

One-carbon metabolite supplementation improves growth of bovine embryonic fibroblasts cultured in divergent glucose media

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INTRODUCTION

One-carbon metabolism is the network of biochemical pathways that regulates amino acid metabolism, nucleotide synthesis, and epigenetic processes, such as methylation and demethylation of DNA (Wu et al., 2006; Ikeda et al., 2012). Deficiency in one-carbon metabolites (OCM) during the periconceptual period in sheep resulted in differential methylation at 4% of CpG sites in fetal liver at d 90 of gestation, altered fat composition in male and female offspring at 12 mo of age, and decreased response to insulin challenge in male offspring at 22 mo of age (Sinclair et al., 2007).

Moderate global nutrient restriction of heifers from breeding to d 50 of gestation decreased glucose, glutamine, and methionine in fetal fluids, whereas homocysteine concentrations increased in maternal serum (Crouse et al., 2019c). In addition, moderate nutrient restriction of heifers increased transcript abundance of histone and histone-modifying genes in fetal liver by d 50 of gestation (Crouse et al., 2019b). Combined, these data suggest that moderate nutrient restriction of heifers reduces energy and OCM substrates to the developing fetus, which may alter the fetal epigenome and lead to developmental programming events in offspring born to heifers that were nutrient restricted during

early pregnancy (Crouse et al., 2019b, 2019c). Therefore, the objective of this study was to determine if supplementing OCM to bovine embryonic fibroblasts cultured in divergent glucose media would improve cellular growth as measured by proliferation rates and total cell counts. We hypothesized that supplementation of OCM (methionine, folate, choline, and vitamin B₁₂) to bovine embryonic tracheal fibroblasts (EBTr) in divergent glucose media would positively affect cell growth and proliferation.

MATERIALS AND METHODS

Cells and Treatments

Bovine EBTr were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Eagle's minimum essential medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA), 1% penicillin–streptomycin (Thermo Fisher Scientific), and 0.11 g/L Na pyruvate (Sigma). Final glucose concentrations of 1 g/L (Low) or 4.5 g/L (High) were achieved by the addition of D-glucose (Sigma). Control (CON) medium contained basal concentrations of folate (0.001 g/L), choline (0.001 g/L), vitamin B₁₂ (4 µg/L), and methionine (0.015 g/L). OCM (folic acid, choline chloride, vitamin B₁₂, and L-methionine [Sigma]) were supplemented to the media to achieve 2.5, 5, or 10 times (2.5X, 5X, or 10X, respectively) the concentrations in the CON

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medium, except for methionine, which was limited at 2X across all supplemented treatments to prevent toxicity. Therefore, the experiment was a completely randomized design with a 2 (glucose) \times 4 (OCM levels) factorial arrangement of treatments.

Cell Growth Rate and Proliferation Analyses

Cells were passaged three times in their respective treatment media before being plated onto one of six Seahorse XF24 microplates (Agilent Technologies, Santa Clara, CA) in triplicate at a cell density of 1,800 cells per well. Cells were plated at 0 h and placed in a humidified incubator (37 °C, 5% CO₂) for 1, 12, 24, 36, 48, or 72 h, after which time the media were aspirated and the cells fixed in 10% neutral buffered formalin (Sigma). Antigen retrieval was performed in 10 mM sodium citrate buffer, pH 6, with 0.05% Tween 20 in a 2100 retriever (Electron Microscopy Sciences, Hatfield, PA). To block nonspecific binding, wells were treated for 1 h with 10% normal goat serum (Vector Laboratories, Burlingame, CA). Each well was stained for cell proliferation with rabbit anti-Ki67 (Abcam, Cambridge, UK) for 1 h and fluorescently labeled with CF633 goat anti-rabbit secondary antibody (Biotium, Fremont, CA) for 1 h. Cells were treated with Pro-Long Gold

with 4,6-diamidino-2-phenylindole (DAPI; Life Technologies, Grand Island, NY) to counterstain all nuclei. Large area (MosaiX, Zeiss) photomicrographs of the wells were taken with a Zeiss Imager M2 epifluorescence microscope using a 5X objective and AxioCam HRm camera. MosaiX images were analyzed using ImagePro Premiere software (Media Cybernetics, Silver Spring, MD) for the total cell number (DAPI stained cells) and proliferating cell number (Ki67 stained cells). Cell growth rate was determined as the slope after natural log transformation of cell number, and cell proliferation with Ki67 was determined by the labeling index (% of cells stained by Ki67).

Statistical Analysis

Cell growth rate was analyzed for early growth (1 to 24 h), late growth (24 to 72 h), and total growth (1 to 72 h) rate using PROC REG of SAS 9.4 (SAS, Cary, NY) followed by PROC MIXED with glucose, OCM, and their interaction as fixed effects.

Labeling index (cell proliferation) was analyzed at 1, 12, 24, 36, 48, and 72 h. Additional analysis included proliferation post-attachment to the plate (12 to 72 h) and total proliferation (1 to 72 h) using PROC MIXED with glucose, OCM, and their interaction as fixed effects.

Table 1. Growth rate of EBTr cells as influenced by glucose and OCM level in Eagle's minimum essential medium (EMEM)¹

Time, h ⁴	Glucose ⁵	One-carbon metabolites ²				Average Glc ⁶	SEM ⁷	P-value ³					
		CON	2.5X	5X	10X			Glc	OCM	Glc \times OCM	L	Q	C
1–24 (early)	Low	1.041	1.033	1.044	1.036	1.039 ^a	0.0044	<0.01	0.41	0.14	0.54	0.77	0.04
	High	1.049	1.049	1.048	1.053	1.050 ^b					0.28	0.50	0.81
	OCM Avg	1.045	1.041	1.046	1.045								
24–72 (late)	Low	1.022 ^{cd}	1.023 ^{cd}	1.028 ^{ef}	1.021 ^c	1.023	0.0017	<0.01	0.01	0.01	0.54	0.01	0.09
	High	1.025 ^{de}	1.029 ^{fg}	1.028 ^{efg}	1.032 ^g	1.029					0.01	0.62	0.17
	OCM Avg	1.023	1.026	1.028	1.026								
1–72 (total)	Low	1.027 ^c	1.027 ^c	1.032 ^d	1.026 ^c	1.028	0.0013	<0.01	0.01	<0.01	0.47	<0.01	0.01
	High	1.031 ^d	1.035 ^{ef}	1.034 ^{de}	1.037 ^f	1.034					<0.01	0.59	0.04
	OCM Avg ⁸	1.029	1.031	1.033	1.031								

¹Rate = slope of the line of cells after natural log transformation.

²CON = basal concentrations of methionine, folate, and choline in EMEM media with 4 μ mol/L vitamin B₁₂. 2.5X, 5X, 10X = 2.5, 5, and 10 times the concentration of folate, choline, and vitamin B₁₂ in CON media. Methionine limited to 2X CON.

³Probability values for the effects of glucose, OCM, and the interaction. Linear (L), quadratic (Q), and cubic (C) polynomial contrasts for the effect of glucose and increasing OCM supplementation on growth rate of EBTr cells within glucose level.

⁴Hours after plating.

⁵Low = 1 g/L glucose and High = 4.5 g/L glucose in culture media.

⁶Average growth rate within glucose level.

⁷Standard error of the mean for the interaction of glucose and OCM level.

⁸Average growth rate within OCM supplementation level.

^{a,b}Means within a column without a common superscript differ.

^{c,g}Means within a time without a common superscript differ.

Polynomial contrasts were performed within the Low and High glucose levels to determine whether increasing OCM affected cell growth rate or proliferation. To account for unequal spacing of OCM treatments, contrast coefficients were determined using PROC IML and analyzed with the general linear model procedure for effects of OCM supplementation within glucose level. All *P*-levels less than or equal to 0.05 were considered significant.

RESULTS

Growth Rate

As shown in Table 1, total growth rate was affected by a glucose \times OCM interaction ($P < 0.01$) with High 10X and 2.5X treated cells having greater cell growth compared with High CON and Low 5X. Furthermore, High CON and 5X as well as Low 5X treated cells were greater ($P < 0.01$) than Low CON, 2.5X, and 10X. Early growth rate was

not influenced by a glucose \times OCM interaction or the main effect of OCM ($P \geq 0.14$); however, High cells had a greater ($P < 0.01$) growth rate compared with Low treated cells. Late growth rate was affected by a glucose \times OCM interaction ($P < 0.01$) with High 2.5X, 5X, and 10X OCM having greater cell growth compared with High CON and Low CON, 2.5X, and 10X. Late and total cell growth in Low treated cells increased quadratically ($P \leq 0.01$) to 5X and decreased at 10X OCM supplementation. In High treated cells, late and total cell growth increased linearly ($P \leq 0.01$) with increasing OCM supplementation

Cell Proliferation

As shown in Table 2, labeling index did not differ between glucose concentrations, OCM, or the interaction for total or after plate attachment (1 to 72 and 12 to 72 h, respectively) and was therefore omitted from the tables and results. At 1 h

Table 2. Labeling index (cell proliferation) of EBTr cells stained with Ki67 as influenced by glucose and one-carbon metabolite level in Eagle's minimum essential medium (EMEM)¹

Time, h ⁴	Glucose ⁵	One-Carbon Metabolites ²				Average Glc ⁶	SEM ⁷	<i>P</i> -value ³					
		CON	2.5X	5X	10X			Glc	OCM	Glc \times OCM	L	Q	C
1	Low	30.67 ^a	32.13 ^a	32.28 ^a	37.86 ^b	33.23	1.85	0.44	0.77	0.05	0.50	0.14	0.27
	High	32.59 ^{ab}	32.50 ^{ab}	35.73 ^{ab}	30.60 ^a	32.85					0.03	0.50	0.67
	OCM Avg ⁸	31.63	32.32	34.00	34.23								
12	Low	9.73	11.69	12.57	12.25	11.56	2.01	0.19	0.60	0.65	0.14	0.19	0.91
	High	10.35	13.91	13.28	10.83	12.09					0.87	0.14	0.55
	OCM Avg	10.04	12.80	12.93	11.54								
24	Low	13.46 ^{ab}	15.34 ^{ab}	14.63 ^{ab}	12.46 ^a	13.97	1.40	0.03	0.07	0.02	0.25	0.09	0.49
	High	12.85 ^a	16.40 ^b	15.03 ^{ab}	18.41 ^c	15.67					0.01	0.78	0.10
	OCM Avg	13.15	13.87	14.83	15.44								
36	Low	10.56 ^{bc}	11.55 ^{bc}	10.32 ^{bc}	8.49 ^a	10.23	0.77	0.75	0.06	0.02	0.01	0.10	0.24
	High	10.14 ^{bc}	10.50 ^c	9.02 ^{ab}	10.75 ^c	10.10					0.62	0.15	0.11
	OCM Avg	10.35	11.02	9.67	9.62								
48	Low	8.88 ^{ab}	7.39 ^a	9.27 ^{ab}	7.98 ^a	8.38	1.02	<0.01	0.56	0.03	0.71	0.86	0.07
	High	10.43 ^{bc}	12.21 ^{cd}	11.00 ^{bc}	13.25 ^d	11.72					0.05	0.80	0.11
	OCM Avg	9.65	9.80	10.13	10.62								
72	Low	5.59 ^d	4.53 ^{abc}	4.99 ^{bcd}	3.87 ^a	4.74	0.48	0.74	0.83	0.01	0.04	0.94	0.19
	High	4.36 ^{ab}	4.78 ^{abcd}	4.63 ^{abcd}	5.41 ^{cd}	4.80					<0.01	0.56	0.19
	OCM Avg	4.97	4.66	4.81	4.64								

¹Labeling index = % of positively stained cells with Ki67.

²CON = basal concentrations of methionine, folate, and choline in EMEM media with 4 μ mol/L Vitamin B₁₂. 2.5X, 5X, 10X = 2.5, 5, and 10 times the concentration of folate, choline, and Vitamin B₁₂ in CON media. Methionine limited to 2X CON.

³Probability values for the effects of glucose, OCM, and the interaction. Linear (L), quadratic (Q), and cubic (C) polynomial contrasts for the effect of glucose and increasing OCM on growth rate of EBTr cells within glucose level.

⁴Hours after plating.

⁵Low = 1 g/L glucose and High = 4.5 g/L glucose in culture media.

⁶Average growth rate within glucose level.

⁷Standard error of the mean for the interaction of glucose and OCM level.

⁸Average growth rate within OCM supplementation level.

^{a-d}Means within an hour without a common superscript differ.

post-plating, labeling index was affected by a glucose \times OCM interaction ($P = 0.05$) being greater in Low 10X compared with all other Low OCM levels and High 10X, with High CON, 2.5X, and 5X being intermediate. There were no differences at 12 h ($P \geq 0.14$). At 24 h, labeling index for High 10X was greater ($P = 0.02$) than all other treatments. Furthermore, labeling index for High 2.5X was greater ($P < 0.01$) compared with High CON and Low 10X and was equal to Low CON, 2.5X, 5X, and High 5X. At 36 h, labeling index was affected by a glucose \times OCM interaction ($P = 0.02$) with High 2.5X and 10X being greater than Low 10X and High 5X. In addition, High CON and Low CON, 2.5X, and 5X were greater than Low 10X. At 48 h, labeling index was affected by a glucose \times OCM interaction ($P = 0.03$) with High 10X being greater compared with all Low treatments as well as High CON and 5X. High CON, 2.5X, and 5X were greater than Low 2.5X and 10X. At 72 h, labeling index was affected by a glucose \times OCM interaction ($P = 0.01$) with Low CON being greater than Low 2.5X and 10X as well as High CON. Low 5X and High 10X were greater than Low 10X and High CON. Labeling index linearly decreased ($P \leq 0.04$) in Low cells at 36 and 72 h with increasing OCM supplementation. In contrast, labeling index linearly increased ($P \leq 0.05$) in High cells at 24, 48, and 72 h with increasing OCM supplementation.

DISCUSSION

These data support our hypothesis that OCM supplementation to bovine embryonic cells cultured in divergent glucose media increases cell growth rate and proliferation. Previously conducted research on OCM supplementation during late gestation and lactation in both beef (Waterman et al., 2007) and dairy (Jayaprakash et al., 2016; Alharthi et al., 2018) cows has demonstrated improved nitrogen retention, dry matter intake, and increased nutrient transport and fetal growth; however, effects during early gestation have been limited to studies in sheep (Sinclair et al., 2007). Although the majority of data have focused on the inclusion of one or two OCM, our data are unique in that they measure the effects of four OCM that may be used in all pathways involved in methylation, nucleotide synthesis, and the remethylation of homocysteine to methionine (the methionine–homocysteine cycle).

An additional experiment completed with the same cell line and treatments (Crouse et al., 2019a) concluded that mitochondrial reserve capacity was greater in High 10X and 2.5X as well as Low 5X

compared with Low CON and 10X and High CON, which is similar to the data reported herein for total growth rate. Combined, these data demonstrate that OCM increased growth and proliferation of bovine embryonic cells, which can be explained in part by improvements in mitochondrial efficiency with OCM supplementation. These data further demonstrate that growth rate is not affected by OCM level early in the growth period but diverges as cells are allowed to proliferate and become more confluent. Furthermore, total proliferation is not different but is time-dependent, with similar trends between 24 to 48 h and differential initial responses at 1 h and final responses at 72 h. Finally, these data indicate that the extreme differences seen between Low and High 10X suggest that there may be either a supranutritional effect of OCM supplementation when additional energy in the form of glucose is provided or cellular requirements for OCM are dependent on energy availability.

IMPLICATIONS

We interpret our observation of increased cellular proliferations in cultured embryonic cells to imply that strategic supplementation with OCM may increase embryonic growth rate *in vitro*. Increased growth may be due in part to greater mitochondrial efficiency, which, if sustained, may affect whole herd production by improving metabolic efficiency in cows throughout the production cycle. Therefore, further studies *in vivo* should be designed to target strategic supplementation during early gestation in beef cows, which may improve pregnancy rates and lead to positive effects on developmental programming.

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Conflict of interest statement. None declared.

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