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Epi-nutrients in the oviductal environment: Folate levels and differential gene expression of its receptors and transporters in the bovine oviduct

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ABSTRACT

Recent studies have demonstrated that the oviductal environment plays an active role in modulating the epigenetic marks of the preimplantation embryo genome, but the molecular factors that mediate this epigenetic effect are unknown. Folate is a well-known epi-nutrient that can impact on cell epigenetic machinery during embryonic and fetal development. However, the study of this epi-nutrient in the oviduct is still limited. The present study was conducted to confirm the presence and physiological concentration of folate in bovine oviductal fluid (OF) and to determine if bovine oviduct epithelial cells (BOECs) are able to regulate the uptake of this micronutrient. Samples of OF from ipsi- and contralateral oviducts were collected at different stages of the estrous cycle and folate levels were determined using a competitive receptor binding immunoassay. In addition, gene expression of folate receptors (*FOLR1*, *FOLR2*) and transporters (*SLC19A1*, *SLC46A1*) were analyzed in BOECs from ampulla and isthmus regions during different stages of the estrous cycle using RT-qPCR. *In vitro* culture assays were also performed to evaluate whether expression of these genes responds to hormonal stimulation. Our results demonstrated presence of folate in the OF, showing changes of its concentration in the ipsilateral oviduct during the estrous cycle and significantly lower levels at the postovulatory stage. Moreover, gene expression of folate receptors and transporters was detected in BOECs, showing regional and cycle-dependent changes. In particular, differential expression of *FOLR1* mRNA was observed in BOECs from the isthmus region, reaching significantly higher levels during the postovulatory stage. Under *in vitro* culture conditions, gene expression of folate receptors and transporters was maintained in BOEC explants and a particular susceptibility to steroid hormone stimulation was observed. In conclusion, the present study confirms the presence of folate in the bovine oviduct and proves the existence of a fine-tuned regulation of the expression of its receptors and transporters, highlighting the importance to expand the knowledge about this epi-nutrient in the oviductal context.

1. Introduction

The mammalian oviduct provides the most appropriate environment for fertilization and early embryo development. Recently, growing evidence has confirmed that the oviduct can also play an important role in modulating the epigenetic landscape of the embryo during early development [1]. It has been shown that the culture of bovine embryos from the zygote to the 16-cell stage in the presence of oviductal fluid (OF) induces DNA methylation changes in specific genomic regions in the resulting blastocysts [2]. Likewise, sequential addition of OF and uterine fluid to embryo culture medium affects DNA methylation marks at the blastocyst stage, showing global methylation patterns similar to those found in *in vivo* porcine blastocysts [3]. However, the molecular factors present in the oviductal milieu that mediate this epigenetic effect are still unknown. The OF has a highly complex composition,

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including amino acids, several proteins, simple and complex carbohydrates, lipids, ions and phospholipids, as well as unknown components [4,5]. Among them, maternal nutrients involved in the regulation of metabolic methylation pathways, known as "epi-nutrients" [6], could be potential constituents mediating the epigenetic effect during the first stages of embryonic development in the oviductal environment.

Epi-nutrients can affect the epigenome to a greater degree than other micronutrients because of their critical role in DNA methylation and histone modification [6,7]. Among them, folate (or its synthetic form folic acid) has been recognized as one of the most important epi-nutrients during pregnancy [8,9]. Folate belongs to the water-soluble B group vitamins (vitamin B9) and it is used by cells as a coenzyme in transfer reactions of single-carbon groups in one-carbon metabolism [10]. Consequently, folates are essential for biosynthesis of purine and thymidine nucleotides, metabolism of amino acids, and regulation of availability of S-adenosylmethionine, the major methyl donor required in the process of DNA and histone methylation [10]. In the folate metabolic pathway, 5-methyltetrahydrofolate (5-mTHF) transfers a methyl group to homocysteine, thereby generating methionine and tetrahydrofolate (THF). Then, THF is recycled by transferring a methyl group from serine to form 5,10-methylene-THF, which is then reduced back to 5-mTHF [11]. Given the biochemical functions of folate, this micronutrient has a critical role in a large number of biological processes, including cellular proliferation and homeostasis, DNA replication and repair, and epigenetic regulation of the genome, which in turn, has an impact on gene expression [12,13].

Folate has important reproductive implications in mammals during the peri- and post-conception periods. Therefore, disruption of folate metabolism or its deficiency leads to impairments in reproductive health. Deficiency in maternal folate levels during embryo and fetal development has clearly demonstrated to be a risk factor for neural tube defects, growth arrest, implantation failure and early pregnancy loss [11,14]. Additionally, some studies have shown that inactivation of genes encoding for folate receptors and transporters in knock out models leads to embryonic death and other often lethal disorders [15]. On the other hand, folate levels in the microenvironment of the maturing oocyte affect the follicular development and reproductive competence of the female gamete [16]. Furthermore, folate-binding proteins have been purified from uterine flushing, and DNA methyltransferase expression in endometrial cells has shown to be regulated by dietary folate levels, suggesting that folates also play a critical role in uterine physiology [17,18].

Most studies to date have been centered on the effect of folate on post-implantation embryonic and fetal development in humans, rodents and some ruminant species [11,19,20]. However, there is little knowledge about the role of folate in the oviductal microenvironment, the maternal site where the early embryo undergoes its initial stages of epigenetic reprogramming. Endogenous folate is essential for the development of bovine embryos [21], and expression of specific folate transporters during the preimplantation period suggests an exogenous requirement of folate by the embryos from their natural environment [21,22]. However, the concentrations of folate to which bovine oviductal cells, gametes, and embryos are exposed *in vivo*, are currently unknown. Considering this, the present study focused on determining the presence and physiological levels of folate in the bovine OF during the estrous cycle. To explore whether oviductal cells could regulate the uptake of this micronutrient modulating its concentration, expression of genes associated to folate uptake was examined in bovine oviduct epithelial cells (BOECs), analyzing differences between anatomic regions during the estrous cycle. In addition, *in vitro* culture assays were performed to determine whether expression of these genes is maintained under *in vitro* culture conditions and if it is susceptible to exogenous addition of 17-β-estradiol (E2) and progesterone (P4).

2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

2.2. Animals and biological samples

Genital tracts from heifers slaughtered in a local abattoir were transported on ice to the laboratory and processed within 2 h after animal death. Only samples from non-pregnant animals without anatomical abnormalities or defects in their reproductive tracts were included in the study. The stage of the estrous cycle was determined by visual examination of the ovarian morphology [23] and the tracts were classified into three groups according to Garcia et al. [24]: (a) Preovulatory phase (Pre-Ov); (b) Postovulatory phase (Post-Ov) and (c) Mid-luteal phase (Mid-L). Once classified, ipsilateral and contralateral oviducts to the ovary containing a preovulatory follicle or an ovulation site were separated from the tracts, washed in sterile ice-cold phosphate buffered saline (PBS), pH 7.4, and transferred to Petri dishes on ice before being dissected to remove blood vessels, connective tissue and adhering fat. The selected oviducts were then processed for collection of OF or epithelial cells as described below.

2.3. Collection of bovine oviductal fluid

Bovine oviductal fluid was collected from ipsilateral and contralateral oviducts during Pre-Ov, Post-Ov and Mid-L phases ($n = 36$; six oviducts per estrous cycle stage and oviductal side). Following the protocol described by Carrasco et al. [25], each oviduct was squeezed gently from the utero-tubal junction toward the ampulla and the OF was collected by aspiration using a 200 μL automatic pipette. Each individual OF sample was centrifuged twice at 7000 × g for 10 min at 4 °C to remove cellular debris and the remaining supernatant (10–25 μL/oviduct) was protected from light and stored at −80 °C until folate measurements. Samples containing blood were discarded. In addition, follicular fluid of preovulatory follicles with a diameter of 15–20 mm ($n = 5$), was collected from bovine ovaries by aspiration with a syringe. Follicular fluid samples from each follicle were centrifuged twice at 7000 × g for 10 min at 4 °C and a volume of 500 μL was thereafter collected for measurement of the folate concentration.

2.4. Analysis of folate concentration in the oviductal fluid

Folate levels were measured in bovine OF samples with chemiluminescent microparticle immunoassay (CMIA) (ARCHITECT Folate, Abbott Diagnostics, Longford, Ireland) [26] using an Architect i1000SR analyzer (Abbott). This technique involves a two-step competitive assay that allows quantitative determination of 5-mTHF, the most abundant form of circulating folate, by using folate binding protein (FBP) coated paramagnetic microparticles that bind to the folate present in the sample. After a washing step, pteroic acid-acridinium labeled conjugate is added, which binds to unoccupied sites of FBP-coated microparticles. After a chemiluminescent reaction, an inverse relationship exists between the amount of relative light units (RLUs) detected by the system and the amount of folate present in the sample. Preliminary experiments showed that a 1:100 dilution of the OF samples was appropriate to obtain values

within the range of sensitivity of the assay (0.003–0.05 μmol/L). Thus, for all determinations, a volume of 5 μL of each OF sample was diluted in 500 μL of physiological solution and immediately quantified. A total of six individual OF samples obtained from oviducts ipsilateral or contralateral to the dominant ovary for each different stage of the estrous cycle were analyzed in duplicate. The intra- and inter-assay coefficients of variation (CV) for all analyses were below 5%. For comparison, folate levels in follicular fluid were also assayed using the biochemical method described above. All concentration values were expressed as μmol/L.

2.5. Isolation of bovine oviduct epithelial cells

Bovine oviduct epithelial cells were obtained *ex vivo* immediately after slaughter as previously described by García et al. [27]. Briefly, oviducts were collected from a local slaughterhouse and transported to the laboratory on ice within 2 h after animal death. The surrounding fat and connective tissues were carefully removed and then each oviduct was ligated and washed three times with sterile PBS (pH 7.4). After washing in PBS, isthmus and ampulla regions were separated and the oviductal mucosa from each anatomical region was obtained by gently squeezing with a sterile glass slide. The oviductal cells were then washed twice with PBS by centrifugation at 300 × g for 10 min and immediately processed for RNA extraction (n = 12; four ipsilateral oviducts/stage of the estrous cycle).

2.6. In vitro culture of bovine oviduct epithelial cells

For *in vitro* culture assays, isthmus and ampulla epithelial cells obtained from ipsilateral oviducts at the Post-Ov stage (n = 12, four ipsilateral oviducts for each experimental replicate) as described in section 2.5 were cultured *in vitro* following the protocol described by Rottmayer et al. [28] with slight modifications. After gently squeezing, the cells obtained from each anatomical region were separately collected in TCM-199 (Gibco product 11150-059; Grand Island, NY, USA) culture medium supplemented with 10% bovine fetal serum (Internegocios, Buenos Aires, Argentina), 25 mM Hepes (Gibco, Life Technology, Burlington, ON, Canada), 0.2 mM sodium pyruvate, and 1% antibiotic-antimycotic solution (Gibco product 15240). The cell suspension was then pipetted 15 times with a 1000 μL filter tip before being passed twice through a 21 gauge syringe needle. Two washing steps were performed, each followed by 15 min sedimentation in a cell culture incubator. After the washing process, cell viability was analyzed by trypan blue staining and by evaluating ciliary activity on an inverted microscope (400× magnification). The cells obtained were pre-cultured during 24 h in 60 mm culture dishes with 5 mL TCM-199 supplemented as described above, at 38.5 °C in a humidified atmosphere with 5% CO_2. As has already been described and validated, during this initial culture period BOECs can grow in suspension forming multicellular spheroids with active cilia on the outer surface that maintain oviductal explants in constant rotational motion. After 24 h of preculture, multicellular spheroids derived from ampulla and isthmus regions were collected and allowed to sediment by gravity for 10 min. As described below, these BOEC explants were then used for gene expression analysis or hormonal treatment assays.

2.7. Hormonal treatment assays

To examine the effect of estradiol (E2) and progesterone (P4) on expression of genes encoding folate receptors and transporters, 10 mg of BOEC spheroids from ampulla and isthmus were separately placed in a 4-well culture plate (Nunclon, Roskilde, Denmark) with 500 μL of TCM-199 medium supplemented as described above and samples were stimulated during 24 h with 100 pg/mL E2 (E1024), 100 ng/mL P4 (P0130) or with a combination of both hormones. Steroid hormone concentrations were selected according to the average concentration of E2 and P4 reported in bovine OF during the postovulatory period [29], in which 100 pg/mL of E2 and 100 ng/mL of P4 were considered physiological concentrations. Before the experiments, a fresh stock solution of E2 and P4 was prepared in absolute ethanol (vehicle). Each stock solution was successively diluted to obtain the working solution. Cells cultured without hormones and treated with an equivalent concentration and dilution of the vehicle were used as control. Culture took place at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. After culture period, cell membrane integrity and viability was confirmed by staining an aliquot of BOEC explants with the nucleic acid stain Hoechst 33342 (25 μg/mL), and the conventional dead-cell stain, propidium iodide (100 μg/mL). Afterwards, cells were processed for RNA extraction. The experiment was carried out three times under the same assay conditions.

2.8. RNA isolation, cDNA synthesis and RT-qPCR analysis

Total RNA of *ex vivo* and *in vitro* BOECs was extracted using TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Reverse Transcription to cDNA was performed using 1 μg of RNA, M-MLV reverse transcriptase (Promega, Madison, WI, USA) and Oligo (dT)17 primers following the protocol previously described [30].

Messenger RNA expression levels of folate receptors (*FOLR1* and *FOLR2*) and transporters (*SLC19A1* and *SLC46A1*) were determined by qPCR using specific primers (see Table 1). All qPCR reactions were performed in a final volume of 20 μL, containing 5 μL of cDNA template (diluted 1:5), 0.25 mM of forward and reverse primers and 10 μL of Fast EvaGreen qPCR Master Mix (Biotium, Hayward, CA) and were run in a CFX96™ Real-Time PCR detection System (Bio-Rad, Hercules, CA, USA). The PCR program consisted of an initial step of 2 min at 95 °C, followed by 48 cycles of 15 s at 95 °C and 30 s at 58 °C for annealing and extension. Melting curve analysis was performed to examine the specificity in each reaction tube. Depending on the experiment, three or four biological repetitions were analyzed and the mean Ct value for each repetition was obtained from a technical duplicate. Also, no-template and no-reverse transcription controls were included. Relative expression levels were quantified by the ΔΔCt method using CFX Manager Software version 3.0 (Bio-Rad Laboratories, Hercules, CA, USA). RT-qPCR data were normalized to the geometric mean of two housekeeping genes, *GAPDH* and *ACTB*. In our determinations, *GAPDH* and *ACTB* produced uniform expression levels varying less than 0.5 Ct between control and treated cDNA samples. The target stability function of CFX96 software determined that the combined M-value for *GAPDH* and *ACTB* for experiments with *ex vivo* obtained BOECs was M = 0.3235 (CV = 0.1120) and for *in vitro* cultured BOECs was M = 0.3257 (CV = 0.1156). The entire study was carried out following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) recommendations [31].

2.9. Statistical analysis

Data analysis was conducted using SigmaStat 3.5 and SigmaPlot 10.0 statistical software (Systat Software, Richmond, CA, USA). One or two-way ANOVA and subsequent multiple pair-wise comparisons using Tukey's test when applicable, were used to detect differences in folate and mRNA levels between samples and treatments. P values < 0.05 were considered statistically significant.

Table 1
Primers sequences used for RT-qPCR analysis.

Gene	Primer sequences (5′- 3′)		Amplicon length (bp)	GenBank accession number
FOLR1	Forward	ACAGATTCGACTGGGACCAC	164	NM_001206532.1
	Reverse	TTTGCAGAGGGGCACATTCA		
FOLR2	Forward	CCAGGAGGTGAACCAGAAGT	185	NM_001075325.1
	Reverse	CAAATGTGCGGCAGATGGTC		
SLC19A1	Forward	ACTGACGACATTTGGTTGTGC	143	NM_001076453.2
	Reverse	AGGAACGTGTTGATGCCGAA		
SLC46A1	Forward	TGGCAGCTGGACTGCTATTC	144	NM_001079585.1
	Reverse	GTGCTCACGTTGCTCCTCTT		
GAPDH	Forward	AGATGGTGAAGGTCGGAGTG	117	NM_001034034.2
	Reverse	GAAGGTCAATGAAGGGGTCA		
ACTB	Forward	GATCATTGCTCCTCCCGA	101	NM_173979.3
	Reverse	ACTCCTGCTTGCTGATCC		

bp: base pairs.

3. Results

3.1. Presence and concentration of folate in bovine oviductal fluid

To evaluate the presence and concentration of folate in the oviductal context, OF samples obtained during the Pre-Ov, Post-Ov and Mid-L phases from ipsi- and contralateral oviducts were quantified using a chemiluminescent microparticle immunoassay (CMIA). As shown in Table 2, presence of folate was detected in all OF samples obtained from ipsi- and contralateral oviducts during the three stages of the estrous cycle analyzed. Folate levels in the ipsilateral OF during the Pre-Ov and Mid-L phase were significantly higher than in the Post-Ov stage ($P < 0.05$). In contrast, the concentration of folate in fluid obtained from contralateral oviducts did not differ significantly among the different phases of the estrous cycle. The folate level was significantly higher in ipsilateral than contralateral OF during the Pre-Ov phase ($P = 0.028$).

On the other hand, to determine a possible contribution of follicular fluid to the OF folate concentration, folate levels in pre-ovulatory follicular fluid were determined. In all the follicular fluid samples analyzed, folate concentration was significantly lower (<0.02 nmol/L) than in OF ($P < 0.001$).

3.2. Gene expression of folate receptors and transporters in bovine oviduct epithelial cells during the estrous cycle

In order to characterize the gene expression pattern of folate receptors and transporters in the bovine oviduct during the estrous cycle, relative mRNA expression of *FOLR1, FOLR2, SLC19A1* and *SLC46A1* was evaluated by RT-qPCR in BOECs obtained from ampulla and isthmus of ipsilateral oviducts during Pre-Ov, Post-Ov, and Mid-L phases.

As shown in Fig. 1A, mRNA expression of the receptors (*FOLR1* and *FOLR2*) and transporters (*SLC19A1* and *SLC46A1*) involved in folate cellular uptake was detected in both anatomic regions during the three phases of the estrous cycle analyzed. Particular differences in the expression levels were observed according to the anatomical region or the stage of the estrous cycle. Expression of *FOLR1* mRNA was significantly higher in epithelial cells of the isthmus than of ampulla in the three stages of the estrous cycle analyzed (Fig. 1A; $P < 0.05$), and the highest transcript levels were observed during the Post-Ov stage in the isthmus epithelial cells (Fig. 1A). In contrast, *FOLR2* mRNA showed a similar expression pattern in both anatomical regions (Fig. 1A) with higher levels during the Mid-L phase and a significant decrease during the Post-Ov stage (Fig. 1A; $P < 0.05$).

Regarding folate transporters, no significant differences were observed in the expression of *SLC19A1* mRNA, showing a similar expression level during the estrous cycle in both anatomical regions (Fig. 1A). In contrast, *SLC46A1* mRNA expression fluctuated markedly during the estrous cycle, increasing from the Pre-Ov until the Mid-L phase (Fig. 1A, $P < 0.05$) in both anatomical regions.

The average expression level, calculated by averaging the expression levels of the genes analyzed throughout the estrous cycle, reflected that only *FOLR1* gene presented an anatomically differential expression pattern in the bovine oviduct (Fig. 1B; $P < 0.001$). Statistical analysis with two-way ANOVA revealed a significant interaction between the estrous cycle and the anatomic region for *FOLR1* ($P = 0.002$) but not for the other genes analyzed.

Table 2
Folate concentration (μmol/L) in oviductal fluid samples from ipsilateral and contralateral oviducts during different stages of the estrous cycle.

Estrous cycle stage	Ipsilateral	Contralateral	P-value
Preovulatory	1.83 ± 0.14^{a}	1.13 ± 0.11	0.028*
Postovulatory	1.11 ± 0.11^{b}	1.20 ± 0.15	0.999
Mid-luteal	1.80 ± 0.21^{a}	1.40 ± 0.18	0.349

Data are presented as Mean ± SEM ($n = 6$ samples for each estrous cycle stage and oviductal side). Different letters denote a significant difference ($P < 0.05$) between estrous cycle stages while asterisk (*) denote a significant difference between ipsilateral and contralateral oviducts (P values are indicated in the table).

3.3. Gene expression of folate receptors and transporters in bovine oviduct epithelial cells under in vitro culture conditions

To evaluate if oviductal cells maintain gene expression of folate receptors and transporters under *in vitro* culture conditions, BOECs obtained from ampulla and isthmus regions during the Post-Ov stage were cultured in a cell suspension culture system. After 48 h of culture (Fig. 2A), gene expression of folate receptors and transporters was evaluated by RT-qPCR (Fig. 2B).

As shown in Fig. 2B, expression of *FOLR1, FOLR2, SLC19A1* and *SLC46A1* mRNA was detected in postovulatory BOEC explants that were obtained from both anatomical regions. As was previously observed in fresh cells directly obtained from the oviduct (*ex vivo*), *FOLR1* mRNA maintained a differential expression profile showing significantly higher expression levels in BOEC explants derived from the isthmus region (Fig. 2B, $P < 0.001$). In the case of *FOLR2*, *SLC19A1* and *SLC46A1,* marked differences in their mRNA levels were observed between BOEC explants derived from the ampulla and isthmus region, but these differences were not previously observed in the *ex vivo* analysis. High expression levels of *FOLR2* mRNA were detected in oviduct explants derived from ampulla (Fig. 2B, $P < 0.05$), while for *SLC19A1* and *SLC46A1* mRNA highest levels were found in BOEC explants obtained from isthmus (Fig. 2B, $P < 0.05$).

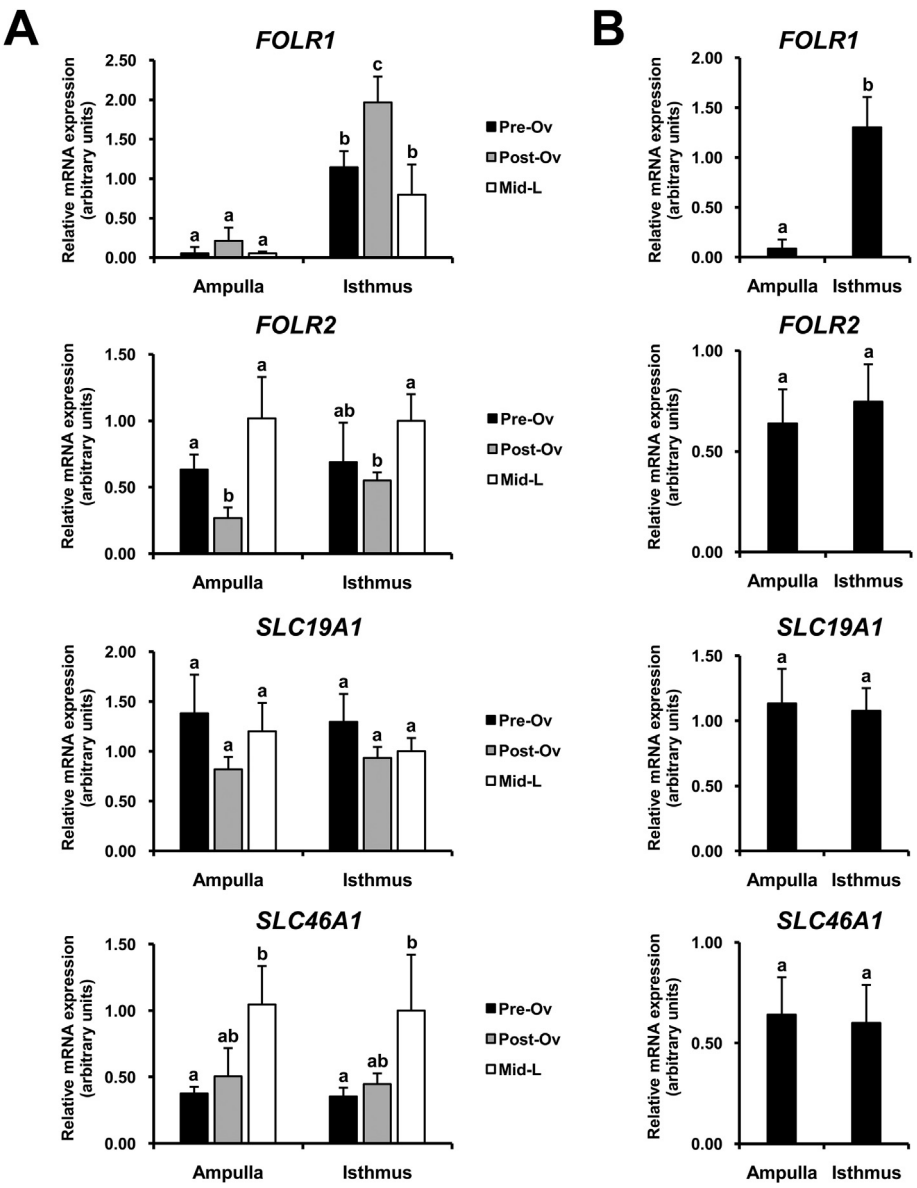

Fig. 1. Relative mRNA expression levels of folate receptors and transporters in bovine oviduct epithelial cells. (A) Bars represent relative mRNA levels of *FOLR1*, *FOLR2*, *SLC19A1* and *SLC46A1* in BOECs obtained from ampulla and isthmus region during different stages of the estrous cycle. (B) Bars represent the average relative mRNA levels for *FOLR1*, *FOLR2*, *SLC19A1* and *SLC46A1* in BOECs from each anatomic region throughout the three estrous cycle phases analyzed. Relative gene expression levels were normalized to the geometric mean of the endogenous *GAPDH* and *ACTB* genes. Results are expressed as mean ± SEM. Significant differences ($P < 0.05$) within oviductal region are indicated with different letters. Data were obtained from four replicates for each anatomic region and cycle stage. Pre-Ov: preovulatory phase; Post-Ov: postovulatory phase and Mid-L: mid-luteal phase.

3.4. Effect of steroid hormones on gene expression of folate receptors and transporters in bovine oviduct epithelial cells cultured in vitro

The following objective was to examine if mRNA expression of folate receptors and transporters responded to hormonal stimulation by evaluating the effect of exogenously applied E2 and P4 to the suspension culture of BOECs from ampulla and isthmus regions. Based on the previous observations that showed anatomic differences in the mRNA expression levels of the studied genes under *in vitro* culture conditions, hormonal assays were separately performed in ampulla and isthmus explants.

Treatment with 100 pg/mL of E2 did not induce changes to *FOLR1* or *FOLR2* compared to the control group in both ampulla and isthmus explants (Fig. 3A and B). However, a significant increase in the expression levels of *SLC19A1* was observed particularly in BOEC explants from ampulla (Fig. 3C, $P < 0.05$), while *SLC46A1* mRNA expression decreased in oviductal explants derived from isthmus region compared with the control groups (Fig. 3D, $P < 0.05$).

Hormonal treatment with 100 ng/mL of P4 demonstrated a significant up-regulation in the expression of *FOLR2* in oviductal explants of both anatomical regions, especially in ampulla (Fig. 3B, $P < 0.05$). A similar behavior was observed for *FOLR1* but the difference was not significant in this case (Fig. 3A). In contrast, P4 treatment induced a significant down-regulation in the expression of *SLC19A1* in explants derived from both anatomic regions (Fig. 3C) and only in isthmus explants for *SLC46A1* (Fig. 3D, $P < 0.05$).

Simultaneous stimulation of BOEC explants with both steroid hormones also had a significant impact on gene expression of folate receptors and transporters. The addition of E2 plus P4 to culture medium induced a significant increase in *FOLR1* transcript abundance compared with the control group, particularly in oviductal

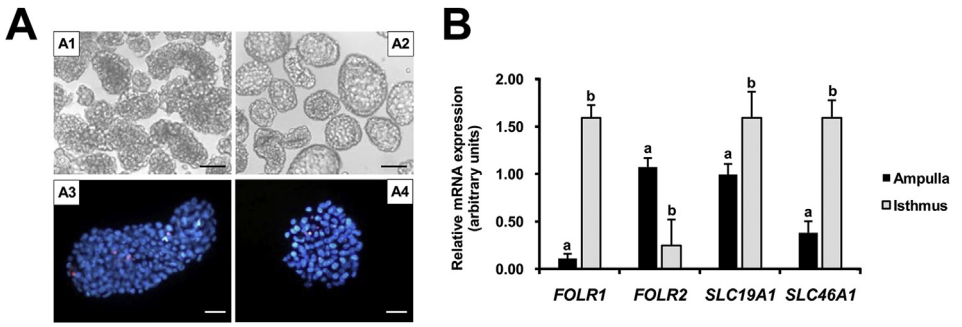

Fig. 2. Expression analysis of folate receptors and transporters in bovine oviduct epithelial cells under *in vitro* culture conditions. (A) Representative inverted microscope image of BOEC explants from ampulla (A1) and isthmus (A2) regions cultured during 48 h in a cell suspension culture system (Scale bar = 100 μm). In addition, representative fluorescent image of Hoechst/PI double staining of BOEC explants from ampulla (A3) and isthmus (A4) after 48 h of culture are shown (Scale bar = 50 μm). (B) Relative mRNA expression levels of folate receptors and transporters in postovulatory BOEC explants obtained from ampulla and isthmus regions. Results are expressed as mean ± SEM. Significant differences ($P < 0.05$) are indicated with different letters. Data were obtained from three experimental replicates for each anatomic region.

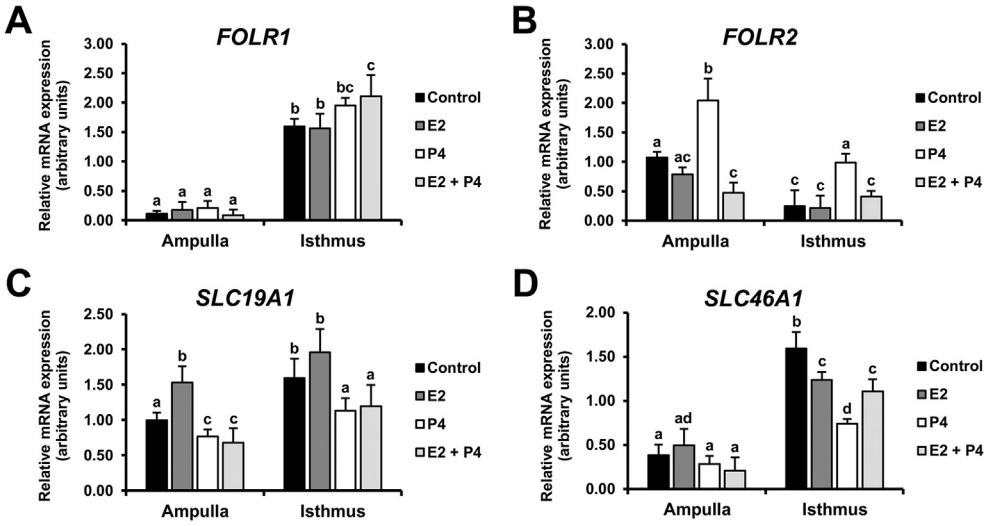

Fig. 3. Relative mRNA expression levels of folate receptors and transporters in bovine oviduct epithelial cells cultured *in vitro* under steroid hormone stimulation. Bars represent the relative mRNA levels of (A) *FOLR1*, (B) *FOLR2*, (C) *SLC19A1* and (D) *SLC46A1* in BOEC explants from ampulla and isthmus regions cultured for 24 h with or without 100 pg/mL E2, 100 ng/mL P4 or 100 pg/mL E2 + 100 ng/mL P4. Relative gene expression levels were normalized to the geometric mean of the endogenous *GAPDH* and *ACTB* genes. Results are expressed as mean ± SEM. Significant differences ($P < 0.05$) within oviductal region are indicated with different letters. Data were obtained from three experimental replicates.

explants derived from the isthmus region (Fig. 3A, $P < 0.05$). However, the treatment with both hormones produced a significant decrease in the expression levels of *FOLR2* in BOEC explants from ampulla, *SLC46A1* in BOEC explants from isthmus and *SLC19A1* in BOEC explants derived from both regions (Fig. 3B, 3C, 3D, $P < 0.05$).

4. Discussion

Even though a large number of constituents have been identified in OF, available information about the presence of different "epi-nutrients" in this biological fluid and their fluctuations during the estrous cycle is scarce. To our knowledge, the present study demonstrates for the first time that folate is present in bovine OF and that the concentration of this micronutrient fluctuates in the ipsilateral oviduct during the estrous cycle. Given the fact that microorganisms present in the cow rumen produce high levels of B vitamins [32,33], it would not be surprising to find significant folate levels in bovine reproductive fluids. Oviductal fluid is formed by secreted components from epithelial cells and blood plasma [4,5]. Therefore, presence of folate in bovine OF could be attributed to transudation from the circulating blood. However, a minor contribution of follicular fluid to local oviductal folate concentrations at the time of ovulation cannot be excluded. Folate concentrations in bovine serum are reported to be around ~8 ng/mL to ~30 ng/mL (approximately equivalent to 20–70 nM) [32,34–38]. Interestingly, according to our results, folate levels were about 30–50 times more concentrated in OF than in bovine serum and the contribution of follicular fluid would be insignificant. Our results are in line with findings by Lamy et al. [29], who determined for example that E2 and P4 levels were 10–50 times higher in bovine OF than in circulating blood. In addition, our results reflect that folate concentrations varied during the estrous cycle, especially in OF derived from the ipsilateral oviduct. Interestingly, folate levels observed during the Post-Ov stage were lower than during the Mid-luteal and Pre-Ov phase. It is well known that the OF composition changes during the estrous cycle and pregnancy under the influence of ovarian steroid hormones through a direct or indirect effect on oviduct epithelial cells [39,40]. Although the production of OF increases during the peri-ovulatory period, in part by modulating the blood flow to the oviduct [40], the lower folate level detected in ipsilateral OF during Post-Ov stage could be related to a greater

uptake of this micronutrient during this specific phase of the estrous cycle. It is well-known that the Post-Ov phase encompasses a period of intense cellular activity in the oviductal epithelium, in which epithelial cells play a preponderant role to maintain and modulate the suitable milieu for fertilization and early embryo development. Indeed, a greater folate uptake could be contributing to regulate the oviduct cellular activity. Nevertheless, additional studies are necessary to examine this hypothesis more thoroughly. While it has been shown that preimplantation bovine embryos express folate transporters [21], up to now, the exogenous folate requirements by the embryo in this species are still unknown. Therefore, knowledge of physiological levels of folate in the bovine oviductal environment merits further investigation in order to better understand the role of this micronutrient in the oviductal physiology and the early embryo development.

Folate uptake across the epithelia occurs via specific receptor and/or transporter proteins. The most studied folate receptors are folate receptors 1 and 2, which are transcribed from *FOLR1* and *FOLR2* genes, respectively [41]. These receptors are attached to the extracellular side of the membrane by a glycosylphosphatidylinositol anchor and they bind 5-mTHF and folic acid with high affinity, allowing folate transport into cells through receptor-mediated endocytosis [42,43]. Other members of the folate receptor gene family that have been identified in mammals are *FOLR3* and *FOLR4* [44,45]. However, unlike *FOLR1* and *FOLR2*, the *FOLR3* gene codes for a protein which is secreted outside the cell [45], while *FOLR4* codes for a protein that is unable to bind to folate and participates in gamete recognition and fertilization [46]. In addition, folate uptake into cells is primarily carried out by two membrane transporter proteins, reduced folate carrier (RFC) and proton-coupled folate transporter (PCFT), which are encoded by *SLC19A1* and *SLC46A1* genes, respectively [47]. RFC is ubiquitously expressed and recognized as the major folate transporter in mammalian cells and tissues [47]. The folate flow through this transporter is coupled to an exchange of organic phosphates, including thiamin monophosphate and pyrophosphate [42]. On the other hand, PCFT functions optimally at acidic rather than neutral pH, co-transporting folate and protons (H^+) into the cell interior [48].

Given the existence of folate in the OF, the gene expression pattern of folate receptors and transporters in ampullary and isthmic derived epithelial cells was examined as an approach to find out whether the bovine oviduct epithelium could be able to internalize this micronutrient from the oviductal lumen. Folate levels, as observed in the present study, significantly vary in the ipsilateral oviduct. Based on this fact, oviducts ipsilateral to the active ovary were selected for this study. Our findings revealed that both folate receptors and transporters are expressed in the bovine oviduct. Interestingly, only *FOLR1* showed significantly different gene expression levels between anatomic regions, with high mRNA abundance in epithelial cells obtained from the isthmus compared to ampulla. A pronounced spatial difference between the transcriptome in ampulla and isthmus has been previously reported [49,50]. In agreement with our results, information derived from microarray analysis performed in the oviduct from cyclic and pregnant cows confirmed that *FOLR1* is one of the differentially expressed genes that show higher mRNA abundance in the isthmus than in the ampulla region [49]. In addition, *FOLR1* mRNA expression seems to increase in oviductal isthmus epithelial cells in response to the presence of the embryo [51]. In a recent study by Pillai et al. [52], the protein product of *FOLR1* was identified in bovine OF and in *in vitro* BOEC secretions, suggesting that FOLR1 can also be synthesized and secreted by the oviduct epithelium. This is consistent with the existence of a soluble form of FOLR1 that can be proteolytically shed from the cell surface into the extracellular milieu [53]. The release of a soluble form may provide an alternative means of controlling surface uptake and bioavailability of this micronutrient. Moreover, different expression levels of *FOLR1* mRNA between the anatomic regions could be indicative of a region-specific regulatory mechanism which may result in a specific action of this receptor in the isthmus region.

Considering that the oviduct undergoes marked changes in morphology, ultrastructure, and gene expression through hormonal influence during the estrous cycle [54,55], fluctuation of mRNA expression of folate receptors and transporters in the bovine oviduct during different stages of the estrous cycle was also analyzed. Our results showed that the relative expression of *FOLR1* mRNA was higher in epithelial cells derived from the isthmus than ampulla region throughout the estrous cycle, with a significantly higher mRNA level during the Post-Ov stage. This result could be correlated with a higher uptake of folate, which would explain the lower levels of this micronutrient detected in the OF during the early time window after ovulation. In contrast, the expression profile of *FOLR2* and *SLC46A1* was similar for both anatomic regions, showing higher relative mRNA levels during the Mid-L phase, while *SLC19A1* mRNA did not show any significant differences in its expression levels during the estrous cycle or between the anatomic regions analyzed. These results reveal the co-expression of folate receptors and transporters in the oviductal epithelium and the existence of a fine-tuned regulation of its gene expression during the estrous cycle. However, further studies are necessary to evaluate the biological significance of these observations.

Solid evidence supports the regulation of gene and protein expression in the oviduct by ovarian steroids such us E2 and P4 [56]. Considering that mRNA expression of folate receptors and transporters is regulated in response to the stage of the estrous cycle, the next objective was to evaluate if the expression levels of these genes in BOECs from ampulla and isthmus are susceptible to hormonal stimulation. For this purpose, an oviduct cell suspension culture system, previously established and characterized, was used, and cells were cultured with or without E2 and/or P4. Under this culture system, BOECs tend to preserve the native features of the oviductal epithelium, a stable gene expression pattern, as well as functional responsiveness to hormonal stimulation [28]. Our results showed the mRNA expression of folate receptors and transporters under *in vitro* experimental conditions. Moreover, anatomical differences in their expression profile were maintained (*FOLR1*) or intensified (*FOLR2*, *SLC19A1* and *SLC46A1*) under these culture conditions, which allowed to confirm that this culture system was a valid model of study. Using this *in vitro* model, hormonal stimulation assays showed a significant increase in *FOLR1* mRNA expression in isthmus explant cultures supplemented with both E2 and P4. This result matches the high transcript abundance of *FOLR1* mRNA observed in *ex vivo* BOECs during the Post-Ov stage of the estrous cycle. It is known that even though *FOLR1* promoters do not contain the classical hormonal response elements, they can be regulated by nuclear receptors and also indirectly activated through the products of upstream target genes [57]. Relative *FOLR2* mRNA abundance increased in BOEC explants from ampulla and isthmus in response to P4 stimulation. This finding correlates with the higher transcript abundance detected during the Mid-L phase. In contrast, mRNA expression of folate transporters increased in both ampulla and isthmus explants cultured under stimulation of E2, but it was significantly decreased in the presence of P4, which proves a different susceptibility to these hormones. To our knowledge, little is known concerning the effect of ovarian steroid hormones on these transporters, particularly at mRNA level. Our results show that E2 and P4 could exert a differential control on gene expression levels of folate receptors and transporters in epithelial cells of the bovine oviduct.

In conclusion, the present study has demonstrated significant folate levels in bovine OF and differential gene expression of folate receptors and transporters in the oviductal epithelium suggesting a possible uptake of this micronutrient during the estrous cycle. The region specific and cycle dependent expression differences together with the susceptibility to steroid hormones provide substantial evidence to suggest the existence of a fine-tuned regulation of expression of folate receptors and transporters and, consequently, a functional importance of this micronutrient in the bovine oviduct. These observations provide new perspectives to thoroughly explore and revalorize the role of maternal folate in the oviductal context.

Conflicts of interest

The authors declare that they have no competing interest.

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