IJAS Iranian Journal of Applied Animal Science

Effect of Adding Different Levels of Folic Acid to the Culture Medium on Developmental Competence of Bovine Oocytes

Research Article

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Received on: 11 May 2020
Revised on: 1 Jul 2020
Accepted on: 15 Jul 2020
Online Published on: Jun 2021

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Online version is available on: www.ijas.ir

ABSTRACT

This study aimed to investigate the effects of adding different levels of folic acid (FoA) in culture medium on bovine embryo developmental competence, reactive oxygen species (ROS) content, and expression of DNA methylation genes. In this study, different levels of FoA including 0, 10, 100, and 1000 ng/mL were added to maturation medium, and then the matured oocytes were subjected to *in vitro* fertilization (IVF) and *in vitro* culture (IVC). To determine the intracellular ROS activity in oocytes, a 20, 70-dichlorofluorescein light assay was used. RNA was extracted from ovaries, denuded mature oocytes and blastocysts using a commercial kit to determine relative expression of DNA methyltransferase 1 (Dnmt1), Dnmt3a, and Dnmt3b. The addition of FoA decreased ($P<0.05$) oocyte ROS content in all the treated groups compared to the control group; meanwhile, the lowest level was noted in FoA100 group ($P<0.05$). The fertilization rate was improved ($P<0.05$) in FoA10 and FoA100 compared to the control group. Transcript abundance of Dnmt1 in the matured oocyte was increased in FoA10 and FoA100 compared to the control ($P<0.05$), but the highest level was noted in FoA100. Folic acid enhanced the expression of Dnmt3a and decreased that of Dnmt3b in bovine oocytes ($P<0.05$). Transcript abundance of Dnmt3a and Dnmt3b in the FoA100 blastocysts indicated an increase and decrease, respectively relative to the control group ($P<0.05$). These findings showed that the addition of 100 ng/mL of FoA to the oocyte maturation culture medium has a beneficial effect on the fertilization rate and developmental competence of produced zygote.

KEY WORDS bovine, folate, homocysteine, IVF, IVM.

INTRODUCTION

Improvement of the potential for higher milk production is negatively associated with risks of infectious diseases, metabolic disorders and low reproductive performance of modern dairy cows (Yousefi *et al.* 2019). Higher metabolic stressors are closely related to poor oocyte and embryo quality (Caraviello *et al.* 2006).

In vitro embryo production with superior genetic quality has been suggested as one of the strategies that can help to improve reproductive performance, especially during heat stress period (Pellegrino *et al.* 2016). As the first stages of *in vitro* maturation (IVM), fertilization (IVF) and embryo development thoroughly depend on the culture media, an appropriate culture media can increase the IVM and IVF success rates and development of the embryo to the blasto-

cyst stage (Camargo *et al*. 2018).

Although several studies have improved the rate of embryos survival and its quality during the pre-implantation period using different culture medium (Farin *et al.* 2001; Hussein *et al.* 2011; Abazarikia *et al*. 2020), the quality of *in vitro* produced embryos still is not comparable with those produced *in vivo* (Ventura-Juncá *et al*. 2015). Several stressors, including medium composition, culture conditions, or oocyte quality, affect the developmental competence and quality of the *in vitro* produced embryos (Balboula *et al*. 2013). Also, the quality of *in vitro* produced embryos is influenced by the culture condition through epigenetic processes that play a major role in productive gametogenesis and growth (Young *et al*. 2001). The suitable establishments of DNA methylation pattern in gametes and early embryos are crucial for healthy development (Steele *et al*. 2005).

Commercially available embryo culture media display a remarkably wide range of methyl donor levels (Steele *et al.* 2005). Nevertheless, common culture media contain no vitamins and methyl donors and the essential compounds for cell growth and survival are just supplied from serum (Eagle, 1955). Folic acid (vitamin B_9) is an important component with several pivotal influences in biological system of the body. It plays a key role in DNA methylation processes via its involvement in the methionine cycle (Van den Veyver, 2002). It has been shown that the level of FoA was inversely related to the level of homocysteine (HSY) in ovarian follicular fluid. HSY can act as an oxidant, while high levels of FoA can reduce the level of cellular HSY, which in turn improves fertilization rate and the quality of oocyte and embryo (Koyama *et al.* 2012). There is also strong evidence indicating a positive role of FoA in the production of energy, the synthesis of DNA, RNA, and proteins (Kwong *et al*. 2010; Pasquale *et al.* 2016). Besides, FoA with antioxidant property effectively scavenges oxidizing free radicals (Nahak *et al.* 2014). Despite the water-soluble characteristic of FoA, its ability to prevent lipid peroxidation has made it as an important component for protecting biocompatible compounds including cell membranes or DNA against free radical damages (Joshi *et al.* 2001). However, several researchers have shown that FoA loss led to significant apoptosis in Chinese hamster ovarian cells, Hep G2 human hepatoma cells, T lymphocytes, fibroblasts, and erythrocytes (Ebisch *et al.* 2007).

As the processes of IVM and IVF are associated with high levels of oxidants, DNA methylation has an impressive effect on activation of critical proteins affecting survival and maintenance of embryo.

A few *in vitro* studies have been conducted on FoA on oocyte quality and embryo development in domestic animals. It has been reported that adding FoA to IVM medium of porcine oocytes increased the concentration of GSH and decreased apoptosis, leading to enhanced improved embryonic production (Kim *et al.* 2008). Another study discovered that supplementation of the IVM medium with FoA improved the development of bovine embryos produced from grade III oocytes (Gennari Verruma *et al.* 2016). However, it has been reported that supplementing 10 to 20 µM of FoA during IVC did not impact embryo growth, but increased the developmental stage at day 6 (Guimarães and Dode, 2016).

Our previous study showed that the concentrations of FoA in maturation medium was lower than larger follicles (diameter >8 mm) or small follicles (≤8 mm diameter) (Baghshahi *et al*. 2019). Therefore, we hypothesized that supplementing oocyte culture media with higher concentrations of FoA rather than those presented in follicles greater than 8 mm in diameter would have a superior effect on fertilization rate and embryo developmental competence. The aim of this research was to evaluate the effects of FoA supplementation to medium culture on bovine embryonic growth, oocyte ROS content, as well as the expression of DNA methylation genes.

MATERIALS AND METHODS

In this study, all chemicals were obtained from Sigma-Aldrich® (USA) except as otherwise indicated.

Oocyte collection

Bovine ovaries were obtained from slaughtered cows at a local abattoir and transported to the laboratory in 0.9% (v/v) saline supplemented with streptomycin and penicillin at 27 to 37 ˚C within 2 hrs. The ovaries were washed several times in sterile saline. Cumulus-oocyte complexes (COCs) were aspirated from follicles of 2 to 8 mm in diameter using an 18-G needle attached to a vacuum pump (MEDAP, Sekretsauger P7040, Tilburg, Netherlands). The follicular fluid containing the COCs was placed in a 50-mL conical tube and held on a warm bath to settle for five minutes and then COCs cells washed three times in oocyte collection medium with tissue culture medium (TCM-199) (m4530) with Hanks' salts supplemented with 2% v/v bovine fetal serum (F0804) and 2 U/mL heparin), 1 mM alanyl-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin.

In vitro maturation (IVM) and experimental design

The IVM procedures were performed in TCM-199 medium supplemented with 10% v/v bovine fetal serum (F0804), 2 µg/mL estradiol-17β (E8875), 20 µg/mL follicle-stimulating hormone (F2293), 22 µg/mL sodium pyruvate, 50 µg/mL gentamicin, 1 mM alanyl-glutamine. The COCs

with at least three layers of surrounding cumulus cells and uniform ooplasm were transferred to 50 µL droplets of pre-warmed maturation medium under mineral oil. The oocytes (5 to 10 oocytes/ droplet) were cultured in maturation medium supplemented with different levels of FoA included 0 (control), 10, 100 and 1000 ng/mL final concentration at 38.5 ˚C for 24 hrs in a humidified atmosphere of 5% (v/v) CO_2. These concentrations were obtained from our previous study based on the concentration of folic acid in the follicular fluid of follicles with a diameter of more than 8 mm.

In vitro fertilization and _in vitro_ culture (IVC)

In vitro fertilization was conducted according to a procedure described by Ortega *et al*. (2017). In brief, before transfer to fertilization drops, oocytes were washed three times in 50-mL droplets of fertilization medium. The components of the fertilization medium included 250 µL of HEPES-buffered Tyrode's Albumin Lactate Pyruvate [TALP]), supplemented with 48 µL of PHE; a 0.9% NaCl solution of dissolving penicillamine, hypotaurine, and epinephrine at concentrations of 0.5 mM, 0.25 mM, and 25 mM, respectively). Oocytes were then transferred into 50 µL drops of HEPES-buffered -TALP covered with mineral oil. After thawing of frozen semen from two bulls in warm water (37 ˚C) for 60 seconds, semen was washed in Sperm-TALP medium and then were separated by centrifuging at 2400 g for 10 min through discontinuous Percoll gradients (45 and 90%; Sigma-Aldrich, E0414). The motile sperm was rinsed by centrifugation at 1100 g for 5 min in HEPES buffered medium and then adjusted to a final concentration of 1×10^6 cells/mL in the culture medium. Oocytes and motile sperms were co-cultured in 270 µL droplets of IVF-TALP in four-well plates under mineral oil and incubate for 18 hours in a humidified atmosphere of 5% CO_2 at 38.5 ˚C. After co-incubation, the cumulus cells and the sperm attached to the zona pellucida were regularly separated by pipetting. Embryos were then cultured in groups of 20 in 100 µL drops of synthetic oviduct fluid [SOF- supplemented with 2% essential amino acids, 1% non-essential amino acids, and 4 mg/mL essentially fatty acid-free BSA (Sigma, A6003)] covered with mineral oil at 38.5 ˚C in a humidified atmosphere of 5% O_2 and 5% CO_2 with the balance N_2 for the next 7 days. After 72 h of incubation, the cleavage rate of the embryo was recorded. The culture medium was renewed by fresh medium every 2 days. To determine the fertilization rate, the presumptive zygotes at 18 hrs post-fertilization were stained with Hoechst 33342 to visualize the sperm head and pronuclei using a fluorescent microscope with a UV filter. The same procedure was utilized for counting the total cells in blastocysts around day 8 after insemination.

ROS measurement

To determine the intracellular ROS activity in oocytes, 20, 70 -dichlorofluorescein (DCF) light assay was used. Consequently, stock solutions of 2-7-dichlorodihydrofluorescein diacetate (H2DCFDA) were ready in dimethyl sulfoxide (DMSO) and held on at -20 ˚C within the dark. Simply before the beginning of every experiment, stock solutions were diluted in phosphate-buffered saline (PBS) and 1 mg/mL of polyvinyl alcohol (PVA).

For each group, a total of 30 to 35 denuded mature oocytes were washed thrice in PBS-0.1% PVA. The oocytes were incubated with PBS-0.1% PVA and 40 µM stock solutions for half-hour within the dark. After incubation, the oocytes were washed with PBS-0.1% PVA and transferred into 10-µL droplets. Finally, washed samples were then exposed to UV light by a fluorescent microscope using a fluorescence microscope equipped with UV filters (460 nm). The light intensities of oocytes were analyzed by Image J software (version 1.46r).

Real-time PCR analysis

RNA was extracted from ovaries, denuded mature oocytes, and blastocysts using a commercial kit (CinnaGen, Iran) following the manufacturer's instructions including the on-column DNase digestion to remove any possible residual DNA contamination. The quantity and quality of RNA were checked by evaluating the optical density measurement (A260/A280 ratio) with NanoDropTM Spectrophotometer (Thermo200c, USA) and agarose gel (1%) electrophoresis. Immediately after RNA extraction, cDNA was synthesized from 1 µg of the total RNA using the first-strand complementary DNA (cDNA) synthesis kit (CinnaGen, Iran) as instructed by the manufacturer, and then stored at –70 ˚C. Quantitative polymerase chain reaction (qPCR) was performed using the Applied Biosystems real-time PCR (StepOne, USA) and RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) to determine relative expression of DNA methyltransferase 1 (Dnmt1), 3A (Dnmt3a) and 3B (Dnmt3b). All primers used were designed using Primer 5.0 software based on the gene sequences in NCBI (Table 1). The reactions were done under the following thermal cycling conditions: 94 ˚C for 15 min, 40 cycles including 94 ˚C for 30 s, 58 ˚C for 30 s, and 72 ˚C for 20 s. Melting curve analysis was performed for each reaction to confirm the specificity of primers. Three replicates were performed for each sample. The expression levels of the target genes were determined relative to endogenous control gene glyceraldehyde 3-phosphate dehydrogenase (Gapdh). The final relative expression was analyzed with the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001).

Table 1 Oligonucleotide primers used in polymerase chain reaction (PCR)

Name	Sequences (5'-3')	Length (bp)	Temperature (°C)
Dnmt1	Forward: CAACCCTGACTCCACCTACG	20	61.40
	Reverse: ACTTGCTCCACCACGAACTG	20	59.35
Dnmt3a	Forward: GCGTGGAATGTGTGGATCTC	20	59.35
	Reverse: GCACATGTAGCAGTTCCAGG	20	59.35
Dnmt3b	Forward: TTACAACAACGGCAAGGACC	20	57.30
	Reverse: TCAAAGAGAGGGTGGAAGGAC	21	59.82
Gapdh	Forward: AGATGGTGAAGGTCGGAGTG	20	59.35
	Reverse: TGACTGTGCCGTTGAACTTG	20	57.30

Dnmt1: DNA methyltransferase 1; Dnmt3a: DNA methyltransferase 3A; Dnmt3b: DNA methyltransferase 3B and Gapdh: glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

In a completely randomized design, data were subjected to analyses of variance (ANOVA) using SAS 9.4 software (SAS, 2004). Gene expression data and levels of ROS were analyzed by GLM procedure; however, binary distributed data such as fertilization, cleavage, 2 to 4 cell, 8 to 32-cell, morula, and blastocyst rates were analyzed using logistic regression and logit odds ratios link function by GENMOD procedure. Means of experimental groups were compared using the Tukey's multiple comparison tests. Data are recorded as least squared means (LSM) and standard error of the mean (SEM). Significant tendencies were registered at $P < 0.05$ and $0.05 < P < 0.10$, respectively.

RESULTS AND DISCUSSION

Measurement of ROS content

The effects of adding different levels of FoA to maturation medium on the concentration of ROS in oocyte are summarized in Figures 1 and 2. The addition of FoA decreased (P<0.05) ROS level in the treated groups compared to the control group (84.96, 76.65, 56.95 and 60.95 for FoA0, FoA10, FoA100 and FoA1000 groups, respectively); however, the lowest level was noted in FoA100 than the other groups (P<0.05).

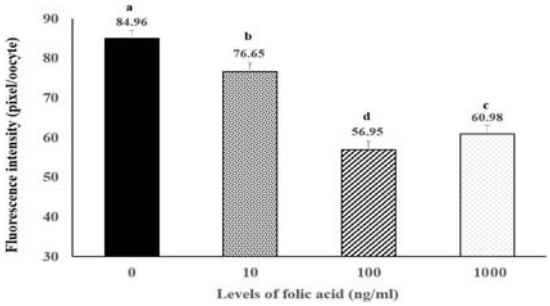

Figure 1 Effect of adding different levels of folic acid to maturation medium on reactive oxygen species (ROS) levels in bovine matured oocytes

Mean fluorescent intensities with different superscript (a-d) are significantly different (P<0.05)

The effects of the supplementing the maturation medium with different levels of FoA on fertilization rate and embryonic development are presented in Table 2. Fertilization rate was improved (P<0.05) in FoA10 and FoA 100 groups compared to the control group (89.68 and 90% vs. 72.97%, respectively), resulting in the odds ratios (ORs) of 3.22 and 3.37 for fertilization success as compared to the control group, respectively (Table 3).

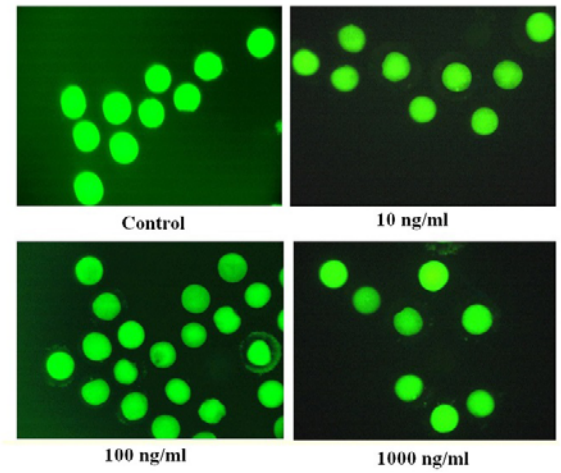

Figure 2 Representative images of *in vitro*-matured oocytes stained with H2DCFDA

The green fluorescence indicates arbitrary fluorescence units of the ROS levels in oocytes matured in culture medium supplemented with different levels of folic acid including 0 (control), 10, 100, 1000 ng/mL

FoA level had no effect on cleavage rate and development of zygote to 2 to 4 cells, 6 to 32 cells, and morula stage (P<0.05). Blastocyst/oocyte and blastocyst/cleaved zygote ratios were increased (P<0.05) in FoA100 compared to the control (17.58% *vs.* 8.64% and 24.24% *vs.* 10.53, respectively), with ORs of 2.67 and 2.72, respectively (Tables 2 and 3). The effects of adding FoA to the maturation medium on relative expression of methylation gene markers in bovine matured oocyte and blastocysts are shown in Table 4. Relative expression of Dnmt1 in matured oocytes increased in FoA10 and FoA100

compared to the control (3.52 and 6.08 *vs*. 1.10, respectively), but the highest levels were noted in FoA100 group (P<0.05). Relative expression of Dnmt3a was enhanced and of Dnmt3b decreased in all FoA-treated oocytes, respectively (P<0.05). Treatment did not affect the expression of Dnmt1 in blastocysts; however, relative expression of Dnmt3a was increased and Dnmt3b was decreased in FoA100 group as compared to the control group (5.28 *vs*. 0.84 and 0.24 *vs*. 1.02, respectively; P<0.05).

Results of the present study report that supplementing oocyte culture media with higher concentrations of FoA rather than those presented in follicles greater than 8 mm in diameter has beneficial effects on *in vitro* bovine fertilization and blastocyst rates. It can be assumed that oocytes in follicles smaller than 8 mm require nutrients to develop, which may not be provided by insufficient nutrients in the culture medium. It is reported that poor oocyte/embryo output is a key factor reducing the final *in vitro* embryo production outcome (Walsh *et al.* 2011).

In recent decades, numerous investigations have been directed to improve the processes involved in the production of embryos, mainly through optimizing culture media (Merton *et al*. 2003; Hussein *et al*. 2011). In an attempt to improve the oocyte quality and subsequent embryonic development, different concentrations of FoA were added to the oocyte maturation medium. The results showed that the addition of FoA has an ability to enhance *in vitro* oocyte and embryo developmental competence through reducing ROS and altering Dnmts expression. Consistently, the FoA supplementation to IVM media has shown to have beneficial effects on oocyte and embryo development in different species such as the mouse (Koyama *et al*. 2012), goat (Saini *et al*. 2018) and porcine (Sato *et al*. 2013).

Our results indicated that most of the evaluated parameters non-linearly responded to increasing levels of FoA, implying that at most 100 ng/mL of FoA can elicit the optimum responses in terms of reducing oocyte ROS content, and improving fertilization rate and embryonic developmental competence.

Baghshahi *et al*. (2019) determined the concentrations of FoA and HCY in commercial maturation medium and follicular fluids obtained from follicles with smaller and larger than 8 mm diameters, and showed that the concentration of FoA in maturation medium was the lowest (2.3 versus 14.5 and 11.9 ng/mL, respectively). However, concentration of HCY was higher in small follicles than larger follicles or commercial maturation medium (10.80 versus 5.00 and 2.3 ng/mL, respectively). This evidence indicated that maturation medium cultures do not adequately supply FoA, to meet requirements of immature oocytes; while, FoA supplementation has shown to improve oocyte maturation and developmental competence (Saini *et al*. 2018). Moreover, the requirement of immature oocytes to FoA is probably higher than the matured ones (Baghshahi *et al*. 2019).

Following the present findings, *in vitro* addition of 500 mM of FoA facilitated oocyte maturation of mouse and Xenopus laevis. (Huang *et al*. 2013). Also, addition of 10 ng/mL FoA to the culture medium improved maturation rate of porcine oocytes (Sato *et al*. 2013). However, Tawatao *et al*. (2015) did not report a significant effect of FoA on a proportion of matured oocytes. This inconsistency may be related to different administrated dosage or media (e.g., type, batches, quantity) used (Tawatao *et al*. 2015). Based on the primary investigation on the levels of FoA presented in follicular fluid (Baghshahi *et al*. 2019), we hypothesized that supplementing oocyte culture media with higher concentrations of FoA rather than those presented in follicles larger than 8 mm in diameter would have had a superior effect on oocyte and embryo developmental competence.

The findings of the present analysis have shown that addition of FoA was associated with decreased oocyte's ROS content. FoA, with antioxidative and considerable ROS scavenging properties (Stanger and Wonisch, 2012), have shown to help acquisition of developmental competence in different species (Koyama *et al*. 2012; Saini *et al*. 2018). Addition of FoA to *in vitro* heat-stressed mouse embryos decreased oxidative stress and ROS levels and recovered embryonic development (Koyama *et al*. 2012).

Also, Lee *et al*. (2008) confirmed that FoA supplementation to porcine oocytes maturation medium decreased the apoptosis of cumulus cells, and thereby, the rate of post-fertilization development such as proportion of embryos reaching the blastocyst stage was improved. These findings support present results, where *in vitro* addition of FoA decreased ROS content, and improved fertilization rate as well as subsequent development of bovine embryos.

It is worth noting that addition of 100 ng/mL of FoA is associated with a 3.77-fold higher odd ratio in fertilization success than the control group, while higher FoA dosages did not increase further fertilization rate. This is probably due to depletion of ROS content as the levels of FoA increased. It has been shown that a level of ROS is needed for many biological processes such as ATP synthesis, while strict limitation of ROS may interrupt these essential processes (Bardaweel *et al*. 2018).

The findings of this research demonstrated that addition of FoA could affect expression of DNA methyltransferases both in matured oocytes and subsequently produced embryos.

Table 2 Effect of different levels of folic acid supplementation to maturation medium on fertilization rate, cleavage rate and different stages of embryonic development (2-4 cells, 6-32 cells, morula, blastocyst and expanded blastocyst) of bovine oocyte

Development status (%)	Levels of folic acid (ng/mL)			
	0 (control)	10	100	1000
Fertilization rate	72.97 (81/111)^{b}	89.68 (113/126)^{a}	90 (91/101)^{a}	73.81 (93/126)^{b}
Cleavage rate	70.37 (57/81)	70.79 (80/113)	72.52 (66/91)	69.89 (65/93)
2-4 cells	20.98 (17/81)	17.7 (20/113)	20.88 (19/91)	18.28 (17/93)
6-32 cells	27.16 (22/81)	30.08 (34/113)	21.98 (20/91)	17.2 (16/93)
Morula	13.58 (11/81)	10.62 (12/113)	12.08 (11/91)	17.2 (16/93)
Blastocyst/oocyte	8.64 (6/81)^{b}	12.39 (14/113)^{ab}	17.58 (16/91)^{a}	17.2 (16/93)^{ab}
Expanded blastocyst	10.53 (6/57)^{b}	17.50 (14/80)^{ab}	24.24 (16/66)^{a}	24.61(16/65)^{a}

The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Table 3 Odds ratios (±SE)[1] of adding different levels of folic acid to the maturation medium on *in vitro* fertilization, cleavage and *in vitro* different stages (2-4 cells, 6-32 cells, morula, blastocyst, expanded blastocyst) of embryonic developmental competence of bovine oocytes

Parameters	Level of folic acid (ng/mL)		
	10	100	1000
Fertilization	3.22±0.36	3.37±0.40	1.04±0.29
Cleavage	1.02±0.31	1.11±0.34	0.98±0.33
2-4 cells	0.81±0.31	0.81±0.37	0.99±0.38
6-32 cells	1.15±0.32	0.76±0.36	0.58±0.37
Morula	0.76±0.45	0.88±0.46	1.32±0.43
Blastocyst/oocyte	1.77±0.51	2.67±0.50	1.29±0.50
Blastocyst/cleaved zygote	1.80±0.52	2.72±0.52	2.78±0.52

[1] Values representing the odd ratios of each evaluated parameter in different experimental groups as compared to the control group.

Table 4 Effect of folic acid supplementation to the maturation medium on relative expression of the methylation gene markers (DNA methyltransferase1 (Dnmt1), Dnmt3a, Dnmt3b) on bovine matured oocytes and blastocysts

Target tissue	Levels of folic acid (ng/mL)	Dnmt1	Dnmt3a	Dnmt3b
Ovary	-	11.20±3.96	6.67±3.07	30.11±10.27
Matured oocyte	0 (control)	1.10^{c}	1.05^{b}	2.44^{a}
	10	3.52^{b}	2.07^{ab}	0.78^{b}
	100	6.08^{a}	4.47^{a}	0.49^{b}
	1000	1.73^{bc}	1.17^{ab}	0.51^{b}
	SEM	0.48	0.38	0.34
Blastocyst	0 (control)	1.00	0.84^{b}	1.02^{a}
	10	1.49	1.01^{b}	0.62^{ab}
	100	2.01	5.28^{a}	0.24^{b}
	1000	1.51	1.99^{b}	0.89^{a}
	SEM	0.49	0.49	0.16

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

Consistently, FoA supplementation has shown to improve developmental competence by altering the expression of genes related to oocyte maturation (Saini *et al*. 2018). It has been demonstrated that FoA acts as an effective compound in many cellular functions such as the de novo nucleotides synthesis, HSY remethylation, protein synthesis and methylation of bioactive molecules (Stover, 2009). The Dnmt1, Dnmt3a, and Dnmt3b are the main enzymes participating in the establishment and preservation of DNA methylation (Hirasawa *et al*. 2008). Expressions of both Dnmt3a and Dnmt3b mRNA were reported in bovine oocytes and blastocysts (Uysal *et al*. 2015). The Dnmt1 is found in mature (MII) bovine oocytes and blastocysts under *in vitro* conditions (Uysal *et al*. 2015).

In vitro culture has shown to interfere the expression Dnmt3a and Dnmt3b in the mature oocytes as opposed to counterparts developed *in vivo* (Song *et al*. 2009). The alteration in gene expression of Dnmt3a most likely results in disrupted oocyte maturation in MII oocytes and successive embryonic development (Fang *et al*. 2016). In mammalian oocytes, Dnmt1's primary role is to preserve methylation in hemi-methylated DNA strands after DNA methylation (Ratnam *et al*. 2002). It has been suggested that factors altering the normal expression of Dnmt1 gene cause abnormal DNA methylation, abnormal imprints, low embryo production, and abnormal oocyte maturation (Whidden *et al*. 2016). As one of the de novo methyltransferases enzymes, Dnmt3a plays a crucial func-

tion in the methylation of unmethylated DNA strands in producing new impressions (Okano *et al*. 1998). However, Dnmt3b is another de novo DNA methyltransferase that specifically works on CpG dinucleotide methylation in the replicated DNA sequence of eukaryotic chromosomes in the pericentric satellite DNA region (Kato *et al*. 2007).

Our results showed that expressions of Dnmts in ovaries were much higher than both the *in vitro* matured oocytes and produced embryos. The high expression levels of methylation genes in the ovary are properly due to the fact that ovary is a very dynamic tissue. Under *in vivo* condition, many essential transcripts and molecules are transferred to the oocyte, immediately before ovulation. The received components from ovulatory follicles help to survive the fertilized oocyte and initiating subsequent development (Schulz and Harrison, 2019). However, *in vitro* matured oocytes most likely do not receive these components and will produce low-quality embryos. In the present study, addition of FoA to the maturation medium unregulated in both de novo (Dnmt3a) and maintenance (Dnmt1) methyltransferases, while down-regulated the Dnmt3b expression in the FoA-treated oocytes and its produced blastocysts. Accordingly, Saini *et al*. (2018) showed that FoA supplementation increased the expression of genes involved in the linked methionine–folate cycles of DNA methylation such as Mtr, Mat2a, Achy, and Dhfr. Since Dnmt3a is synthesized from maternally stored mRNAs (Hirasawa *et al*. 2008; Uysal *et al*. 2015), the higher Dnmt3a transcript abundance observed in this study implies that addition of FoA increased the expression of this gene in the blastocyst, probably by affecting expression of oocyte's Dnmt3a. However, the mechanisms and consequences beyond the effect of FoA on the down-regulation of the Dnmt3b are not clear.

CONCLUSION

We conclude that addition of 100 ng/mL FoA to the culture medium of bovine oocyte reduced ROS content and changed transcript abundance of Dnmt1, Dnmt3a, and Dnmt3b, associating with an improvement in fertilization and blastocyst rates.

ACKNOWLEDGEMENT

The authors would like to thank Prof. A. Nejati-Javaremi and Zargene Alborz Co., a member of Mobarakandish Group for providing frozen bovine semen.

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