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—Original Article—

Methionine Requirements for the Preimplantation Bovine Embryo

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Abstract. The early embryo's nutritional environment plays an important role in establishing its developmental potential. However, little is known about the specific nutrient requirements of the embryo. The objective of the present study was to determine requirements of the *in vitro* produced bovine embryo for the essential amino acid methionine. In addition to serving as a precursor for polypeptides, methionine plays roles in regulation of translation, DNA methylation, and antioxidant balance. In the first experiment, embryos were cultured in potassium simplex optimized medium - bovine embryo modification 2 containing 0, 35, 50, 100, 200 or 400 μmol/l L-methionine for 8 days. There was no effect of methionine concentration on cleavage rate. The percent of oocytes that developed to blastocyst was lower for embryos without methionine at Day 7 and 8 than other groups but was similar for embryos cultured with 35–400 μmol/l. Neither total cell number, allocation of cells to trophectoderm or inner cell mass, or frequency of apoptosis was affected by methionine concentration. In the second experiment, embryos were cultured with 0, 7, 14, 21, 28 or 35 μmol/l methionine. There was no effect of methionine concentration on cleavage rate. The percent of oocytes that developed to blastocyst was lower for embryos without methionine at Day 7 and 8 but was not different between embryos cultured with 7–35 μmol/l methionine. However, the proportion of blastocysts that were expanded, hatching or hatched on Day 7 was reduced at lower concentrations of methionine (7 and 14). DNA methylation of blastocyst nuclei was unaffected by methionine concentration but intracellular glutathione content was higher for embryos cultured without methionine. In conclusion, the methionine requirement for preimplantation development is between 14 and 21 μmol/l. These concentrations are lower or similar to those found in the reproductive tract and suggest that methionine deficiency is not a common cause of embryonic mortality.

Key words: Blastocyst, Methionine, Preimplantation embryo

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The fate of the preimplantation embryo is determined to a large extent by the environment in which it executes its developmental program. Perturbations in that environment, such as caused by abnormal patterns of steroid hormone exposure [1], asynchrony between embryo and mother [2], and exposure to high contracentrations of urea in the reproductive tract [3] can disrupt embryonic development and lead to infertility. The importance of the environment for proper development is illustrated by the comparison of embryos produced *in vivo vs*. those produced by *in vitro* fertilization. In cattle, *in vitro* produced embryos have reduced competence for establishing pregnancy after transfer into recipients, altered biochemical, molecular, and ultrastructural characteristics, and reduced ability to survive cryopreservation [4]. Culture conditions during the period of embryonic development, rather than changes in the process of oocyte maturation or fertilization, are responsible for at least some of these alterations since placement of bovine embryos produced by *in vitro* maturation and fertilization into the oviduct improves embryo cryopreservation [5].

One aspect of the early embryo's microenvironment that can affect its development is amino acid availability. In cattle, the proportion of cultured embryos that develop to the blastocyst stage is affected by amino acid composition of the culture medium [6–8]. However, little is known about the specific requirements for individual amino acids. One amino acid that might be important for embryonic development and survival is the essential amino acid methionine. Shown to be transported into one or more cellular compartments of the preimplantation bovine embryo [9–11], methionine may be particularly important during the preimplantation period because, in addition to serving as a precursor for polypeptides, methionine plays roles in regulation of translation, DNA methylation, and antioxidant balance [12].

Concentrations of methionine in culture media are typically slightly higher than concentrations in the reproductive tract. The most common amino acid supplement used in culture media, Basal Medium Eagle essential amino acid mixture, results in a final methionine concentration of 50 μmol/l [13]. Concentrations of methionine in cattle average 32–49 μmol/l in oviductal fluid, 31–46 μmol/l in uterine fluid, and 16–35 μmol/l in blood plasma [14]. It may be possible, however, to improve embryo survival by increasing methionine concentration above these concentrations. Feeding a rumen-protected methionine supplement to lactating dairy cows, which can increase circulating concentrations of methionine from 22–78% [15–17], tended to increase fertility [18, 19].

The purpose of this experiment was to establish the methionine requirement for the bovine preimplantation embryo with respect to competence to develop to the blastocyst stage, cell number and allocation, apoptosis, intracellular glutathione concentration, and DNA methylation.

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Materials and Methods

Materials

Unless otherwise stated, reagents were from Sigma (St. Louis, MO, USA) or Fisher (Pittsburgh, PA, USA). HEPES-Tyrodes albumin lactate pyruvate (TALP), IVF-TALP, and Sperm-TALP were prepared as described previously [13]. Oocyte collection medium (OCM) consisted of Tissue Culture Medium-199 (TCM-199) with Hank's salts without phenol red (HyClone, Logan, UT, USA) supplemented with 2% (v/v) bovine steer serum containing 2 U/ml heparin (Pel-Freez, Rogers, AR, USA), 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium consisted of TCM-199 (Invitrogen, Carlsbad, CA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 μg/ml estradiol 17-β, 20 μg/ml bovine follicle stimulating hormone (Folltropin-V; Bioniche Life Sciences, London, ON, Canada), 22 μg/ml sodium pyruvate, 50 μg/ml gentamicin sulfate, and 1 mM glutamine. Frozen semen from various bulls was donated by Southeastern Semen Services (Wellborn, FL, USA). Percoll was from GE Healthcare Bio-Sciences AB (Uppsala, Sweden).

The embryo culture medium was KSOM-BE2 that was modified to exclude methionine. The medium was prepared as a customized medium by the Specialty Media Division of Millipore (Billeca, MA, USA). Methionine was added to the requisite concentration on the day of use.

The In Situ Cell Death Detection Kit (TMR green) was from Roche Diagnostics Corporation (Indianapolis, IN, USA), Hoescht 33342 was from Sigma and polyvinylpyrrolidone (PVP) was from Eastman Kodak (Rochester, NY). Propidium iodide was obtained from Sigma. Calbiochem (San Diego, CA, USA) provided anti-5-methylcytosine (mouse IgG1; clone 162 33 D3) and Invitrogen Molecular Probes (Eugene, OR, USA) was the source of the Zenon Alexa Fluor 488 mouse IgG1 labeling kit 488.

Embryo culture

Bovine embryos were produced from oocytes harvested from ovaries of various breeds at a local abattoir. The procedures for oocyte collection, maturation and fertilization are as described elsewhere [13]. Fertilization was ~1×10^6 Percoll-purified spermatozoa from a pool of frozen-thawed semen from three bulls (various breeds) for 6–8 h at 38.5 C in an atmosphere of 5% CO_2 in humidified air. A different pool of three bulls was used for each replicate and some bulls were used for more than one replicate. Fertilized oocytes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in 1 m*l* of 1000 U/m*l* hyaluronidase and placed in groups of 30 in 50-μl drops of KSOM-BE2 containing various concentrations of methionine overlaid with mineral oil. Embryos were cultured at 38.5 C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 until Day 8 after insemination. Cleavage was assessed at Day 3 and the proportion of embryos developing to the blastocyst stage was assessed at Days 7 and 8. Blastocysts were harvested at Day 8 for additional morphometric or biochemical analysis.

Cell number and apoptosis

Procedures for determination of numbers of inner cell mass (ICM) and trophectoderm (TE) cells in combination with TUNEL labeling of nuclei for determination of apoptosis was performed as described by Fouladi-Nashta *et al*. [20]. Briefly, blastocysts at Day 8 after insemination were harvested and then incubated in a 4-well plate containing 10 mM KPO_4, pH 7.4, 0.9% (w/v) NaCl and 1 mg/ml PVP (PBS-PVP) with 50 μg/ml propidium iodide and 0.5% (v/v) Triton X-100 for 30 sec at 37 C. Embryos were washed twice in PBS-PVP and incubated in a 50 μl microdrop of PBS-PVP with 4% (w/v) paraformaldehyde and 1 μg/ml Hoescht 33258 for 15 min, Embryos were washed twice in PBS-PVP, permeabilized in a 50 μl microdrop of PBS-PVP containing 0.5% (v/v) Triton X-100 for 5 min at room temperature, washed again twice in PBS-PVP, and incubated in a 50 μl microdrop of TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and the enzyme terminal deoxynucleotidyltransferase) according to manufacturer's instructions for 1 h at 37 C in the dark. Positive and negative control embryos were incubated in 50 U/ml RQ1 RNase-free DNase for 1 h at 37 C in the dark. In addition, negative control embryos were incubated in 50 μl microdrops of fluorescent label in the absence of terminal deoxynucleotidyltransferase. Embryos were washed in PBS-PVP medium and immediately mounted on glass slides and examined under a Zeiss Axioplan 2 epifluorescence microscope using red, green, and blue filters. Each embryo was analyzed for total cell number, inner cell mass cells, trophectoderm cells and number of TUNEL-labeled nuclei using ImageJ 1.43 g software (http://rsb.info.nih.gov/ij/)

Glutathione content

Total glutathione content (reduced and oxidized) was measured using a modification of a previously published procedure [21] with a glutathione assay kit from Sigma. Briefly, blastocysts were harvested at Day 8 after insemination and stored in Eppendorf microtubes in groups of 20 in 10 μl water at –20 C. After thawing, embryos were lysed by freezing and thawing 3 times. Samples were transferred to a 96 well plate and 150 μl of a working mixture containing assay buffer [100 mM potassium phosphate with 1 mM EDTA, pH 7.0, 0.162 U/ml glutathione reductase and 0.04 mg/ml 5,5'-dithiobis (2-nitrobenzoic acid) previously diluted in dimethyl sulfoxide] was added to each sample and incubated for 5 min. Then 50 μl of 0.16 mg/m*l* NADPH was added and the absorbance was monitored at 412 nm at 1 min intervals for 5 min with a microplate spectrophotometer (SpectraMax 340PC384, Molecular Devices, Sunnyvale, CA). The concentration of glutathione in each sample was determined by comparison with a standard curve of 7.8–500 pmol/well prepared in the same plate. The amount of glutathione per embryo was determined by dividing the total glutathione per well by the number of embryos in the sample.

DNA methylation

The degree of methylation of nuclear DNA was determined by labeling with anti-methylcytosine tagged with Alexa Fluor 488 as described elsewhere [22]. Nuclei were counterstained with propidium iodide. Microscopic images were captured and analyzed by ImageJ to determine fluorescent intensity of methylcytosine

Table 1. Effect of methionine concentration (0–400 μmol/l) on developmental competence of bovine embryos produced *in vitro*[a]

[Methionine], μmol/l	No. of oocytes	Cleaved/ oocyte (%)	Blastocysts/oocyte, Day 7 (%)	Advanced blastocysts/ blastocyst, Day 7 (%)[d]	Blastocysts/ oocyte, Day 8 (%)
0	160	59.3[b] ± 3.6	4.9[b] ± 3.4	8.3[b] ± 9.1	13.5[b] ± 3.4
35	161	72.1[b] ± 3.6	23.2[c] ± 3.4	42.4[c] ± 9.1	36.1[c] ± 3.4
50	160	63.6[b] ± 3.6	18.2[c] ± 3.4	46.2[c] ± 9.1	30.9[c] ± 3.4
100	161	72.5[b] ± 3.6	21.5[c] ± 3.4	55.9[c] ± 9.1	33.6[c] ± 3.4
200	161	63.3[b] ± 3.6	16.3[c] ± 3.4	64.6[c] ± 9.1	29.8[c] ± 3.4
400	160	69.1[b] ± 3.6	21.3[c] ± 3.4	56.3[c] ± 9.1	33.0[c] ± 3.4
Concentration effect, P		0.10	0.02	0.02	0.005

[a] Data are the least-squares means ± SEM of results from 4 replicates. [b,c] Means with different superscripts differ (P<0.05 or less). The overall effect of concentration is given in the last row. [d] Advanced blastocysts were those that were expanded, hatching or hatched.

Table 2. Effect of methionine concentration (0–400 μmol/l) on characteristics of blastocysts produced *in vitro*[a]

[Methionine], μmol/l	No. of embryos	Total cell number	Ratio of cell number, trophectoderm: inner cell mass	TUNEL-positive blastomeres (% of total cells)	TUNEL-positive trophectoderm cells (% of total)	TUNEL-positive inner cell mass cells, (% of total)
0	7	146 ± 13.4	1.4 ± 0.41	2.8 ± 0.50	1.0 ± 0.06	5.1 ± 1.29
35	26	149 ± 6.1	1.7 ± 0.19	1.9 ± 0.23	1.1 ± 0.03	3.3 ± 0.59
50	17	163 ± 7.4	2.1 ± 0.23	1.7 ± 0.27	0.8 ± 0.03	3.7 ± 0.71
100	22	153 ± 7.3	1.6 ± 0.27	2.3 ± 0.27	1.4 ± 0.03	3.9 ± 0.70
200	21	168 ± 6.4	1.8 ± 0.20	1.6 ± 0.24	0.8 ± 0.03	3.3 ± 0.61
400	18	165 ± 7.3	2.0 ± 0.22	1.9 ± 0.27	1.1 ± 0.03	3.6 ± 0.70

Treatment effects were non-significant.

(green) and nuclei (red). The ratio of green to red fluorescence was used to estimate amount of DNA methylation.

Experimental design and statistical analysis

Two experiments were conducted to evaluate the effect of methionine concentration on the proportion of embryos that developed to the blastocyst stage. For the first experiment, fertilized oocytes were placed in microdrops of KSOM-BE2 containing 0, 35, 50, 100, 200, or 400 μmol/l methionine. Cleavage was assessed on day 3 after insemination and development was assessed on Day 7 and 8. At day 8, blastocysts were harvested and evaluated for cell number, cell allocation to trophectoderm and inner cell mass, and for the proportion of cells that were apoptotic (i.e., labeled with the TUNEL reagent). The experiment was performed using a minimum of 30 embryos per treatment for each replicate; treatments were replicated on 4 occasions. The second experiment was conducted similarly except methionine concentrations were 0, 7, 14, 21, 28 and 35 μmol/l. The experiment was replicated 9 times. For 5 replicates, blastocysts at Day 8 were harvested at random and subjected to analysis for glutathione content. For 3 replicates, Day 8 blastocysts were randomly chosen and analyzed for DNA methylation.

Data were analyzed statistically by least-squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS for Windows, Release 9.00; Cary, NC). Percentage data (for example, percent cleavage and percent blastomeres that are apoptotic) were transformed before analysis using the arcsine transformation because of potential problems of normality with percentage data. The mathematical model included main effects due to replicate (a random effect), and concentration of methionine (fixed effect). The methionine × replicate interaction was used to test the effect of methionine concentration. For all analyses, differences between concentrations were determined using the pdiff procedure of SAS.

Results

In the first experiment, embryos were cultured with concentrations of methionine ranging from 0–400 μmol/l methionine on Day 0–8 after fertilization. As compared to other treatments, embryos cultured in the absence of methionine had aberrant development (Table 1). There was a tendency (P=0.10) for the proportion of embryos that cleaved to be lowest in embryos cultured in methionine-deficient medium. Moreover, embryos in methionine-deficient medium were less likely to become blastocysts at Day 7 (P=0.02) or 8 (P=0.005) and a fewer proportion of blastocysts were advanced in development at Day 7 (P=0.02). While the absence of methionine reduced development, there was no difference in cleavage or blastocyst development rates between embryos cultured in 35, 50, 100, 200 or 400 μmol/l methionine (Table 1).

As shown in Table 2, there was no significant effect of methionine concentration on several characteristics of blastocysts, including total cell number, ratio of trophectoderm to inner cell mass, or frequency of apoptotic cells. While not significant, blasto-

Table 3. Effect of methionine concentration (0–35 μmol/l) on developmental competence of bovine embryos produced *in vitro*[a]

[Methionine], μmol/l	No. of oocytes	Cleaved/ oocyte (%)	Blastocysts/oocyte, Day 7 (%)	Advanced blastocysts/ blastocyst, Day 7 (%)[f]	Blastocysts/oocyte, Day 8 (%)
0	455	$72.7^{b} \pm 1.9$	$11.4^{b} \pm 3.4$	$27.2^{b} \pm 5.8$	$20.4^{b} \pm 2.3$
7	456	$74.6^{b} \pm 1.9$	$27.0^{c} \pm 3.4$	$45.0^{c} \pm 5.8$	$38.0^{c} \pm 2.3$
14	456	$71.1^{b} \pm 1.9$	$28.4^{c} \pm 3.4$	$54.9^{cd} \pm 5.8$	$37.1^{c} \pm 2.3$
21	457	$72.6^{b} \pm 1.9$	$27.7^{c} \pm 3.4$	$72.4^{e} \pm 5.8$	$38.9^{c} \pm 2.3$
28	461	$76.7^{b} \pm 1.9$	$27.6^{c} \pm 3.4$	$60.8^{cde} \pm 5.8$	$38.2^{c} \pm 2.3$
35	453	$75.1^{b} \pm 1.9$	$24.7^{c} \pm 3.4$	$69.2^{de} \pm 5.8$	$35.6^{c} \pm 2.3$
Concentration effect, P		> 0.10	0.0001	0.0001	0.0001

[a] Data are the least-squares means ± SEM of results from 9 replicates. [b–e] Means with different superscripts differ (P<0.05 or less). The overall effect of concentration is given in the last row. [f] Advanced blastocysts were those that were expanded, hatching or hatched.

Table 4. Effect of methionine concentration on total glutathione content of blastocysts collected at Day 8 after insemination[a]

[Methionine], μmol/l	Glutathione concentration, pmol/embryo
0	$0.69^{b} \pm 0.004$
7	$0.51^{c} \pm 0.004$
14	$0.49^{c} \pm 0.004$
21	$0.48^{c} \pm 0.004$
28	$0.45^{c} \pm 0.004$
35	$0.50^{c} \pm 0.004$

[a] Data are the least-squares means ± SEM of results from 5 pools of 20 blastocysts. [b,c] Means with different superscripts differ (P<0.05 or less). The overall effect of concentration was P=0.005.

Table 5. Effect of methionine concentration on DNA methylation as determined by intensity of fluorescence after labeling with anti-methylcytosine[a]

[Methionine], μmol/l	No. of blastocysts	Fluorescent intensity, ratio
0	10	0.96 ± 5.0
7	23	0.99 ± 4.2
21	25	1.01 ± 3.1

[a] Data are the least-squares means ± SEM. There were no effects of concentration of methionine.

cysts produced without methionine had a numerically lower ratio of trophectoderm to inner cell mass cells. The proportion of cells undergoing apoptosis was higher for the inner cell mass than for trophectoderm.

In this first experiment, the lowest concentration of methionine tested was equivalent to concentrations seen in the reproductive tract. To further define the requirement for methionine and to ascertain whether a deficiency in methionine in the reproductive tract might compromise development, a second experiment was conducted in which embryos were cultured with concentrations of methionine ranging from 0–35 μmol/l. As in the previous experiment, the proportion of oocytes that became blastocysts at Day 7 or 8 after insemination was lower for embryos cultured in the absence of methionine than for embryos cultured with methionine (P= 0.0001) but there was no difference in blastocyst development between embryos cultured with 7, 14, 21 28, or 35 μmol/l methionine (Table 3). The proportion of blastocysts at Day 7 that were at advanced states (expanded, hatching, or hatched) was also lowest (P=0.0001) for embryos cultured without methionine. For this variable, however, there was a dose-dependent response to addition of methionine. The percent of blastocysts that were advanced increased as concentrations of methionine rose from 7–21 μmol/l and then reached a plateau so that there was no difference in percent advanced blastocysts between embryos cultured at 21, 28 and 35 μmol/l.

In the second experiment, the effect of methionine concentration on blastocyst glutathione concentration (Table 4) and DNA methylation (Table 5) was evaluated. There was an increase in glutathione content of embryos cultured without methionine as compared to embryos cultured with methionine (P=0.005). The degree of DNA methylation, as determined by immunohistochemical labeling of methylcytosine, was similar among embryos at all concentrations of methionine tested (0, 7 and 21 μmol/l).

Discussion

Results from this study suggest that the methionine requirement of preimplantation bovine embryos is between 14 and 21 μmol/l. Although addition of more than 7 μmol/l methionine did not improve development to the blastocyst stage, more blastocysts were classified as advanced at Day 7 after insemination when cultured with methionine at ≥21 μmol/l than for embryos cultured with 7 or 14 μmol/l methionine. Establishing a requirement for methionine of 14–21 μmol/l means that concentrations of methionine in the reproductive tract (32–49 μmol/l in oviductal fluid and 31–46 μmol/l in uterine fluid) [14] and in culture media (typically 50 μmol/l) are optimal for development.

It is not known whether there are physiological or nutritional situations that would lead to methionine concentration in the reproductive tract declining to concentrations below 21 μmol/l.

Even in cows fed a diet low in low crude protein (14%), however, methionine concentrations in blood plasma (which are close to concentrations in the reproductive tract; ref. 14) averaged 21.6 μmol/l [23]. It is likely, therefore, that methionine concentrations in the reproductive tract are adequate for embryonic development in most cases.

While not compromising the competence of an embryo to become a blastocyst, low concentrations of methionine (7 and 14 μmol/l) reduced the proportion of blastocysts classified as advanced in development, i.e., that had either expanded or was hatching or hatched from the zona pellucida. Since expansion and hatching both involve an increase in the size of the blastocoelic cavity, it is possible that methionine is important for regulation of blastocoelic volume. Such a role could be exerted through conversion of methionine to S-adenosylmethionine, which is produced solely from methionine and can serve as a methyl donor to cause methylation of membrane lipids and increase Na/K ATPase activity [24].

S-adenosylmethionine also provides methyl groups for DNA methylation. Extracellular methionine does not appear to be limiting for DNA methylation in the bovine preimplantation embryo, however, since elimination of methionine from culture medium had no effect on labeling of nuclei with anti-methylcytosine. The bovine embryo does not undergo widespread DNA methylation until after the 8-cell stage [25]. Perhaps, in contrast to the situation for membrane lipids (see above), requirements of the preimplantation embryo for S-adenosylmethionine for DNA methylation are satisfied by stores from the oocyte or regeneration of methionine through conversion of S-adenosylhomocysteine, the demethylated product of S-adenosylmethonine, to homocysteine and then to methionine [26, 27]. A recent study indicates that transcripts for enzymes for S-adenosylmethionine synthesis are present in the bovine oocyte and preimplantation embryo and that transcripts for methionine adenotransferase 1A, one of the enzymes converting methionine to S-adenosylmethionine, are reduced after the 8-cell stage [28]. There may therefore be a reduction in capacity for S-adenosylmethionine synthesis as development proceeds.

The most surprising result was that intracellular concentrations of glutathione, the major water-soluble antioxidant in cells, were higher in embryos cultured in the absence of methionine than in embryos receiving methionine in the culture medium. Methionine is a precursor of cysteine, which is an amino acid with its own antioxidant properties and which is one of the three amino acids used for biosynthesis of glutathione [12]. A reduction in intracellular glutathione concentrations must be caused by either reduced glutathione biosynthesis or increased oxidation of glutathione by reactive oxygen species. The latter possibility seems more likely since oxidation of methionine can lead to formation of methionine reactive oxygen species [29]. The fact that there was not a concentration-dependent decrease in glutathione content with increasing methionine concentration may mean that the chemical systems leading to methionine free radical synthesis are saturated at low concentrations of methionine. One measure of redox status is the incidence of apoptosis [30]. Since the frequency of apoptotic blastomeres was not affected by methionine status, it is likely that the reduction in glutathione concentrations among embryos receiving methionine was not sufficient to compromise cellular function. As reported elsewhere [20, 31], the incidence of apoptosis was lower for trohectoderm than inner cell mass, indicating differences between these two cell types in either the amount of reactive species generation or antioxidant defenses.

There is likely to be some variation in absolute methionine requirement for the preimplantation embryo. Concentrations of other amino acids could affect methionine transport and metabolism. In addition, methionine concentrations varied somewhat during culture depending upon uptake or production of methionine by the embryo. This effect is likely to be small because there was either a slight uptake (~0.5 pmol/embryo/h) [10] or no net uptake [11] of methionine by the cultured bovine blastocyst.

In conclusion, these results suggest that methionine requirements for development of the preimplantation embryo are between 14 and 21 μmol/l. These concentrations are lower than methionine concentrations typically seen in the reproductive tract and suggest that deficiencies in methionine compromising embryonic development are uncommon. The fact that methionine is required for development at lower concentrations than existing in the reproductive tract suggests that the utilization of methionine is low during preimplantation development. Additional evidence for this idea comes from the observation that the uptake of methionine by the preimplantation bovine embryo is lower than for most other amino acids [10, 11]. The lack of effect of methionine deficiency on DNA methylation in the embryo suggests extracellular methionine is not required for DNA methylation in the early preimplantation period. Moreover, protein synthesis remains low throughout early development and does not increase substantially until the blastocyst stage of development [32].

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