Folic acid supplementation for bovine oocyte maturation and fertilization in vitro

Karlo M. Tawatao[1], Fely V. Manaois II[1], Lerma C. Ocampo[2] and Marlon B. Ocampo [1,2*]

[1]College of Veterinary Science and Medicine, Central Luzon State University, Science city of Munoz, Nueva Ecija, Philippines 3120
[2]Reproductive Biotechnology Unit, Philippine Carabao Center, Science City of Munoz, Nueva Ecija, Philippines 3120

The study aimed to evaluate the effect of folic acid supplementation on the maturation and development of bovine oocytes and embryos in vitro, respectively. In Experiment 1, oocytes were matured in mSOF or TCM-199 medium with/without FA supplementation. No significant difference was observed in the nuclear maturation rates but the cytoplasmic maturation (MPN formation) was higher significantly in TCM-199 groups. In Experiment 2, cleaved embryos were cultured in mSOF with/without FA supplementation. Significantly higher blastocyst formation rate was observed in treatment without FA (47.33±8.44). In Experiment 3, LC addition significantly improved the blastocyst formation rate of bovine embryos than with the presence of FA. Overall, the results showed that FA supplementation is not beneficial in the acquisition of developmental competence of bovine oocytes and embryos in vitro.

Key words: Folic acid, maturation, fertilization, oocyte, male pronucleus formation, embryos

Introduction

In the field of reproductive and developmental biology, understanding on what constitutes oocyte developmental competence remains a challenge especially towards full developmental potential to term. Embryo production from ovaries of slaughtered females using oocyte in vitro maturation (IVM) technique is now a routine procedure and becomes an important technology for artificial breeding. Basically, it involves the aspiration of oocytes from antral follicles and culturing them in standard cell culture conditions for maturation (metaphase-2 [M-2] stage). However, only a small proportion of matured oocytes have full developmental potential to term (Schroeder and Eppig, 1984; Hyttel *et al.*, 1997). The choice of medium for maturation plays an integral part

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*Corresponding author: Marlon B. Ocampo; **Email:** ocampomarlon29@yahoo.com

on the success of embryo production in vitro. Moreover, oocyte quality is closely related to developmental competence and its acquisition accompanies spatiotemporal changes in histone acetylation and DNA methylation (Bui *et al*., 2007: Mason *et al*., 2012). The methylation level of DNA increases with oocyte growth, eg., oocytes derived from large antral follicles have higher DNA methylation levels than oocytes taken from small antral follicles (Endo *et al.*, 2005: Lodde *et al*., 2009: Fagundes *et al.*, 2011). In mice, DNA methylation continues up to approximately M-2 stage (Imamura *et al.*, 2005). Thus, the presence of a methyl donor (eg., folic acid) in culture profoundly influences the DNA methylation state of an oocyte. Oocytes that develop under methyl-donor deficient conditions have low developmental competency and decreased methylation state of their imprinted genes (Anckaert *et al.*, 2010). Moreover, one major event in early embryonic development, a zygotic genome activation, is associated with global histone modification including the acetylation of histones and the demethylation of DNA (Maalouf *et al.*,2008). The acetylation levels of histones in early stage embryos are reported to be related to the acquisition of developmental competence of porcine embryos (Yamanaka *et al.*, 2009). That, folic acid supplementation of the maturation medium increased the global DNA methylation of the oocytes and the development rate to the blastocyst stage (Sato *et al.,* 2013). Also, it decreased the amount of reactive oxygen species in matured oocytes and increases the level of GSH, hence improved the blastocyst formation rate (Kim *et al*.,2009). It has been suggested that folic acid also acts as an antioxidant, thereby improving oocyte quality (Lazalde-Ramos *et al*., 2012). In this study, we examined the effect of FA supplementation on the acquisition of developmental competence of bovine oocytes matured and fertilized in vitro.

Materials and Methods

Media

The basic media for maturation of oocytes was tissue culture medium (TCM-199; Gibco Co., Grand Island, N.Y., USA) with Earle's salts and L-glutamine (Krisher et al., 1999), for fertilization was a Brackett-Oliphant medium (BO; Brackett and Oliphant, 1975) and for culture was the modified synthetic oviductal fluid medium (mSOF, Tervit et al.,1972).

Oocyte collection and maturation

Bovine ovaries were collected immediately postmortem at local abattoirs and transported to the laboratory in 0.9 % saline solution at 30 - 35º C within 4 - 6 hr. The ovaries were pooled irrespective of the donors estrus cycle. COCs were aspirated from antral follicles (3 - 5 mm in diameter) by using 18- gauge needle attached to a 10- ml sterile plastic syringe, washed three times in the maturation medium before selecting using a stereomicroscope based on the criteria described by Ocampo et al., (1993). A group of 10 - 15 COCs were transferred into 50 µl droplets of maturation medium under mineral oil (Sigma Chem Co., St. Louis, USA) in a 35 x 10 mm Falcon polysterene culture dish (Becton and Dickinson Labware, N.J., USA) which had been previously pre-incubated to equilibrate for at least 2 hr in a CO_2 incubator. COCs were cultured at 39°C under an atmosphere of 5 % CO_2 and 95 % air with high humidity. After culture for 22 hr, the cumulus and corona cells were removed by pipetting.

Sperm preparation

Straws of locally processed frozen semen from the Sperm Processing Unit of the Philippine Carabao Center at Central Luzon State University Ranch in Digdig, Carranglan, Nueva Ecija were thawed at 39°C water bath for 15 sec and washed twice with fertilization medium (Brackett and Oliphant; BO medium) containing 1 mg/ml bovine serum albumin (BSA, Initial fractionation by heat shock, Sigma, St. Louis, MO, USA) by centrifugation at 800 rpm for 5 min. After the final wash, the sperm was re-suspended at 2×10^6 sperm/ml in fertilization medium with 5 mM theophylline and 6 mg/ml BSA, and pre-incubated for 1 hr at 39°C; 95 % air atmosphere in a tightly capped test tube.

In vitro fertilization and culture

Selected oocytes were transferred to the fertilization droplets (50 µl) with 15 oocytes per drop under mineral oil. A portion of sperm suspension was added to the droplets giving a final sperm concentration of 1×10^6 cells/ml, 2.5 mM theophylline and 3 mg/ml BSA. The motility rate of sperm during insemination was more than 50%. After sperm-oocyte co-culture for 24 hr, the extra sperm cells attached to the oocytes were removed by pipetting using a finely drawn glass pipette. Subsequently, the oocytes were washed twice with culture medium before transferring to 50 µl drops of mSOF medium and cultured for 7

days in a humidified incubator with a gas phase of 5% O_2, 5% CO_2 and 90% N_2 level at 39°C.

Cell counting

Blastocyst appearing on the 7th day were collected and subjected to a differential staining protocol for embryos (Thouas et al., 2001) with modification. Briefly, blastocyst were washed in PBS-PVP, then placed in 1 ml of Hoechts working solution (0.75 ml of 2.3% Na citrate dehydrate solution; 0.25 ml of ethanol; 10 µl of Hoechts 33342 stock solution of 1 mg/ml concentration dissolved in ethanol) in an Effendorf tube, wrapped in aluminum foil and stored in the refrigerator (4°C) for at least 24 hrs. Subsequently, the blastocyst were recovered and washed in glycerol, mounted on a glass slide, flattened in glycerol by a cover slip to a level where all nuclei appeared at the same focal plane and examined by using a fluorescent microscope (Eclipse E-600; Nikon) under ultraviolet light. A digital image of each embryo was taken and the total cells (both inner cell mass and trophectoderm) counted.

Experimental design

Experiment 1. Effect of folic acid supplementation on nuclear and cytoplasmic maturation. COCs were matured in IVM medium supplemented with folic acid (0, 10, 50 or 100 µM) for 22 hr. Nuclear and cytoplasmic maturation of the oocytes was based on the presence of 1st polar body and male pronucleus formation (MPN), respectively. The experiment was replicated four times.

Experiment 2. Effect of folic acid supplementation on embryo development. Early- stage embryos (2- to 4- cell stage) resulting from IVF were selected randomly and assigned to droplets of IVC medium with or without folic acid (0, 10, 50 or 100 µM) supplementation. Resulting blastocyst were recorded and their cell count analyzed. The experiment was replicated three times.

Experiment 3. Effect of folic acid and L-carnitine supplementation on the maturation and development rate to the blastocyst stage. COCs were matured in IVM medium with or without folic acid or L-carnitine (LC; 0.5 mg/ml) supplementation or a combination of both and analyzed for their ability to mature and support development to the blastocyst stage following fertilization. The experiment was replicated five times.

Statistical analysis

Statistical comparisons of percentage data were by ANOVA (P<0.05).

Results

In Experiment 1, the percentage nuclear maturation rate (M-2 stage) in both maturation medium used with or without FA supplementation showed no significant difference. The penetration rate was higher significantly in TCM-199 alone than with FA addition and in mSOF with/without FA addition. The cytoplasmic maturation (MPN formation) was higher significantly in mSOF alone than when FA was used as a supplement. Also, TCM-199 medium alone or with FA supported MPN formation better than mSOF medium (Table 1).

Table 1. Effect of FA addition in maturation medium on the rate of nuclear and cytoplasmic maturation

FA	No. of	No. of oocytes					
(µM)	replicates	cultured	matured	penetrated	MPN	UF	others
mSOF medium							
Control	4	70	54 (77.38±6.4)[a]	29 (53.64±7.8)[b]	27 (94.37±3.3)[d]	25	15
10	4	56	44 (77.21±9.4)[a]	14 (31.27±8.5)[b]	12 (68.75±2.6)[e]	32	10
50	4	57	44 (76.18±4.3)[a]	10 (24.64±8.5)[b]	5 (56.28±2.5)[e]	35	12
100	4	56	46 (79.00±5.0)[a]	25 (51.76±3.3)[b]	15 (48.35±2.5)[e]	23	8
TCM-199 medium							
Control	4	40	42 (84.00±2.5)[a]	31 (74.44±6.2)[c]	28 (90.95±3.7)[d]	17	2
10	4	58	47 (85.31±4.6)[a]	24 (49.23±5.1)[b]	18 (86.36±3.6)[d]	23	10
50	5	81	68 (82.82±6.2)[a]	29 (52.73±9.6)[b]	31 (90.18±7.8)[d]	35	7
100	4	61	42 (71.56±6.2)[a]	19 (43.75±9.5)[b]	19 (100.0±00)[d]	27	18

[a,b,c,d,e] Values with different subscript in the same column differ significantly (P<0.05)

In Experiment 2, the developmental rate to the blastocyst stage of cleaved embryos was higher significantly in mSOF alone than when FA was added as a supplement (Table 2). The total cell count of the blastocyst stage embryos derived from all treatments showed no difference.

Table 2. Effect of FA addition in mSOF (IVC) on embryonic development

FA (µM)	No. of replicates	No. of embryos (%)				Cell count
		cultured	16- to 32-	morula	blastocyst	
Control	3	50	43	33	16 (47.33±8.44)[a]	95.6±7.8
10	3	44	36	32	7 (22.86±2.86)[b]	85.8±3.4
50	3	40	36	32	6 (18.81±4.52)[b]	82.5±7.3
100	3	49	43	36	5 (13.33±10.18)[b]	86.2±4.3

[a,b]Values differ significantly (P<0.05)

In Experiment 3, the percentage nuclear maturation of oocytes cultured in the maturation medium with FA, LC or in combination had no significant difference. However, the blastocyst formation rate was higher significantly in LC supplemented condition than with FA. The total cell count showed no significant difference (Table 3).

Table 3. Effect of FA and LC addition on the maturation and embryonic development

Treatment	No. of replicates	No. of oocytes						Cell count
		cultured	matured	inseminated	cleaved	blastocyst	count	
FA	3		81	68 (84.0)	68	40 (58.8)	6 (8.8)[a]	82.5±7.3
LC	5		185	161 (87.0)	105	60 (57.1)	31 (29.2)[b]	97.4±4.9
FA + LC	3		48	40 (83.3)	38	17 (44.7)	4 (10.5)[a]	88.1±4.1

[a,b] Values differ significantly (P<0.05).

Discussion

The ability to achieve full developmental competence of oocytes up to the blastocyst stage after fertilization in vitro requires a carefully regulated environment which is dependent on the compositions of the medium used. Variability in the results under in vitro system can occur on account of the differences on the media (eg., type, batches, lot) used and its supplementations. Thus, a need for a single culture system that would support the oocytes acquisition of competence for complete maturation, the resulting zygotes development past the "cell-block" stage up to the blastocyst stage is imperative. In this study, mSOF medium was used on the basis of the results reported on its usefulness as an "all in one medium" for oocyte maturation, fertilization and embryo development (Takahashi and First, 1993; Gandhi et al., 2000). Also,

the supplementation of mSOF with FA was done in an attempt to further optimize the chances of oocytes for acquisition of developmental competence (Kim et al., 2009; Sato et al., 2013) by increasing the global DNA methylation of the oocytes. Our findings showed that FA addition has no influence on the nuclear maturation rate of bovine oocytes but has detrimental effect on the cytoplasmic maturation. Most of penetrating spermatozoa in the ooplasm of FA supplemented oocytes failed to decondense owing to the minimal presence of a full male pronucleus after 24 hr of sperm-oocyte co-incubation. This observation was further supported by the significantly lower blastocyst formation of early- stage embryos cultured in mSOF with FA as a supplement. These resultswas in contrast with earlier reports using porcine oocytes (Kim et al., 2009; Sato et al., 2013). Interestingly, when FA was added in TCM-199 medium, both the nuclear and cytoplasmic maturation of the oocytes improved. Similarly, the use of North Carolina State University 37 solution (Petters and Wells, 1993), containing 0.6 mM cysteine and 10% follicular fluid with FA resulted to improved developmental competence of the oocytes (Sato et al., 2013).One plausible explanation for this difference could be associated with the synergistic effect of cysteine, which is not present in mSOF, with FA in enhancing the oocytes developmental competence. It has been reported that addition of homocysteine or N-acetyl-cysteine in the maturation medium improved the MPN formation of penetrated oocytes and its blastulation ratio (Whitaker et al., 2012).

The addition of LC significantly improved the blastocyst formation of bovine oocytes post fertilization compared to FA or LC + FA as a supplement. The use of LC has been claimed to have a beneficial role in the cellular metabolism and embryonic development of some mammalian species (Kruip et al., 1983; Fergusson and Leese, 2006; Abdelrazik et al., 2009) due to its involvement in lipid metabolism, an alternative source for energy production. Also, LC plays a role in reducing oxidative stress by enhancing the activity of numerous antioxidant enzymes, eg., superoxide dismutase, catalase and glutathione peroxidase (Rizzo et al., 2010). As demonstrated in bovine and porcine, supplementation of maturation medium with LC increased the gluthathione and decreased the reactive oxygen species levels in the oocytes which has deleterious effect on embryo development (Wu et al., 2011; Manzano et al., 2014). The combination of FA and LC showed no positive influence on the blastocyst formation of bovine oocytes. In conclusion, FA supplementation in the maturation and embryo culture medium has no beneficial influence in the acquisition of developmental competence of bovine oocytes in vitro.

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