of dietary choline and folate deficiencies caused growth retardation and fatty liver that were stronger than those caused by choline deficiency alone.[33] This joint effect of choline and folate deficiencies is in line with our results and could be due to hypomethylation, and other mechanisms that collectively lead to inhibition of cell growth and differentiation.

Earlier studies have shown that prenatal deficiency of choline caused upregulation of choline transporters.[34] Thus, it is possible that adding HC-3 to media containing choline could cause upregulation of choline receptors which may partly maintain choline content in the cells. In addition, it is possible that the HTR-8/SVneo cells can synthesize phosphatidylcholine from phosphatidylethanolamine, especially when folate is present in the medium.

Earlier studies have shown that choline supplementation in third trimester pregnant women lowered plasma sFLT-1 levels.[14] Moreover, sEGFR protein has been shown to be lowered in plasma of women with preeclampsia (later in pregnancy).[15] Our results on sFLT-1 (i.e., not significantly higher under choline and folate deficient conditions) and sEGFR (unchanged at low choline or upregulated in the presence of HC-3) could be due to variations between the experiments that results in wide standard deviations, the cell model we used or the methods of measuring protein levels. Moreover, the model we used here (early pregnancy trophoblast cells) is not directly comparable with third trimester pregnant women and the protein levels might change when the pregnancy progresses or might be differentially expressed by different placental cell types that are not present in the HTR-8/SVneo cell line.

The finding that LRP2 and CDX2 were upregulated in HTR-8/SVneo cell line under choline and folate deficiency could reflect adaptation of the cells to extreme conditions. LRP2 protein is a multi-ligand receptor with a role in development of the retina.[35] CDX2 is a transcription factor that binds preferably to methyl-containing CpG sequences on DNA[21] and regulates the transcription of genes expressed in the intestinal epithelium.

The present study has some limitations. The HTR-8/SVneo cell line may not resemble the function of the placenta as a whole organ with different types of cells. Moreover, we have used commercially available ELISA methods to measure concentrations of the target proteins. These methods have limitations with regard to sensitivity and the results need to be confirmed by future studies or in vivo animal models. Finally, we did not investigate the concentrations of one-carbon metabolites. Therefore, we cannot confirm the mechanisms by which choline and folate deficiencies modulated the concentrations of the proteins. We cannot either confirm that the deficiency caused upregulation of the LRP2 and CDX2 genes.

Taken together, we identified LRP2 and CDX2 (both downregulated) as potential molecular targets for combined choline and folate deficiencies in the HTR-8/SVneo cell line of human trophoblast. Isolated choline deficiency and folate deficiency was not associated with significant changes of the concentrations of these proteins in HTR-8/SVneo cells. The concentrations of sFLT-1, IGF-1, sEGFR, and EOMES proteins were not significantly influenced by choline and folate deficiencies. The functional relevance of downregulation of LRP2 and CDX2 in placental cells under choline and folate deficiencies during early development needs to be investigated in vivo.

AUTHOR CONTRIBUTIONS

Karin Engelhart and **Inka Pfitzner**: Planning, experimental design and methodology, laboratory work, and data analyses. **Rima Obeid**: Study design, interpretation, and drafting the manuscript. The sponsor had no role in the design, interpretation, data analyses, or drafting of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interests for this article.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article (and its supplementary information files). Additional data regarding any part of this article can be made available for researchers upon request to the corresponding author(s).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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