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Impact of extracellular folic acid levels on oviductal gene expression

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ABSTRACT

Folate plays a specific role as methyl donor for nucleotide synthesis and genomic methylation patterns, which in turn are important epigenetic determinants in gene expression. Previous studies have revealed the presence of folate in bovine oviductal fluid as well as the existence of a fine-tuned regulation of the gene expression of folate receptors and transporters in bovine oviduct epithelial cells (BOECs). However, the functional implications of folate in the oviduct remain unknown. The present study aimed to assess the effect of folic acid (FA) on expression levels of selected genes that potentially respond to the folate status in *in vitro* BOECs. To obtain an insight into the optimization of a culture system for assays, gene expression of folate receptors and transporters was compared between BOECs grown in monolayers and in suspension. The results showed that BOECs from isthmus and ampulla in suspension culture better preserved the region-dependent gene expression profile than in monolayers. Subsequently, BOECs from both anatomical regions were separately cultured in suspension for 24 h assaying different FA concentrations: I) TCM-199 (control); II) TCM-199 + 1 μM FA (similar to the oviduct concentration); III) TCM-199 + 10 μM FA and IV) TCM-199 + 100 μM FA. Expression analysis of genes related to important cellular processes including folate transport, DNA methylation, cell-cell interaction, antioxidant activity and signaling pathways was performed in BOECs using RT-qPCR. Our data demonstrated that addition of 1 μM FA did not affect mRNA levels of most genes analyzed. In contrast, BOECs cultured with 10 μM FA exhibited increased mRNA expression levels of genes involved in folate intake, DNA methylation and antioxidant protection. It is worth noting that at 100 μM FA, transcriptional response in BOECs mainly resulted in decreased mRNA levels of the majority of the genes assayed. Interestingly, cytotoxicity analysis showed a similar LDH activity in the culture media

of the experimental groups, indicating that cell integrity was not affected by the FA concentrations assayed. In conclusion, our findings suggest that folate can affect BOECs, promoting changes in gene activity in a framework of functional readjustments in response to environmental conditions.

Keywords: folic acid, micronutrient, oviduct, oviductal cells, transcriptional response

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1. INTRODUCTION

Folate is a water-soluble vitamin B which functions as a coenzyme in transfer reactions of methyl groups in one-carbon metabolism. This micronutrient is essential for cellular biochemical processes, encompassing nucleotide synthesis and methylation of DNA and histones [1]. Consequently, folate-mediated pathways impact on cell division, cell growth and proliferation, as well as epigenetic modification mechanisms, which can affect gene expression [2-3].

Given the biochemical functions of folate, this micronutrient plays an important role in pregnancy outcomes in humans and other mammalian species. There is a large body of evidence that maternal folate availability is critical to normal embryonic and fetal development in the uterine environment as a result of the increased demand for one-carbon transfer reactions that are required for DNA synthesis and cell replication [4-5]. Previous studies suggest transplacental folate transport through folate receptors and transporters [6], demonstrating that folate is also linked to normal development and regulation of placental functioning [7]. Inadequate folate status during the early gestation period clearly constitutes a risk factor of neural tube closure defects, implantation failures, early pregnancy loss or intrauterine growth retardation [5, 8-9]. Although all evidence aforementioned supports the importance of folate during the intrauterine gestational period, there is little information about its role in the oviductal environment. The oviduct provides the first maternal milieu with which the developing embryo interacts and it has recently been demonstrated that oviductal secretions can modulate the epigenetic landscape of the embryo, particularly inducing changes in the DNA methylation pattern [10-11]. Given the fact that nutrients like folate can act as potential epigenetic modulators, we decided to examine the implications of this micronutrient in the context of the bovine oviduct.

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In previous studies, significant levels of folate were detected in the bovine oviductal fluid (bOF), reaching a concentration ≈50 times higher than that found in bovine serum [12]. Interestingly, folate levels fluctuate in the ipsilateral oviduct during the estrous cycle, showing lower levels during the postovulatory stage (≈1.1 μM) compared to the mid-luteal and preovulatory stages (≈1.8 μM) [12]. This information raised the possibility that bovine oviduct epithelial cells (BOECs) could internalize this micronutrient, modulating its levels in the bOF. In fact, a fine-tuned regulation of mRNA expression of folate receptors (*FOLR1*, *FOLR2*) and transporters (*SLC19A1*, *SLC46A1*) was observed in BOECs from ampulla and isthmus regions. Expression levels depend on the stage of the estrous cycle and are particularly susceptible to steroid hormone stimulation [12]. Most notably, *FOLR1* mRNA showed a differential expression pattern between the two anatomical regions, reaching significantly higher levels in BOECs from isthmus during the early time window after ovulation [12]. In consonance with this, *FOLR1* was identified within the 27 up-regulated genes in BOECs from ipsilateral oviducts during the postovulatory period [13]. In addition, information derived from transcriptome analysis indicates that *FOLR1* is up-regulated in the isthmus region in response to the presence of the embryo [14]. Collectively, these findings suggest a possible functional importance of folate in oviductal cells, particularly during the period of transit of the embryo through the oviduct. However, further studies are needed to know the specific role of folate in the oviductal context.

The epithelial lining of the oviduct plays a key role in fertility, contributing to the preparation of an efficient microenvironment for early reproductive and pregnancy success [15-16]. Oviduct epithelial cells undergo changes in gene expression depending on the ovarian cycle [17], the anatomical region [18], the presence of gametes and embryo [19] or in response to signaling molecules [20-21]. As an adaptive response to

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their physiological needs, oviductal cells must experience rapid and dynamic transcriptional changes. Indeed, gene expression can be affected by several factors and nutrients connected to epigenetic regulation may have particular relevance [22].

Considering the presence of folate in bOF, the differential expression of folate receptors and transporters in BOECs and implications on gene expression caused by extracellular folate levels, it was assumed that folate could have a functional impact on BOECs by inducing transcriptional changes. Therefore, the present study first compared expression levels of genes associated with folate uptake between two *in vitro* BOEC cultures, a cell suspension or monolayer culture system, in order to select the most suitable culture model. Subsequently, *in vitro* culture assays were performed to determine the effect of different folic acid (FA) concentrations on expression levels of a set of genes that potentially respond to the folate status in BOECs. In addition, the effect of FA treatments on cell integrity was assessed by measuring LDH activity in the medium in which BOECs were cultured.

2. MATERIALS AND METHODS

2.1 Chemicals

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

2.2 Isolation of bovine oviduct epithelial cells

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

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visual examination of the ovarian morphology [23] and oviducts ipsilateral to the ovary containing a corpus hemorrhagicum (day 1-4 of the estrous cycle) 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 washed three times with sterile PBS (pH 7.4) and isthmus and ampulla regions were separated. The oviductal mucosa from each anatomical region was obtained by gently squeezing with a sterile glass slide. The oviductal cells of both anatomical regions were then washed twice with PBS by centrifugation at 300 x g for 10 min and immediately used for *in vitro* culture experiments.

2.3 Cell culture conditions

Isthmus and ampulla epithelial cells obtained from ipsilateral oviducts at the postovulatory stage (n=12, four ipsilateral oviducts for each experimental replicate) were cultured in TCM-199 medium (Gibco 11150-059; Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Internegocios, Buenos Aires, Argentina), 25 mM Hepes (Gibco, Life Technology, Burlington, ON, Canada), 0.2 mM sodium pyruvate, and 1% antibiotic-antimycotic solution (Gibco 15240). During an initial approach, two different culture systems were assayed: i) monolayer and ii) cell suspension cultures. For monolayer cultures, as previously described by Garcia *et al.* [24], cells were passed 10 times through a syringe with a 25 gauge needle to obtain a single cell suspension. Then, two washing steps were performed and the cell concentration was determined using a hemocytometer camera after cell staining with Trypan blue to assess cell viability. A final concentration of 2×10^6 viable cells/mL was cultured in four-well plates at 38.5°C, 5% CO_2 and saturated humidity until confluence.

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Half of each medium was replaced every 48 h. For suspension cultures, oviductal cells were pipetted 15 times with a 1,000 µL filter tip before being passed twice through a syringe with a 21 gauge needle. After two washing steps, cells 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. During this initial culture period, BOECs are able to grow in suspension forming multicellular spheroids with active cilia on the outer surface that maintain the oviductal explants in constant rotational motion as previously described by Rottmayer *et al.* [25]. After 24 h of pre-culture, BOEC explants derived from ampulla and isthmus regions were collected and used for gene expression analysis or FA treatment assays.

2.4 Folic acid treatment

To evaluate the effect of FA supplementation, 10 mg of BOEC explants from ampulla and isthmus were separately distributed in 4-well culture plates, containing 500 µL of TCM-199 medium (Gibco) per well. Cells were cultured for 24 h under different treatments: I) Culture medium without addition of FA (Control Group), which contained 20 nM FA (concentration of FA provided by the basal culture medium) ; II) Culture medium supplemented with 1 µM of FA (Group 1 µM), representing the concentration of folate detected in bOF during the postovulatory period [12]; III) Culture medium supplemented with 10 µM of FA (Group 10 µM), an elevated concentration which is 10 times higher than normally present in the intraoviductal lumen and IV) Culture medium supplemented with 100 µM of FA (Group 100 µM), a concentration 100 times higher than the oviductal concentration. It is important to remark, that currently the levels between which the folate concentration in bOF fluctuates under different physiological/pathological conditions (or even through

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exogenous administration to the diet) are unknown. Therefore, 10 μM and 100 μM concentrations were chosen in order to determine how oviductal cells respond to an excess of FA. Addition of 10% FBS provided a minimal and non-significant background contribution of 1.5 nM of folate in the culture medium. Prior to the experiments, a fresh stock solution of light-protected FA (2 mM) was prepared in TCM-199 medium, which was successively diluted to obtain the different working solutions. In all cases, cultures were carried out at 38.5 °C under an atmosphere with 5% CO_2 and 100% humidity; in total, three experimental replicates were performed under the same assay conditions. After the incubation period, cell membrane integrity and cell viability were confirmed by staining aliquots of BOEC explants of each treatment with the nucleic acid stain Hoechst 33342 (25 μg/mL) and the conventional dead-cell stain, propidium iodide (100 μg/mL), respectively. Afterwards, cells were processed for gene expression analysis and their culture medium was used to measure LDH activity.

2.5 LDH activity assay

Lactate dehydrogenase (LDH) activity in the cell medium was used to determine cell damage/death and the cytotoxic potential of the different FA concentrations to which BOECs were exposed. As described previously, the culture medium of each experimental group was collected for analysis. In addition, two controls were included in the experimental setup: i) a background control (culture medium without cells) and ii) a lysate control to determine the maximum release of LDH activity in cells of which 10 mg of BOEC explants from ampulla and isthmus were incubated with 0.1% Triton X-100 for 45 min prior to harvesting the culture medium. The culture medium of each experimental well was centrifuged twice at 1,000 x g for 5 min to remove cells and then 400 μL of the supernatant was transferred to 1.5 mL tubes. LDH activity was measured

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UV-spectrophotometrically using a Hitachi Cobas® 6000 (c501 module) automated analyzer (Roche Diagnostics, Inc.). The activity level detected in the background control was subtracted from the samples and the lysis control. Results were obtained from three independent cell culture assays and are expressed as IU/L.

2.6 RNA isolation, cDNA synthesis and RT-qPCR analysis

Total RNA of *in vitro* cultured BOECs (monolayers, explants and explants treated with FA) was isolated using TriReagent (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 a protocol described by Garcia *et al.* [12].

Messenger RNA expression levels of folate receptors (*FOLR1*, *FOLR2* and *FOLR3*), folate transporters (*SLC19A1* and *SLC46A1)*, DNA methyltransferases (*DNMT1*, *DNMT3A* and *DNMT3B*) and genes associated with cellular functions (*CDH1*, *SOD2*, *TGFB1* and *MTOR*) were determined by qPCR using specific primers (see Table 1). The last group of genes was chosen because 1) their transcriptional activity is at least partly controlled by DNA methylation, one of the main epigenetic mechanisms influenced by folate metabolism, and these genes presumably respond to FA treatment [26-31] and 2) previous reports have evidenced their expression in BOECs [14, 18, 32-34]. In addition, these genes are related to critical cell functions such as cell adhesion (*CDH1*) [35], antioxidant protection (*SOD2*) [34], multifunctional implications regulating cell proliferation, differentiation and embryo development (*TGFB1*) [32, 36], and nutrient-sensing signaling pathways (*MTOR*) [37]. All qPCR reactions were performed in a final volume of 20 µL, containing 5 µL of cDNA

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template (diluted 1:5), 0.25 mM of forward and reverse primers and 10 μL of Fast EvaGreen qPCR Master Mix (Biotium, Hayward, CA, USA) and run in a CFX96^{TM} 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. Three biological repetitions were analyzed and the mean Ct value for each repetition was obtained from a technical duplicate. No-template and no-reverse transcription controls were also 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 entire study was carried out following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [38].

2.7 Statistical analysis

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

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3. RESULTS

3.1 Gene expression analysis in BOEC suspensions and monolayer cultures

In order to determine which cell culture system best reproduces the gene expression profile of folate receptors and transporters previously observed *ex vivo* [12], BOECs obtained from ampulla and isthmus regions were cultured *in vitro* under two different conditions: multicellular aggregates of three-dimensional nature in suspension (BOEC explants) and monolayers.

Once both culture systems were obtained, expression levels of genes encoding folate receptors and transporters were assessed using RT-qPCR. Studies were performed with 24-h BOEC cultures of explants and with 7-day-old BOEC monolayers from both anatomical regions. As shown in Figure 1, gene expression of *FOLR1*, *FOLR2*, *SLC19A1* and *SLC46A1* was detected in BOECs derived from ampulla and isthmus regions in both culture systems.

In the case of BOEC explants, *FOLR1* mRNA showed a differential expression profile with a higher transcriptional level in BOEC explants derived from isthmus (Fig. 1A, $P<0.001$). *FOLR2* showed significantly higher expression levels in BOEC explants derived from the ampulla region (Fig. 1B, $P<0.05$), while *SLC19A1* and *SLC46A1* genes exhibited higher expression levels in BOEC explants derived from the isthmus region (Fig. 1C and 1D, $P<0.05$).

In the case of BOEC monolayers, *FOLR1* mRNA expression showed significant differences between isthmus and ampulla (Fig. 1A, $P<0.05$). In contrast, no differences were found between oviductal cells of both anatomical regions for the other genes analyzed (Fig. 1B, 1C and 1D).

These results demonstrate that BOEC explant cultures in suspension maintained or even increased the differential gene expression patterns of folate receptors and

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transporters observed previously in *ex vivo* samples in both anatomical regions. Based on these results, the suspension culture system was chosen as a study model to carry out further experiments.

3.2 Effect of extracellular folic acid levels on gene expression of folate receptors and transporters in BOECs

In order to examine the potential impact of FA on mRNA levels of folate receptors and transporters, relative mRNA expression of *FOLR1*, *FOLR2*, *FOLR3*, *SLC19A1* and *SLC46A1* was determined by RT-qPCR in BOEC explants of ampulla and isthmus regions cultured in the presence of different concentrations of FA.

Culture medium supplemented with an FA concentration similar to that in the oviduct (1 μM) showed a significant increase in *FOLR3* transcript abundance in isthmus explants, when compared with the control group (Fig. 2C). In addition, a significant decrease in the expression levels of *SLC46A1* was observed in BOEC explants from isthmus (Fig. 2D, $P<0.05$). However, most of the genes analyzed did not show significant changes in relative mRNA levels in BOECs derived from both anatomical regions in response to an FA concentration similar to that found in the intraoviductal environment ($P>0.05$, Fig. 2A, 2B and 2D).

In contrast, presence of an elevated FA concentration (10 μM) during *in vitro* culture produced a significant increase in expression levels of *FOLR1* mRNA in ampulla explants, *FOLR2* mRNA in explants of both anatomical regions, and *FOLR3* and *SLC19A1* mRNA in explants derived from the isthmus region ($P <0.05$; Fig. 2).

Treatment with 100 μM FA induced a different transcriptional response demonstrating increased expression levels of *FOLR1* in explants of both anatomical regions and of *FOLR3* in isthmus explants, while *FOLR2* expression levels in ampulla

306 explants and *SLC19A1* and *SLC46A1* in isthmus explants significantly decreased (P
307 <0.05; Fig. 2).
308
309 ***3.3 Effect of extracellular folic acid concentrations on expression levels of DNA***
310 ***methylation related genes in BOECs***
311 Addition of FA to the culture medium at the intraoviductal concentration (1 μM)
312 did not induce significant changes in the expression levels of *DNMT1*, *DNMT3A* and
313 *DNMT3B* mRNA in ampulla and isthmus explants when compared with the control
314 group (Fig. 3). Addition of FA at a concentration of 10 μM produced a significant
315 increase in relative expression levels of *DNMT1* and *DNMT3A* (P<0.05; Fig. 3).
316 However, *DNMT3B* demonstrated a different behavior; transcriptional levels were
317 reduced in BOEC explants from isthmus in response to an elevated FA concentration
318 (P<0.05; Fig. 3).
319 Interestingly, BOEC cultures in the presence of 100 μM FA induced a
320 significant down-regulation in the expression of the three genes analyzed compared to
321 the control group: *DNMT1* mRNA levels decreased in BOEC explants from ampulla,
322 while *DNMT3A* and *DNMT3B* mRNA significantly diminished in BOEC explants from
323 both anatomical regions (P<0.05; Fig. 3).
324
325 ***3.4 Expression of genes involved in cell adhesion, antioxidant protection and cell***
326 ***signaling pathways in BOECs treated with folic acid***
327 Treatment with FA at an intraoviductal concentration (1 μM) produced a
328 significant decrease in the expression level of *CDH1* mRNA in BOEC explants from
329 isthmus and *SOD2* mRNA in BOEC explants from ampulla (P<0.05; Fig. 4A and 4B).

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When the BOEC culture medium was supplemented with 10 μM or 100 μM FA, down-regulation of *CDH1* mRNA levels in BOEC explants from isthmus was observed and up-regulation of *SOD2* in BOEC explants for both concentrations ($P<0.05$; Fig. 4A and 4B).

Messenger RNA levels of *TGFB1* and *MTOR* in groups treated with FA were not different from those in the control group ($P>0.05$; Fig. 4C and 4D).

3.5 Lactate dehydrogenase (LDH) activity in BOEC culture medium supplemented with folic acid.

To evaluate the cellular integrity under the three different FA concentrations assayed, LDH activity released into BOEC culture medium was measured.

Our results showed a similar LDH activity in the culture media of cells treated with FA and control cells from both ampulla and isthmus (Fig. 5), indicating that cellular integrity was not affected after exposure to high concentrations of FA during 24 h.

4. DISCUSSION

The present study demonstrates that under *in vitro* culture conditions extracellular levels of FA can induce gene expression changes in BOECs from ampulla and isthmus in a region-dependent manner.

Different primary cell culture models have been established to perform *in vitro* studies with oviductal epithelial cells, and monolayer and suspension systems are the ones most commonly employed [39-40]. Even though monolayer cultures are widely used, one of the main disadvantages is the dedifferentiation process and the concomitant loss of morphological characteristics [39, 41]. These cell alterations can be accompanied

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with modifications in the mRNA expression patterns during the transition from *in vivo* to *in vitro* conditions and even through consecutive culture passages [42]. In order to avoid these issues, cell culture strategies have been devised to maintain morphological and functional characteristics. Suspension systems allow cells to remain free and do not require an extreme adaptation process, preserving morphological, ultrastructural and molecular characteristics such as the presence of cilia on the luminal surface, active ciliary beating and a stable mRNA expression pattern during short-term cultures [25]. Hence, mRNA expression levels for folate receptors and transporters were assessed in two culture systems (monolayer and suspension) prior to performing *in vitro* assays with FA. Our experiments showed that mRNA expression of *FOLR1*, *FOLR2*, *SLC19A1* and *SLC46A1* was conserved in BOECs of the two anatomical regions using both techniques. However, as expected, oviductal cells cultured in suspension exhibited a gene expression profile that was more similar to *ex vivo* BOECs than cells cultured in monolayers, maintaining the differential expression of *FOLR1* mRNA in BOECs from isthmus [12]. Considering this, cell suspension was selected to carry out the subsequent experiments with BOECs.

Bearing in mind that folate can directly affect gene transcription in cells via DNA methylation changes or other molecular mechanisms [3, 30]; the objective of the present study was to determine how oviductal cells respond *in vitro* to exogenous addition of FA. The focus was placed on the expression of genes associated with different cellular processes including folate uptake, DNA methylation, cell-cell adhesion, antioxidant activity and intracellular signaling pathways. Experiments were conducted using BOEC explants that were exposed for 24 h to increasing concentrations of FA. In general, no marked effects were observed at transcriptional level in BOECs supplemented with 1 μM FA, which mimics the folate concentration found in the bOF

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during the postovulatory period [12]. Because folate is a micronutrient necessary for normal cell growth and basal cell functions, maintaining cells in conditions similar to their physiological context, fulfilling their minimal requirements (including micronutrient demand) for normal functioning, substantial changes that reflect a noticeable effect will probably not occur or may not be detectable. For this reason, the effects of this kind of factors usually become noticeable in case of deficiency or excess [43].

Studies performed with different cell types have demonstrated that expression of folate receptors/transporters is modified by intra- and extracellular folate levels, suggesting that one of the mechanisms through which their transcriptional levels can be controlled is linked to cellular demand [44-45]. However, it is unknown whether a similar regulation mechanism occurs in BOECs. In the present study, expression levels of *FOLR1* mRNA increased in BOEC explants from ampulla in the presence of 10 and 100 μM FA, while in BOEC explants from isthmus this effect was only observed with 100 μM FA. One plausible explanation for these findings is that given the significantly lower basal *FOLR1* expression in explants from ampulla than from isthmus, *FOLR1* is likely to be more susceptible to transcriptional activation in response to a lower FA concentration in ampulla explants. Indeed, mRNA levels of *FOLR1* increased in isthmus explants when BOECs were cultured in the presence of 100 μM FA, suggesting that higher levels of this micronutrient are necessary to promote a transcriptional response in BOECs derived from this oviductal region.

In humans, the folate receptor gene family includes a third functional member named *FOLR3* [46]. Even though the bovine orthologue of human *FOLR3* has been identified, there is still limited information on the biological implications of this gene. Our results showed increased mRNA levels of *FOLR3* in BOECs from isthmus when

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oviductal explants were cultured with intraoviductal or higher FA concentrations. These results seem logical taking into account that *FOLR3* encodes for an isoform of the folate receptor which is secreted and could allow BOECs to take up FA from their surroundings [47].

Additionally, mRNA expression levels of *FOLR2* and *SLC19A1* increased in BOEC explants of both anatomical regions cultured in the presence of a high FA concentration (10 μM). These results suggest that both genes as well as *FOLR1* and *FOLR3*, can be activated at the transcriptional level in response to an increasing concentration of extracellular FA. However, it is worth noting that addition of 100 μM FA induced a different transcriptional response; mRNA expression levels of *FOLR1* and *FOLR3* increased, whereas the levels of *FOLR2*, *SLC19A1* and *SLC46A1* decreased. This may be a result of a reduction in the demand of the cell for folate or a desensitization mechanism at the transcriptional level of proteins associated with folate uptake. Desensitization is an important mechanism of homeostatic capacity in cellular activation processes and it has marked physiological and even pathological effects [48]. It implies the loss of cellular responses when ligands are in excess [48] and it could determine a protective mechanism against excessive exposure to FA. However, the mechanism does probably not affect transcriptional regulation of *FOLR1* and *FOLR3* since the receptor encoding for *FOLR1* (FR-α) can also acquire a soluble form after separating from the glycosylphosphatidylinositol (GPI) membrane anchor, whereas *FOLR3* codes for a secreted form of the receptor protein (FR-γ) [49].

Folate metabolism is essential for biosynthesis of S-adenosylmethionine (SAM), the universal methyl group donor which is used by DNMT1, DNMT3A and DNMT3B enzymes in DNA methylation reactions [50]. The enzymes DNMT3A and DNMT3B are responsible for *de novo* DNA methylation, while DNMT1 is involved in

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preservation of methylation patterns in hemi-methylated CpG dinucleotides [50]. Our results show that transcriptional levels of DNMTs in BOEC explants are susceptible to fluctuations in extracellular FA concentrations. Relative expression levels of *DNMT1* and *DNMT3A* mRNA increased in BOEC explants from ampulla and isthmus when the culture medium was supplemented with 10 μM FA. Our hypothesis is that a greater methyl donor availability in BOECs, as a consequence of elevated levels of exogenous FA, can influence methylation reactions in oviductal cells. Therefore, increased mRNA of *DNMT1* and *DNMT3A* could be a response to cellular demand. In line with our observations, other authors have demonstrated that fortification of grain products with FA has an impact on expression of DNA methylation related genes, increasing mRNA levels of *DNMT1* in human cervical cells [51]. In addition, it has been shown that folate depletion in the culture medium led to a significant down-regulation of *DNMT3A* and *DNMT3B* in a cervical cancer cell line, suggesting that the methyl donor status of these cells affect gene expression levels of DNA methyltransferases [52].

Interestingly, mRNA expression analysis showed that exposure of oviductal explants to 100 μM FA produced a significant decrease in expression levels of the three DNMT genes. However, the mechanism behind this gene repression remains unknown. S-adenosylmethionine is converted into S-adenosylhomocysteine (SAH) by DNMTs during DNA methylation and it has been reported that intracellular SAH accumulation can act as an inhibitor of DNMTs [53]. *In vitro* culture of normal human cells with high levels of folate led to a decreased SAM:SAH ratio and global DNA hypomethylation [54], suggesting that an excess of FA may actually decrease the establishment of genomic methylation marks. These observations support our findings and a decreased expression of genes encoding key enzymes for DNA methylation probably constitutes a protection mechanism against hypermethylation in cells.

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The *CDH1* gene encodes Cadherin 1 (E-cadherin) which is essential for cell-cell and cell-extracellular matrix adhesion in epithelial tissues. In the present study, mRNA expression levels of *CDH1* were assessed in BOEC explants cultured in the presence of different FA concentrations. Our results revealed that physiological and elevated concentrations of FA induced a significant decrease in expression levels of *CDH1* mRNA in isthmus explants. Previous reports showed that mRNA levels of *CDH1* diminished with decreasing folate concentrations in an epithelial cell line [30]. In addition, folate down-regulated expression of E-cadherin in cancer cells [55]. All these data indicate that folate can have an impact on the expression of *CDH1* mRNA. The *CDH1* gene promoter is regulated by methylation marks [29], and considering that DNA methylation patterns can be influenced by extracellular FA levels, high FA concentrations could lead to low transcriptional levels of *CDH1* in BOECs. Cadherin 1 has been proposed as a biomarker for embryo implantation and its odd expression affects this crucial reproductive process [56]. Given that our study revealed changes in gene expression of *CDH1* in isthmus explants, future studies should focus on whether the decreased expression levels of this gene in response to the folate concentration could be linked to a mechanism that avoids ectopic implantation in the oviduct.

It has been demonstrated that folate exhibits important antioxidant effects at cellular level [57]. Antioxidant enzymes play a key role in the removal of reactive oxygen species (ROS) in cells and thus maintain the intracellular homeostasis. Superoxide dismutase (SOD) catalyzes the modification of the superoxide radical into molecular oxygen and hydrogen peroxide. Our results showed that *SOD2* gene expression levels decreased in ampulla explants exposed to intraoviductal FA concentrations. However, their expression levels increased when ampulla explants were cultured with elevated FA concentrations, suggesting a differential transcriptional

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response of *SOD2* mRNA to FA supplementation. Folate was found to diminish intracellular production of superoxide both *in vitro* and *in vivo* [58-59], and therefore influence of FA on mRNA levels of *SOD2* may be correlated with a protective effect against oxidative stress in ampulla epithelial cells.

In the case of the genes involved in signaling pathways that are linked to cell proliferation, growth and nutritional status (*TGFB1* and *MTOR*) [37, 60], no changes in mRNA levels were found in BOECs incubated with different FA concentrations. However, further research is needed to determine if there is any effect at another level in these signaling pathways.

Given the decreased mRNA levels for most genes analyzed in BOEC explants cultured with an elevated FA concentration (100 μM), it was decided to assess whether high FA levels can affect cell integrity. The assay was based on measuring the activity of cytosolic LDH in the culture medium which constitutes a useful tool to estimate cell damage *in vitro* [61]. Our results showed similar LDH activities in the extracellular environment in the experimental groups of BOEC explants from both anatomical regions, indicating that cell integrity of BOECs was not affected by a high FA concentration. Consequently, the results suggest that a high FA dose influences the transcriptional status of BOECs without having a cytotoxic effect on cells. Considering that our study was focused on mRNA expression analysis, further studies on the effects of folate on cell viability, proliferation and genomic stability of oviductal cells are required. In addition, it is important to remark that the transcriptional effects observed with an excess of FA provide valuable information since it shows that bovine oviductal cells can be used as a model to extrapolate studies to different physiological/pathological states and to conditions particularly observed in humans.

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Currently women are widely supplemented with FA, and the effect of an excess of FA is not as thoroughly explored as a deficiency during early pregnancy.

In summary, our results emphasize that folate inside the oviduct could be an important contributing factor to establish molecular signatures in BOECs. These oviductal cells can respond to extracellular FA levels through gene expression changes in a region-dependent manner. Messenger RNA expression levels of key genes that are related to cellular uptake of folate, DNA methylation and antioxidant activity increased in response to an elevated FA concentration (10 μM). On the contrary, supplementation with a 10-fold higher amount of FA (100 μM) caused a decrease in mRNA levels for most genes analyzed, suggesting a differential transcriptional response of BOECs to the extracellular FA levels. These findings shed light on the functional importance of folate for bovine oviductal cells and constitute the basis for further studies to obtain a better insight into the implications of this micronutrient on the early embryo environment.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHOR CONTRIBUTIONS

E.V.G. performed experiments, interpreted data and helped draft the manuscript. M.J.M. performed experiments. J.G.L. contributed to the critical revision of the manuscript. A.D.B. designed and directed the project, performed all experiments, participated in the interpretation of the results and wrote the manuscript. All authors have read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. Relative mRNA expression levels of folate receptors and transporters in bovine oviduct epithelial cells (BOECs) cultured *in vitro*. (A) Bars represent relative mRNA levels of (A) *FOLR1*, (B) *FOLR2*, (C) *SLC19A1* and (D) *SLC46A1* in BOECs obtained from ampulla and isthmus regions and cultured for 24 h in suspension or for 7 days in monolayer. Relative gene expression levels were normalized to the geometric mean of endogenous *GAPDH* and *ACTB* genes. Results are expressed as mean ± SEM. Significant differences ($P<0.05$) between anatomic regions are indicated with asterisks. Data are obtained from three experimental replicates.

Figure 2. Relative mRNA expression levels of folate receptors and transporters in folic acid-treated bovine oviduct epithelial cells. Bars represent relative mRNA levels of (A) *FOLR1*, (B) *FOLR2*, (C) *FOLR3*, (D) *SLC19A1* and (E) *SLC46A1* in BOEC explants from ampulla and isthmus regions and cultured for 24 h in the presence of different folic acid concentrations: 20 nM (control), 1 μM, 10 μM or 100 μM. 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 ($P<0.05$) between treated cells compared to control cells are indicated with asterisks. Data are obtained from three experimental replicates.

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Figure 3. Relative mRNA expression levels of DNA methylation related genes in folic acid-treated bovine oviduct epithelial cells. Bars represent relative mRNA levels of *DNMT1*, *DNMT3A* and *DNMT3B* in BOEC explants from ampulla and isthmus regions and cultured for 24 h in the presence of different folic acid concentrations: 20 nM (control), 1 μM, 10 μM or 100 μM. 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 ($P<0.05$) between treated cells compared to control cells are indicated with asterisks. Data are obtained from three experimental replicates.

Figure 4. Relative mRNA expression levels of genes associated with cell adhesion (*CDH1*), antioxidant protection (*SOD2*) and cell signaling pathways (*TGFB1* and *MTOR*) in folic acid-treated bovine oviduct epithelial cells. Bars represent relative mRNA levels of (A) *CDH1*, (B) *SOD2*, (C) *TGFB1* and (D) *MTOR* in BOEC explants from ampulla and isthmus regions and cultured for 24 h in the presence of different folic acid concentrations: 20 nM (control), 1 μM, 10 μM or 100 μM. 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 ($P<0.05$) between treated cells compared to control cells are indicated with asterisks. Data are obtained from three experimental replicates.

Figure 5. LDH activity in culture media of bovine oviduct epithelial cells cultured under folic acid treatment. Bars represent LDH activity released into the culture supernatant of control and treated cells. Basal BOEC culture medium containing the usual folic acid concentration (20 nM) was used as control (white bar). Medium

742 obtained after cell lysis with 1% Triton X-100 was used as maximum LDH release
743 control (hatched bar). Data are the average of three independent cell culture experiments
744 ± SEM. Asterisks indicate significant differences (P<0.05).

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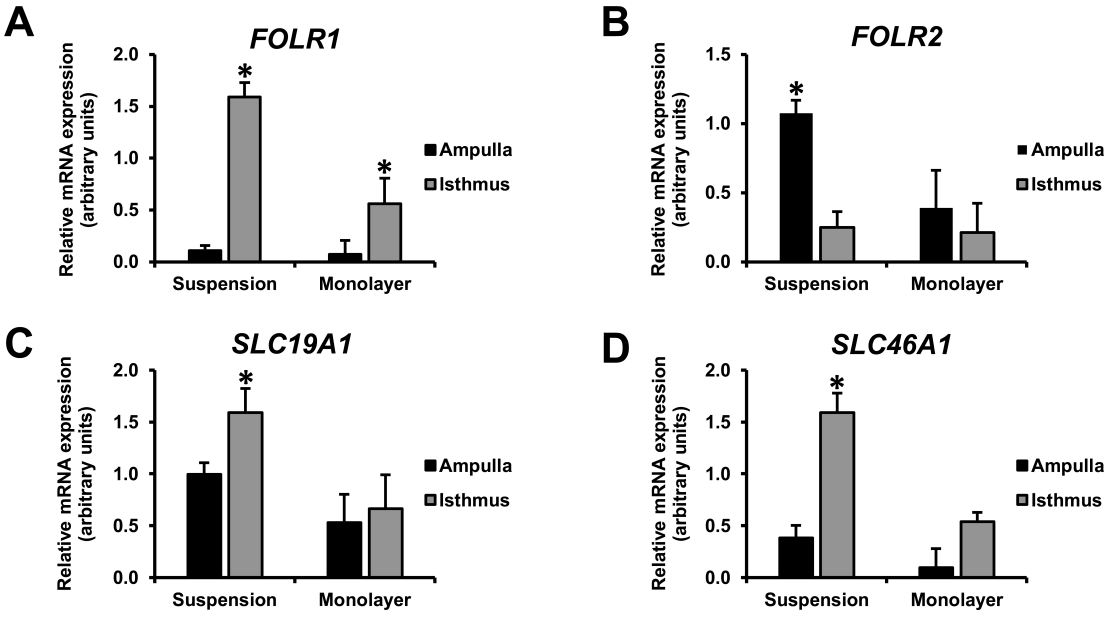
Table 1. List of primers used for RT-qPCR analysis

Genes		Primer sequences (5´- 3´)	Amplicon size (bp)	GenBank accession number
FOLR1	Forward Reverse	ACAGATTCGACTGGGACCAC TTTGCAGAGGGGCACATTCA	164	NM_001206532.1
FOLR2	Forward Reverse	CCAGGAGGTGAACCAGAAGT CAAATGTGCGGCAGATGGTC	185	NM_001075325.1
FOLR3	Forward Reverse	CAGAGGACAGTCTACATGAGCAG GTGAGCACTCGTAGAGACAGGT	186	NM_001206527.1
SLC19A1	Forward Reverse	ACTGACGACATTTGGTTGTGC AGGAACGTGTTGATGCCGAA	143	NM_001076453.2
SLC46A1	Forward Reverse	TGGCAGCTGGACTGCTATTC GTGCTCACGTTGCTCCTCTT	144	NM_001079585.1
DNMT1	Forward Reverse	GTACCAGTGCACCTTTGGCGT GTGCGAACACATGCAACGGCT	134	NM_182651.2
DNMT3A	Forward Reverse	CTCCATAAAGCAGGGCAAG TCATGTTGGAGACGTCGGTA	128	NM_001206502.1
DNMT3B	Forward Reverse	AGACCGGCCTTTCTTCTGGATGT TGTGAGCAGCAGACACTTTGATGG	129	NM_181813.2
CDH1	Forward Reverse	CGTATCGGATTTGGAGGGAC CGAGGAACAAGAGCAGGGTG	192	NM_001002763.1
SOD2	Forward Reverse	GCTTACAGATTGCTGCTTGT AGGTAATAAGCATGCTCCCA	100	NM_201527.2
TGFB1	Forward Reverse	CCTGGACACCAACTACTGCT AATTGGCGTGGTACCCCTTG	125	NM_001166068.1
MTOR	Forward Reverse	GGCTCCAGACTATGACCACC TCGATCAAACCACACCTCGG	136	XM_002694043
GAPDH	Forward Reverse	AGATGGTGAAGGTCGGAGTG GAAGGTCAATGAAGGGGTCA	117	NM_001034034.2
ACTB	Forward Reverse	GATCATTGCTCCTCCCGA ACTCCTGCTTGCTGATCC	101	NM_173979.3

bp: base pairs.



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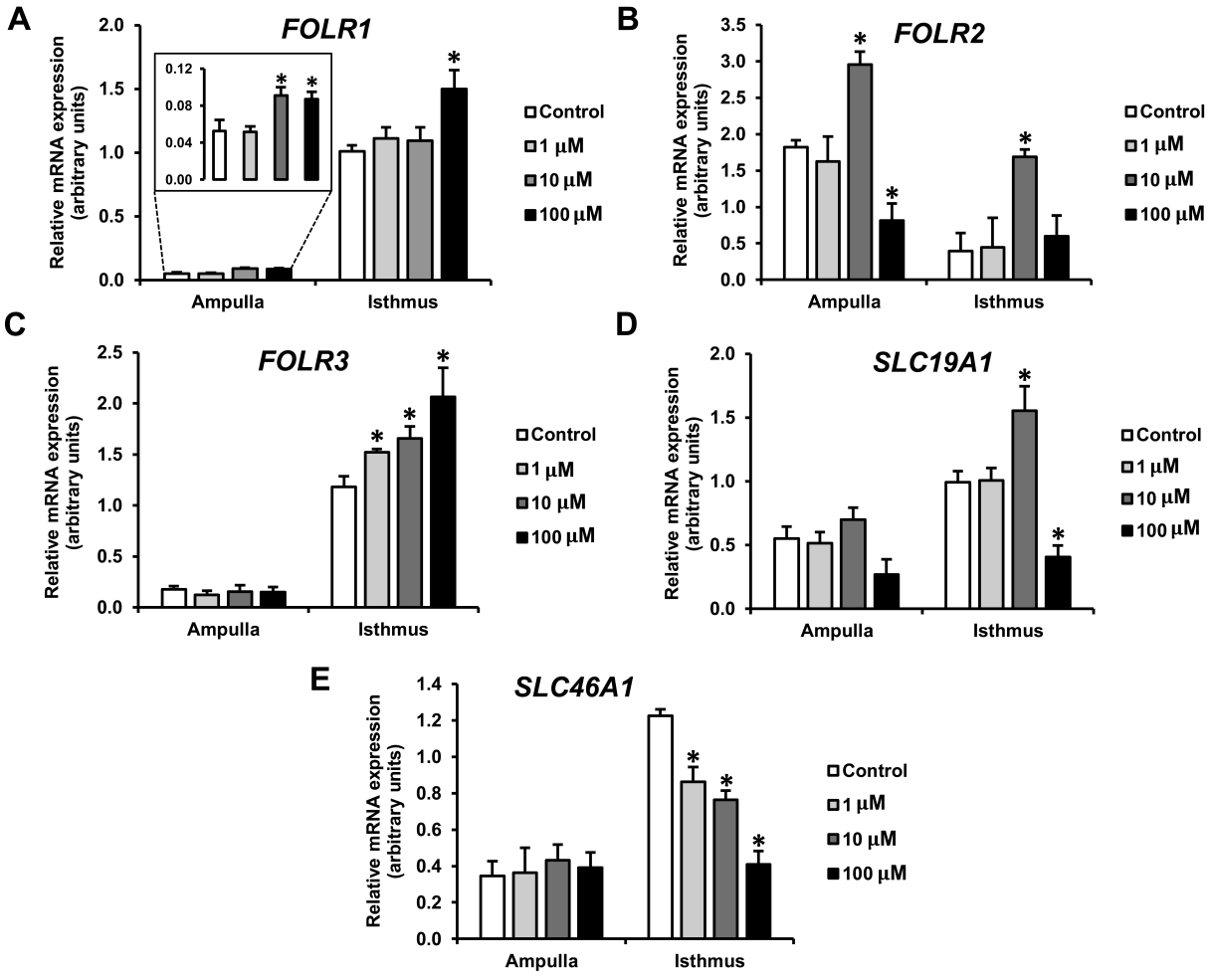
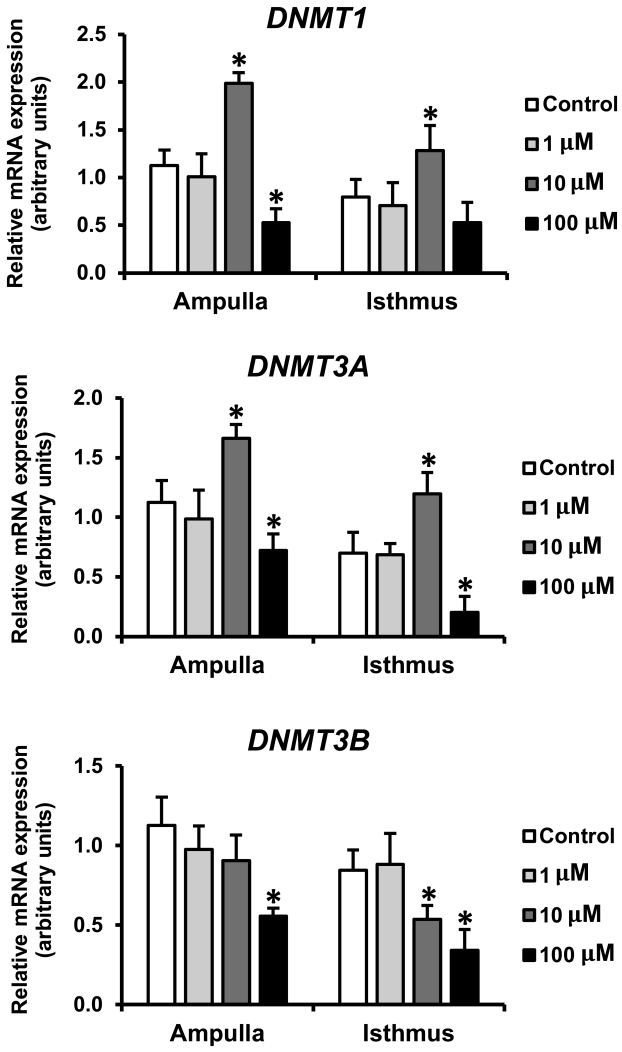
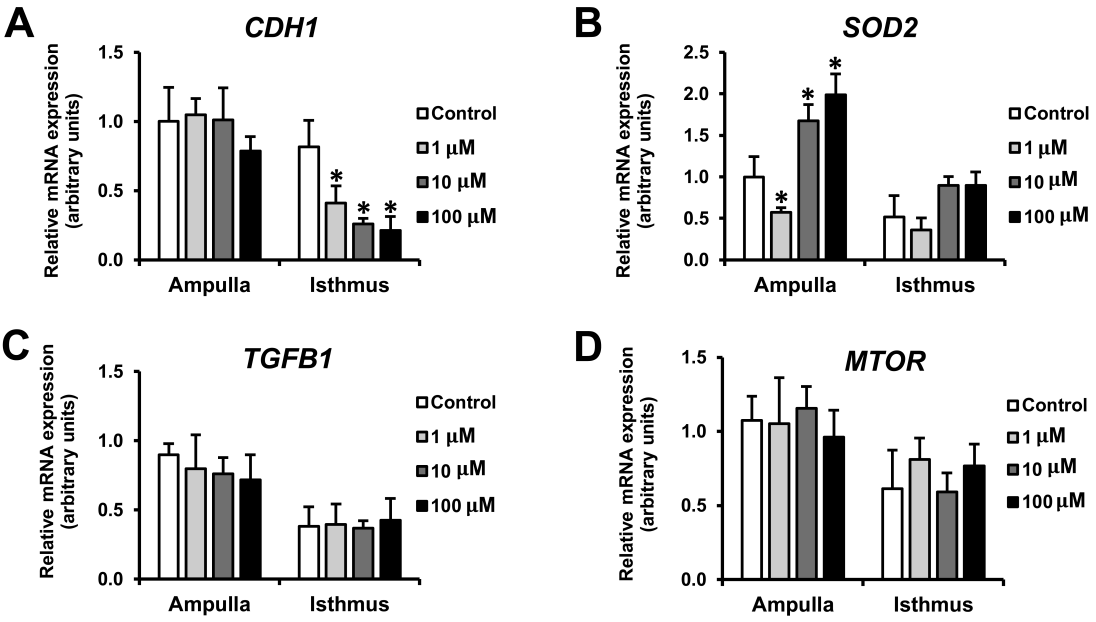

A
FOLR1
Relative mRNA expression (arbitrary units)
2.0
1.5
1.0
0.5
0.0
0.12
0.08
0.04
0.00
Ampulla
Isthmus
Control
1 μM
10 μM
100 μM
B
FOLR2
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C
FOLR3
2.5
2.0
1.5
1.0
0.5
0.0
D
SLC19A1
2.0
1.5
1.0
0.5
0.0
E
SLC46A1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0


DNMT1
Relative mRNA expression (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
Ampulla
Isthmus
Control
1 μM
10 μM
100 μM
DNMT3A
Relative mRNA expression (arbitrary units)
2.0
1.5
1.0
0.5
0.0
Ampulla
Isthmus
Control
1 μM
10 μM
100 μM
DNMT3B
Relative mRNA expression (arbitrary units)
1.5
1.0
0.5
0.0
Ampulla
Isthmus
Control
1 μM
10 μM
100 μM



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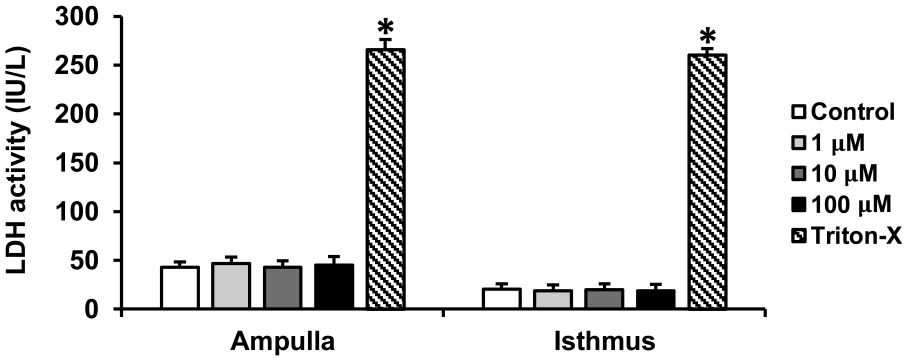

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Highlights

- The effect of folic acid (FA) on expression of selected genes was explored in BOECs cultured *in vitro*
- The addition of 1 μM FA did not affect mRNA levels of most genes analyzed
- Expression levels of genes related to folate transport, DNA methylation and antioxidant protection increased with 10 μM FA
- Elevated concentrations of FA (100 μM) decreased mRNA levels of the majority of the genes assayed
- LDH activity in the culture medium reflected that cell integrity was not affected by the FA concentrations assayed

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