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Effects of different oocyte retrieval and *in vitro* maturation systems on bovine embryo development and quality

Sandra Milena Bernal Ulloa[2,3,4], *Julia Heinzmann*[2], *Doris Herrmann*[2], *Bernd Timmermann*[5], *Ulrich Baulain*[2], *Rudolf Großfeld*[6], *Mike Diederich*[7], *Andrea Lucas-Hahn*[2] *and Heiner Niemann*[1,2]

Institute of Farm Animal Genetics, Biotechnology, Friedrich-Loeffler-Institut (FLI), Mariensee, Germany; Facultad de Ciencias Agropecuarias, Universidad de Ciencias Aplicadas y Ambientales, Bogotá, Colombia; Adaptation Physiology Group, Wageningen, Wageningen University, The Netherlands; Max Planck Institute for Molecular Genetics, Berlin, Germany; Minitube GmbH, Tiefenbach, Germany; and Weser-Ems-Union eG, Rodenkirchen, Germany

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Summary

Cyclic adenosine monophosphate (cAMP) modulators have been used to avoid spontaneous oocyte maturation and concomitantly improve oocyte developmental competence. The current work evaluated the effects of the addition of cAMP modulators forskolin, 3-isobutyl-1-methylxanthine (IBMX) and cilostamide during *in vitro* maturation on the quality and yields of blastocysts. The following experimental groups were evaluated: (i) slicing or (ii) aspiration and maturation in tissue culture medium (TCM)199 for 24 h (TCM24slicing and TCM24aspiration, respectively), (iii) aspiration and maturation in the presence of cAMP modulators for 30 h (cAMP30aspiration) and *in vivo*-produced blastocysts. *In vitro*-matured oocytes were fertilized and presumptive zygotes were cultured *in vitro* to assess embryo development. Cleavage, blastocyst formation, blastocyst cell number, mRNA abundance of selected genes and global methylation profiles were evaluated. Blastocyst rate/zygotes for the TCM24aspiration protocol was improved (32.2 ± 2.1%) compared with TCM24slicing and cAMP30aspiration (23.4 ± 1.2% and 23.3 ± 2.0%, respectively, $P<0.05$). No statistical differences were found for blastocyst cell numbers. The mRNA expression for the *EGR1* gene was down-regulated eight-fold in blastocysts that had been produced *in vitro* compared with their *in vivo* counterparts. Gene expression profiles for *IGF2R*, *SLC2A8*, *COX2*, *DNMT3B* and *PCK2* did not differ among experimental groups. Bovine testis satellite I and *Bos taurus* alpha satellite methylation profiles from cAMP30aspiration protocol-derived blastocysts were similar to patterns that were observed in their *in vivo* equivalents ($P > 0.05$), while those from the other groups were significantly elevated. It is concluded that retrieval, collection systems and addition of cAMP modulators can affect oocyte developmental competence, which is reflected not only in blastocyst rates but also in global DNA methylation and gene expression patterns.

Keywords: cAMP modulators, *In vitro* maturation, Oocyte competence, Preimplantation embryo

Introduction

In modern cattle production, *in vitro* maturation (IVM) of oocytes is widely used as part of the *in vitro*-production systems for bovine embryos. Recent figures have indicated that in 2011 there were 374,000 transfers that used *in vitro*-produced embryos (Stroud, 2012). Nevertheless, numerous studies have found that IVM

[1]All correspondence to: Heiner Niemann. Institute of Farm Animal Genetics, Biotechnology, Friedrich-Loeffler-Institut (FLI), Mariensee, Höltystraße 10, Mariensee, 31535 Neustadt, Germany. Tel: +49 5034871136. Fax: +49 5034871143. e-mail: heiner.niemann@fli.bund.de
[2]Institute of Farm Animal Genetics, Biotechnology, Friedrich-Loeffler-Institut (FLI), Mariensee, Neustadt, Germany.
[3]Facultad de Ciencias Agropecuarias, Universidad de Ciencias Aplicadas y Ambientales, Calle 222 No. 55–37, Bogotá, Colombia.
[4]Adaptation Physiology Group, Wageningen University, De Elst 1 (Building 122) 6708 WD Wageningen, The Netherlands.
[5]Max Planck Institute for Molecular Genetics, Ihnestraße 63–73, 14195, Berlin, Germany.
[6]Minitube GmbH Hauptstrasse 41, Tiefenbach, Germany.
[7]Weser-Ems-Union eG, Aussenstelle Rodenkirchen, Bullenmutterstation, Mittenfelder Weg 11, 26935 Rodenkirchen, Germany.

can be associated with changes in gene expression and methylation profiles that result in deterioration in the developmental competence of oocytes and early embryos (Wrenzycki *et al.*, 2005a, 2007; Heinzmann *et al.*, 2011; Diederich *et al.*, 2012).

When IVM is performed, the oocyte is released mechanically from its antral follicle, this release in turn triggers meiotic resumption and results in maturation of the oocyte and arrest at metaphase II (Mehlmann, 2005; Zhang *et al.*, 2010). This process has been designated 'spontaneous' or 'pseudo' maturation and has been attributed to the removal of inhibitory factors from the follicle rather than active processes (Pincus & Enzmann, 1935; Tsafriri & Pomerantz, 1986). Several suggestions have been made to improve IVM that included matching the IVM medium to requirements of the cumulus–oocyte complexes (COCs), inhibition or delay of spontaneous maturation and acquisition of oocyte capacitation. Delay or inhibition of spontaneous maturation has been employed successfully and several molecules have been applied to better simulate the physiological pathways of oocyte maturation.

Different biochemical pathways are involved in oocyte maturation, which culminates in cumulus expansion and cytoplasmic and nuclear maturation, reflected by acquisition of complete developmental competence (Tripathi *et al.*, 2010). Cyclic adenosine monophosphate (cAMP) is critical for the maintenance of meiotic arrest *in vivo*. Elevated intra-oocyte levels of cAMP maintain oocytes in meiotic arrest by suppression of maturation promoting factor (MPF) and by stimulation of cAMP-dependent protein kinase A (PKA) [reviewed by Bilodeau-Goeseels (2011)]. The application of different approaches has been reported to increase cAMP content in oocytes and the surrounding cumulus cells during IVM. Albuz *et al.* (2010) reported that bovine COCs matured in the presence of cAMP modulators forskolin, 3-isobutyl-1-methylxanthine (IBMX) or cilostamide, before and during extended IVM (simulated physiological oocyte maturation (SPOM)), should increased bovine embryo production and quality based on blastocyst cell numbers. However, to our knowledge, no reports have been published on the expression profile of selected genes and global DNA methylation status of SPOM-derived bovine blastocysts, which are important parameters of embryo quality assessment. The current work set out to characterize SPOM-derived blastocysts in detail. Specifically, the effects of supplementation of the basic culture medium with forskolin, IBMX and cilostamide before and during extended IVM on the developmental potential of bovine COCs, mRNA abundance of genes related to cell physiology, methylation and imprinting, and also DNA methylation were studied in two satellite repeat sequences of bovine preimplantation embryos.

Materials and methods

The original SPOM method was performed in connection with aspiration of bovine ovarian follicles from slaughtered females (Albuz *et al.*, 2010). The method of oocyte recovery used routinely in our laboratory is slicing of ovaries rather than aspiration, therefore slicing was used as a 'control' in the current work. We consider that slicing mimics the ovum pick-up clinical approach sufficiently well, in which follicular fluid is diluted during oocyte recovery. Experiments were designed that included oocytes retrieved either via aspiration or by slicing. Therefore, three different IVM protocols for bovine oocytes were used in the current study, and the developmental potential of the selected COCs as well as blastocyst quality were examined. The protocols varied in the method of oocyte retrieval (slicing and aspiration), addition of cAMP modulators and length of the IVM period of the COCs. *In vivo*-produced expanded blastocysts were used as a physiological standard for comparison with their counterparts produced *in vitro*. All media used in the different procedures of *in vitro* and *in vivo* blastocyst production were prepared in-house in accordance with the manufacturer's recommendations.

Oocyte recovery

Bovine ovaries from a local slaughterhouse were transported at 30°C in saline solution supplemented with 6 μg/ml penicillin G (AppliChem, Darmstadt, Germany) and 50 μg/ml streptomycin sulphate (AppliChem).

To obtain COCs, the ovaries were either submitted to aspiration or slicing. For aspiration, follicles 3–8 mm in diameter were punctured using 18G×1½ needles; follicular fluid that contained the oocytes was collected in sterile 50 ml centrifuge tubes (Corning®, New York, USA), connected to a vacuum pump that had been adjusted to a negative pressure of 100 mmHg (VM AR-5100, Cook, Queensland, Australia; Holker *et al.*, 2005). Immediately after collection, COCs were examined under a stereomicroscope in undiluted follicular fluid to avoid a drop in cAMP levels.

Ovaries with follicles of 3–8 mm in diameter were sliced to release COCs, which were examined in Dulbecco's phosphate-buffered saline (PBS; AppliChem) supplemented with 2.2 IU/ml heparin (AppliChem), 1 mg/ml bovine serum albumin (BSA; Sigma-Aldrich), 6 μg/ml penicillin G (AppliChem) and 50 μg/ml streptomycin sulphate (AppliChem; Wrenzycki *et al.*, 2001).

For both retrieval protocols, only COCs with homogeneous cytoplasm and at least three layers of cumulus cells were selected (categories I and II) for IVM (Looney *et al.*, 1994, Goodhand *et al.*, 1999).

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In vitro maturation (IVM) protocols

Standard IVM (TCM24slicing)

After slicing, oocytes were collected and maintained in tissue culture medium (TCM) 199 (TCM199, Sigma-Aldrich), enriched with 50 μg/ml gentamicin sulphate (Sigma-Aldrich), 0.2 mM Na-pyruvate (Sigma-Aldrich), 4.2 mM $NaHCO_3$ (Honeywell Riedel de Haën, Seelze, Germany) and 1 mg/ml BSA-FAF (Sigma-Aldrich; Eckert & Niemann, 1995), here called TCM-air medium.

For IVM, selected COCs were washed three times in TCM199 that contained 0.2 mM Na-pyruvate (Sigma-Aldrich), 25 mM $NaHCO_3$ (Honeywell Riedel-de Haën), 50 μg/ml gentamycin (Sigma-Aldrich) and 1 mg/ml FAF-BSA (Sigma-Aldrich). This medium is termed TCM-culture in the present study. Groups of 15–20 COCs were incubated in 100 μl TCM-culture drops under silicone oil that contained 10 UI/ml of equine chorionic gonadotropin (eCG) and 5 IU/ml of human chorionic gonadotropin (hCG; Suigonan®, Intervet, Unterschleissheim, Germany; Heinzmann *et al.*, 2011). Oocytes were incubated in a humidified atmosphere at 39°C, 5% CO_2 in air for 24 h.

TCM24aspiration (retrieval control)

Following aspiration, COCs were collected in TCM-air medium and subsequently treated as described for the standard IVM protocol (TCM24slicing).

Modified extended IVM system (cAMP30aspiration)

For the cAMP30aspiration protocol, the selected COCs were maintained in TCM-air medium additionally supplemented with 500 μM 3-isobutyl-methylxanthine (IBMX, Sigma-Aldrich) and 100 μM forskolin (FSK, Sigma-Aldrich) to ensure consistently high cAMP levels (Albuz *et al.*, 2010). The COCs were washed three times in TCM-air that contained FSK and IBMX and were then cultured prior to IVM (pre-IVM) for 2 h in groups of 15–20 COCs in 100 μl drops under silicone oil at 39°C in air.

After the pre-IVM phase, COCs were washed three times in TCM-culture medium that contained 20 μM cilostamide (Sigma-Aldrich), and matured *in vitro* in 100 μl drops of TCM-culture medium, supplemented with 20 μM cilostamide and Suigonan® in a humidified atmosphere at 39°C, 5% CO_2 in air for 30 h.

Evaluation of in vitro *maturation success*

Cumulus cells were removed from subsets of COCs for every protocol by incubation in PBS supplemented with 0.1% hyaluronidase (Sigma-Aldrich) and 1 mg/ml BSA (Fraction V, Sigma-Aldrich) for 5 min at 38°C and subsequent centrifugation for 5 min at 1400 rpm. Any remaining cumulus cells were removed by gentle pipetting. Oocytes were washed in TCM-air and evaluated under a stereomicroscope. Meiotic resumption was determined by the presence of the first polar body after IVM.

In vitro fertilization (IVF) and culture (IVC)

After maturation, COCs from the different protocols were processed in accordance with Heinzmann *et al.* (2011)). Oocytes were washed three times in fertilization medium (Fert-TALP; Parrish *et al.*, 1986, 1988) that contained 6 mg/ml BSA (fraction V, Sigma-Aldrich), 0.05 mg/ml gentamicin (Sigma-Aldrich) and 0.028 mg/ml Na-pyruvate (Sigma-Aldrich), and transferred into 100 μl drops under silicone oil of 100 μl of Fert-TALP enriched with 10 μM hypotaurine (Sigma-Aldrich), 0.1 IU/ml heparin (AppliChem), 1 μM epinephrine (Sigma-Aldrich). Frozen–thawed sperm from one bull with proven performance in IVF was used. Motile spermatozoa were obtained by centrifugation of thawed sperm (30°C, 2 min) in 1 ml of 90% Bovipure™ (Labotect, Göttingen, Germany) at 300 *g* for 10 min. Spermatozoa were washed twice using Fert-TALP and centrifuged at 400 *g* for 3 min. Spermatozoa were added to reach a final concentration of 1×10^6 cells/ ml and co-incubated with COCs for 19 h for the TCM24 protocols and 24 h for the COCs submitted to the cAMP30aspiration protocol, in a humidified atmosphere of 5% CO_2 in air at 39°C. Immediately after fertilization, presumptive zygotes from all protocols were denuded by vortexing for 5 min in TCM-air medium. Completely denuded zygotes were washed twice in synthetic oviductal fluid (SOF) medium enriched with 4 mg/ml of BSA-FAF (Sigma-Aldrich; Wrenzycki *et al.*, 2001; Heinzmann *et al.*, 2011). Groups of five zygotes were cultured in 30 μl droplets of SOF under silicone oil at 39°C, 5% CO_2 and 5% O_2. Post-fertilization, embryo development was evaluated for cleavage (day 2) and blastocyst formation (day 8).

Expanded blastocysts on days 7 and 8 were either directly subjected to differential staining to assess cell numbers or frozen at –80°C in PBS that contained 0.1% polyvinyl alcohol (PVA; PBS–PVA) in pools of three for gene expression analysis and pools of five for methylation analysis.

In vivo production of embryos

Holstein Friesian cows from the experimental herds of the Institute of Farm Animal Genetics in Mariensee (Germany) were superovulated using a single dose of 2500–3000 UI eCG (Intergonan®; Intervet, Tönisvorst, Germany). A single dose of cloprostenol was administered 48 h after eCG (3 ml; Estrumate®, Essex, Munich, Germany). Two artificial inseminations were performed, with sperm from the same bull used for IVF, 48 h after cloprostenol injection with a 12 h interval. Embryos were recovered from the uterine

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horns by non-surgical means on days 7 and 8 as described by Bungartz & Niemann (1994). Dulbecco's PBS medium (AppliChem) supplemented with 1% fetal calf serum (FCS; Invitrogen, Karlsruhe, Germany) was used for uterine flushing. Expanded blastocysts were frozen in groups of three for gene expression analysis and groups of five for methylation analyses in accordance with the protocol for *in vitro*-produced embryos. Additional blastocysts were processed for cell number counting by differential staining.

Differential staining

Cell numbers of the inner cell mass and trophectoderm from *in vitro*- and *in vivo*-derived expanded blastocysts were counted using a protocol described by Thouas *et al.* (2001), with minor modifications. Briefly, expanded blastocysts were washed in PBS–PVA and subsequently permeated and stained by incubation in PBS without Ca and Mg and containing 0.25% Triton X-100 (Packard Instruments Company, USA) and 0.2 mg/ml propidium iodide (Sigma, Munich Germany) for 30 s. Thereafter, the embryos were washed twice in PBS–PVA and placed for 4 min in PBS that contained bisbenzimide (Hoechst 33258, Sigma, Munich Germany) dissolved in 4% formaldehyde (Honeywell Riedel de Haën, Seelze, Germany) for a final working concentration of 0.026 mg/ml. The stained embryos were mounted on microscope slides in a small drop of glycerol (Carl Roth GmbH, Karlsruhe, Germany). In order to determine the number and type of cells, the embryos were examined immediately under a fluorescence microscope (Olympus BX60F, Tokyo, Japan) at ×400 magnification equipped with an ultraviolet filter and an attached digital camera (Olympus DP71). The trophectoderm (TE) nuclei appeared red and the inner cell mass (ICM) nuclei were blue.

Determination of the relative mRNA abundance of developmentally important genes

The relative abundance of selected genes was determined in expanded blastocysts using semi-quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Analysed genes included the imprinted gene insulin-like growth factor 2 receptor (*IGF2R*), DNA methyltransferase 3b (*DNMT3b*), solute carrier family 2 (facilitated glucose transporter) member 8 (*SLC2A8*), prostaglandin G/H synthase-2 (*COX2*), mitochondrial phosphoenolpyruvate carboxykinase 2 (*PCK2*) and early growth response 1 (*EGR1*). Pools of three blastocysts from four sources (TCM24slicing, TCM24aspiration, cAMP30aspiration and *in vivo*) were submitted to poly(A)$^+$ mRNA extraction using the Dynabeads® mRNA DIRECT™ kit (Invitrogen, Carlsbad, CA, USA; Niemann *et al.*, 2010; Heinzmann *et al.*, 2011). Lysis of the cells was performed using 40 µl lysis binding buffer (100 mM Tris–HCl pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% lithium dodecyl sulphate, 5 mM dithiothreitol) and incubation for 10 min at room temperature, followed by addition of 1 pg/reaction rabbit globin mRNA (BRL, Gaithersburg, MD, USA) as an external standard (Cheng *et al.*, 1986) and 5 µl of prewashed Dynabeads® oligo(dT)$_{25}$. After a 15 min incubation at room temperature the poly(A)$^+$ RNAs bound to the beads were captured magnetically, washed in accordance with the manufacturer's instructions and resuspended in 11 µl of water. Finally, the poly(A)$^+$ mRNAs were released from the beads by heating at 68°C for 2.5 min and were used immediately for reverse transcription. Reverse transcription reactions were performed in a total volume of 20 µl that contained 2 µl of 10× reaction buffer (Invitrogen), 10 mM dNTPs solution (Amersham Biosciences, Piscataway, NJ, USA), 2.5 µM random hexamer primers (Applied Biosystems, Darmstadt, Germany), 20 U/µl ribonuclease inhibitor RNasin® (Applied Biosystems), 50 U/µl murine leukemia virus reverse transcriptase (MuLV, Applied Biosystems), the extracted RNA sample and water to 20 µl. The reverse transcription reaction was performed in a thermocycler (MJ Research PTC-200) and the program used was 10 min at 25°C, 60 min at 42°C, and 5 min at 99°C.

After reverse transcription, the cDNA obtained was submitted to semi-quantitative real-time qRT-PCR. The reactions were performed in 96-well optical reaction plates (Applied Biosystems, Carlsbad, California, USA; Niemann *et al.*, 2010; Heinzmann *et al.*, 2011). The final volume for the reaction in each well was 20 µl that contained 10 µl of 2× Power SYBR®Green PCR Master Mix (Applied Biosystems), 0.8 µl of 5 µM forward and reverse specific primers, 6.4 µl water and 2 µl cDNA. The details of the primers for the genes under evaluation are shown in Table 1. Standard curves of mRNA from pooled blastocyst cDNA were performed for each gene to assess the relative amount of the target gene in each sample. Values were normalized to the signal from the exogenous standard (rabbit globin) for each sample. The qRT-PCR reactions were carried out in an ABI 7500 Fast Real-Time System cycler (Applied Biosystems). The program used was 10 min at 95°C followed by 40 cycles of 15 s at 95°C, 60 s at 60°C, 15 s at 95°C and 1 min at 60°C followed by a slow heating cycle to obtain the dissociation curves. Sequence Detection Software 1.3.1 was used to perform quantification.

Analysis of DNA methylation

The methylation status of the sequences of the bovine testis satellite I (BTS) and *Bos taurus* alpha satellite

Table 1 List of primers used for qPCR for the specific selected genes

Gene identification	Primer sequences (forward and reverse, 5′→3′)	Location	Accession number	Product length (bp)	References
DNMT3B (DNA (cytosine-5)-methyltransferase 3B)	F: GGCCTCGGAAGTGTGTGA; R: TGATGTTGCCCTCGTGCTTA	DNMT3B_fw (1779–1796); DNMT3B_rev (1821–1840)	NM_181813.2	62	Heinzmann *et al.* (2011)
IGF2R (insulin-like growth factor 2 receptor)	F: CTACGACCTGACCGAGTG; R: TGACAGCCTCCCAGTTG	IGF2R_fw (4268–4285); IGF2R_rev (4346–4362)	NM_174352	95	Heinzmann *et al.* (2011)
SLC2A8 (GLUT8) (solute carrier family 2 facilitated glucose transporter, member 8)	F: GCATCTTCGGTGTCCTTTTCA; R: CAAAATGGGCTGTGATTTGCT	GLUT8_fw (1441–1461); GLUT8_rev (1501–1521)	AY208940	80	Heinzmann *et al.* (2011)
COX2 (prostaglandin G/H synthase-2 (PGHS-2))	F: ATCTACCCGCCTCATGTTCCT; R: GGATTAGCCTGCTTGTCTGGA	*COX2*_ fw (887–907); *COX2*_rev(1053–1073)	AF031698	187	El-Sayed *et al.* (2006)
PCK2 (phosphoenol pyruvate carboxykinase 2 (mitochondrial))	F: AACTTTGGGCGCTACCTTT; R: ACCAGTTGACGTGGAAGATG	PCK2_fw (1633–1652); PCK2_rev (1699–1718)	NM_001205594.1	85	
EGR1 (early growth response 1)	F: GTGCAATTGTGAGGGATGTG; R: TCCTTGTTTTGGCTCCCAAAG	EGR1_fw (2695–2714); EGR1_rev (2763–2782)	BC118328	87	
Globin (rabbit alpha-1 globin)	F: GCAGCCACGGTGGCGAGTAT; R: GTGGGACAGGAGCTTGAAAT	Globin_fw (241–260); Globin_rev (548–569)	X04751	256	Cheng *et al.* (1986)

I (BTαS) was evaluated to gain an overview of the global methylation status of the bovine genome in preimplantation embryos derived from the SPOM system. In BTS, 12 highly conserved CpG sites were evaluated in a 211-bp fragment. For the BTαS sequence, a fragment of 154 bp that contained nine CpG sites was analysed (Kang *et al.*, 2001). Genomic DNA from pools of five blastocysts each from every IVM protocol and *in vivo*-produced blastocysts was subjected to bisulphite conversion using the EZ DNA Methylation-Direct™ Kit (Zymo Research, Freiburg, Germany) as described previously by Diederich *et al.* (2012). Briefly, blastocysts were digested with 13 µl 1 M digestion buffer, 1 µl proteinase K, and 12 µl H_2O at 50°C for 20 min and subsequently centrifuged for 5 min at 10,000 *g* in a bench top centrifuge. Bisulphite conversion was performed using the CT Conversion Reagent provided by the kit at 98°C for 8 min followed by 64°C for 3.5 h in a thermal cycler. The converted DNA was washed and cleaned using the Zymo-Spin™ IC Column and submitted to PCR amplification using satellite specific primers in accordance with Kang *et al.* (2005; Table 2). Subsequently, the PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Mannheim, Germany). Fragments were ligated into the pGEM®-T-Easy Vector (Promega) and plasmids were transformed into *Escherichia coli* XL-10 Gold ultracompetent cells (Stratagene, Santa Clara, CA, USA). Successful ligation and transformation was confirmed by screening of the obtained colonies by PCR using primers SP6 and T7 (Table 2) and positive clones were submitted to sequencing analysis using the same primers. Sequences were analysed using the BiQ Analyzer program (MPI for Informatics, Saarland, Germany; Bock *et al.*, 2005). The sequences were compared with the specific genomic sequence from the bovine genome for each satellite. Clones with a conversion rate lower than 90% or with a high number of sequencing errors in the alignment were excluded from the analysis. The methylation status per treatment for each satellite was evaluated counting the total methylated CpG sites of the total number of analysed CpG.

Statistical analysis

Non-parametric one-way analysis of variance (ANOVA) was performed to evaluate gene expression and one-way ANOVA was implemented to analyse the number of cells, using JMP Statistical Discovery software™ version 8.0. (SAS Institute Inc., Cary, NC, USA, 1989–2007). Maturation, blastocyst, cleavage and methylation rates were evaluated using the Glimmix procedure from SAS/STAT® software version 9.2. The experimental groups were always run in parallel. In

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Table 2 Primer sequences used for analysis of repeat sequences

Repeat/binding site	GenBank accession no.	Primer sequences (5′→3′)	Fragment size (bp)	References
Bovine testis satellite I (BTS)	J00032.1	AATACCTCTAATTTCAAACT TTTGTGAATGTAGTTAATA	211	Kang *et al.* (2005)
Bos taurus alpha satellite I (BTαS)	AJ293510.1	GATGTTTTYGGGGAGAGAGG CCRATCCCCTCTTAATAAAAACC	154	Kang *et al.* (2005)
T7		ACTCACTATAGGGCGAATTG		
SP6		ATTTAGGTGACACTATAGAATACTC		

Table 3 Effect of oocyte retrieval method and maturation *in vitro* system on cleavage and blastocyst rates

Treatment	Total zygotes (*n*)	Total blastocysts day 8 (*n*)	2-cell embryos at day 2 (%)[c]	Blastocysts/presumptive zygotes (%)[c]
TCM24slicing[d]	1173	275	58.9 ± 1.5[b]	23.4 ± 1.2[b]
TCM24aspiration[e]	485	156	63.0 ± 2.3[a,b]	32.2 ± 2.1[a]
cAMP30aspiration[f]	476	111	70.1 ± 2.4[a]	23.3 ± 1.9[b]

[a,b]Mean values followed by different superscript letters in the same column differ statistically among maturation systems ($P < 0.05$). The column 'Blastocysts/presumptive zygote' includes all oocytes that were initially placed into culture.
[c]Data are expressed as mean ± standard error of the mean (SEM). [d]19 replicates. [e]10 replicates. [f]12 replicates.

Table 4 Effect of oocyte retrieval method and maturation *in vitro* system on oocyte meiosis resumption

Treatment	Total (*n*)	Metaphase II (*n*)	Maturation rates (%)[b]
TCM24slicing[b]	158	112	70.1 ± 3.6
TCM24aspiration[b]	84	67	79.8 ± 4.3
cAMP30aspiration[a]	98	73	74.5 ± 4.4

[a]Data are expressed as mean ± standard error of the mean (SEM). [b]Three replicates were performed.

some cases there was not enough biological material to run all groups in one experimental set-up. This factor explains the different number of replicates for the various experimental groups in Table 3 and this difference was taken into account for the statistical analysis. For all tests statistical significance was set at $P < 0.05$.

Results

In vitro oocyte maturation and embryo production

No statistical differences were found among the three groups of IVM oocytes with regard to success of oocyte maturation as determined by presence of the polar body. The highest maturation rate was obtained in the TCM24aspiration group (79.8 ± 4.3%), followed by the group supplemented with cAMP modulators (74.5 ± 4.4%); and the TCM24slicing system (70.1 ± 3.6%; mean ± SEM (standard error of the mean); Table 4).

Statistical differences were observed in cleavage rates among treatments. The cAMP30aspiration protocol showed the highest cleavage rate (70.1 ± 2.4%), a result that differed significantly ($P < 0.05$) from that of TCM24slicing (58.9 ± 1.5%), whereas TCM24aspiration (63.0 ± 2.3%) did not differ significantly from either treatment. When blastocyst rates per presumptive zygote were calculated, the TCM24aspiration group showed a significantly ($P < 0.05$) higher percentage (32.2 ± 2.1%) of development compared with TCM24slicing and cAMP30aspiration (23.4 ± 1.2% and 23.3 ± 1.9%, respectively; Table 3). Embryos from the three *in vitro* treatments and *in vivo*-produced embryos were compared using differential staining. In contrast with the blastocyst rates, no statistical differences were found among the different sources of blastocysts with regard to cell numbers (Table 5).

Gene expression analysis

The expression levels for *EGR1* were significantly lower ($P < 0.05$) in all groups of *in vitro*-produced embryos compared with the *in vivo*-produced blastocysts, whereas similar expression patterns were observed among the different *in vitro* protocols. The relative

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Table 5 Effect of oocyte retrieval method and maturation system on expanded blastocyst cell numbers

Treatment	Total (*n*)	Total cells	ICM	TE	ICM/Total cells (%)
TCM24slicing[a]	10	129 ± 5.0	29.4 ± 3.5	100 ± 5.0	23.3 ± 2.4
TCM24aspiration[a]	16	132.2 ± 4.0	37.4 ± 2.7	95 ± 4.0	27.7 ± 1.9
cAMP30aspiration[a]	7	129 ± 6.0	34 ± 4.2	95 ± 6.0	26.8 ± 2.9
In vivo	9	134.1 ± 5.3	–	–	–

Data are expressed as mean ± standard error of the mean (SEM). ICM, inner cell mass; TE, trophectoderm.
[a]Embryos from three different replicates were evaluated.

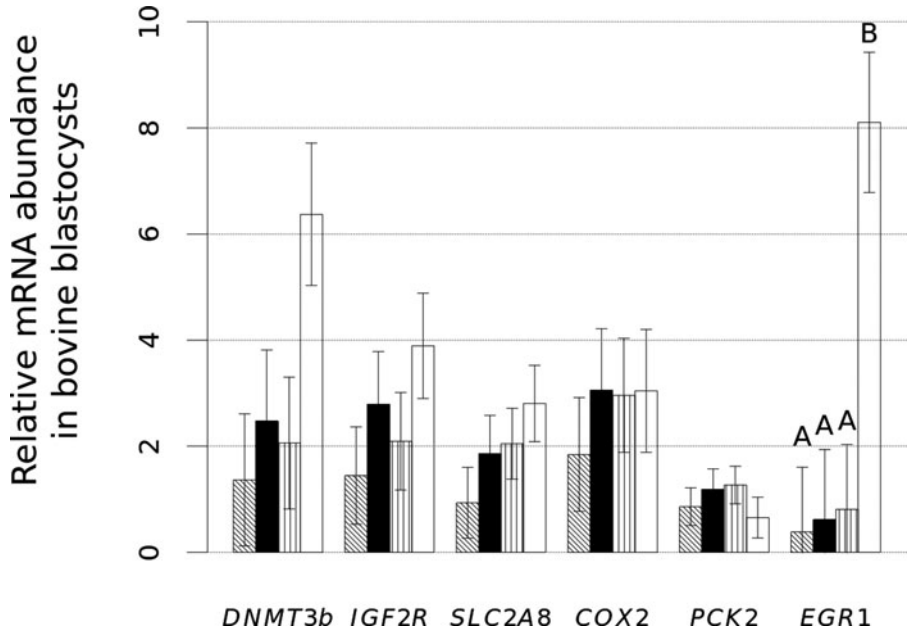

Figure 1 Effect of the oocyte *in vitro* maturation system on gene expression in day 8 expanded blastocysts compared with *in vivo*-produced embryos. Genes *DNMT3b*, *IGF2R*, *SLC2A8*, *COX2*, *PCK2*, *EGR1* were analyzed. TCM24slicing (shaded), TCM24aspiration (black), cAMP30aspiration (vertically lined), *in vivo* (white). Normalized transcription levels are shown as mean ± standard error of the mean (SEM). [A,B]Different superscripts indicate statistical differences among treatment groups ($P < 0.05$). Data were obtained from seven replicates.

abundance of transcripts for *DNMT3b*, *IGF2R* and *SLC2A8* in embryos produced *in vivo* was not statistically different compared with controls. Similar mRNA expression profiles were observed in blastocysts from the four different sources for *COX2* and *PCK2* genes (Fig. 1).

Analysis of DNA methylation

The number of CpGs analysed and the mean percentages of methylated CpGs for each protocol and satellite are shown in Table 6. Embryos produced either by the SPOM-like system or *in vivo* generally showed a lower DNA methylation than embryos produced in the two other *in vivo*-produced groups. No statistical differences were found between TCM24slicing (30.5 ± 2.2%) and TCM24aspiration for BTS (32.0 ± 2.3%) and BTαS (49.1 ± 2.3% and 52.7 ± 3.0%, respectively) while the DNA methylation in cAMP30aspiration and *in vivo*-produced embryos was significantly lower ($P < 0.05$) (Table 6). This finding indicates that *in vitro*-produced embryos derived without the extended maturation period and supplementation of cAMP modulators were hypermethylated at the CpG sites compared with embryos produced by the modified SPOM system (cAMP30aspiration) or *in vivo* embryos. Embryos produced *in vivo* and those from the cAMP30aspiration protocol showed significantly ($P < 0.05$) reduced methylation for the two satellites (16.4 ± 1.4% and 8.1 ± 1.3% for BTS, and 40.1 ± .2.1% and 32.2 ± 2.5% for BTαS, respectively), when compared with the other experimental groups (Table 6).

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Table 6 Number of CpGs evaluated and level of methylation in bovine expanded blastocysts

Satellite	Protocol	CpGs evaluated (*n*)	CpGs methylated (*n*)	Methylation level (%)
Bovine testis satellite I (BTS)	TCM24slicing	426	130	30.5 ± 2.2^{A}
	TCM24aspiration	394	126	32.0 ± 2.3^{A}
	cAMP30aspiration	404	33	8.1 ± 1.3^{B}
	In vivo	620	102	16.4 ± 1.4^{C}
Bos taurus alpha satellite I (BTαS)	TCM24 slicing	442	217	49.1 ± 2.3^{a}
	TCM24aspiration	271	143	52.7 ± 3.0^{a}
	cAMP30aspiration	357	115	$32.2 \pm 2.5^{b,c}$
	In vivo	518	208	40.1 ± 2.1^{c}

^{A,B,C,a,b,c}Rows with different superscript letters per satellite are significantly different ($P < 0.05$),
Data are expressed as mean ± standard error of the mean (SEM).

Discussion

The current work investigated for the first time the effects of oocyte retrieval and IVM systems using cAMP modulators on the mRNA expression profiles of bovine blastocysts for one imprinted and four non-imprinted genes, and unravelled its effects on global DNA methylation dynamics represented by two satellite DNA repeat sequences. Furthermore, embryos derived from the SPOM maturation system were characterized with regard to mRNA expression and satellite DNA methylation profile.

In vitro oocyte maturation is critical for the acquisition of full developmental competence. To induce a more physiological maturation process, biphasic IVM protocols have been proposed, by combination of an initial inhibitory culture period, using various cAMP modulators, followed by the conventional IVM phase that included hormone supplementation (Downs *et al.*, 1986; Hashimoto *et al.*, 2002). Recently, Albuz *et al.* (2010) proposed the SPOM system that is based on a two-step protocol and includes cAMP modulators before and during IVM.

Total blastocyst cell number and, specifically, the allocation of cells to the ICM or trophectoderm (TE) are considered to be important criteria for the assessment of blastocyst quality (Mori *et al.*, 2002; Ushijima *et al.*, 2008; Velazquez *et al.*, 2012). In contrast with that of Albuz *et al.* (2010), the present study did not reveal differences in total cell numbers and ICM/total cells ratio for cAMP modulators and extended maturation-derived blastocysts. The figures were similar to those of *in vivo*-produced blastocysts, which are considered to be the standard control.

No increase in blastocyst production was observed in the current study, a finding that is in contrast with the values reported by Albuz *et al.* (2010) after an extended (30 h) maturation period in the presence of cilostamide. However, an increased percentage was obtained when oocytes were aspirated from follicles rather than collected by slicing and when COCs were maintained in follicular fluid followed by a conventional maturation protocol (TCM24aspiration). These observations could suggest that oocyte maintenance in follicular fluid during and after recovery is beneficial to oocyte competence, presumably related to the maintenance of the cAMP levels in the COCs, modulated by the follicular fluid. It has been also stated that not only cAMP, but also other pathways such as AMP-activated protein kinase, cGMP, and Ca^{2+} play a major role in the maintenance of meiotic inhibition (Bilodeau-Goeseels, 2011).

It has been shown that the use of several assisted reproductive technologies (ARTs), which include IVM, can affect gene expression and epigenetic marks in oocytes and embryos (Wrenzycki *et al.*, 2005b, 2007; Niemann *et al.*, 2010). With the exception of *EGR1*, no significant differences were observed in the current study with regard to mRNA expression levels of the selected developmentally important genes *DNMT3b*, *IGF2R*, *SLC2A8*, *COX2*, *PCK2* between the different *in vitro* treatments and *in vivo*-derived blastocysts. This situation indicates that *in vitro* methods were compatible with physiological expression levels. However, significant down-regulation of *EGR1* mRNA levels in all *in vitro*-produced blastocysts compared with their *in vivo* counterparts was discovered. *EGR1* has previously been shown to be closely related to oocyte developmental competence; the expression level correlated with successful follicular maturation (Robert *et al.*, 2001). The present study showed that expression in embryos produced *in vivo* was eight-fold higher than for *in vitro*-produced embryos, which indicates that *EGR1* mRNA expression could serve as a marker of embryo quality.

Two types of DNA methyltransferase (DNMT) activity have been described in vertebrates, related to *de novo* and maintenance of methylation levels. The current results, as previously reported in mice by Horii *et al.* (2011), showed slightly lower transcript

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levels for the *de novo* DNA methylation enzyme *DNMT3b* in the *in vitro* - produced blastocysts in comparison with their *in vivo*-produced counterparts. A physiological expression level of *COX2* has been related to successful pregnancy establishment and calf delivery, probably due to its crucial function in differentiation, proliferation and anti-apoptosis (Smith *et al.*, 2000; El-Sayed *et al.*, 2006; Pakrasi & Jain, 2008). In the current study, it was observed that *COX2* expression profiles were closer to that of *in vivo*-produced embryos in the IVM protocols using aspiration rather than retrieval method. The imprinted *IGF2R* gene is critically involved in the control of fetal growth by acting as a scavenger receptor for the imprinted fetal mitogen insulin-like growth factor (IGF; Suteevun-Phermthai *et al.*, 2009). Moore *et al.* (2007) reported a higher level mRNA expression pattern for *IGF2R* in embryos produced *in vivo* compared with *in vitro* embryos. The relative abundance of solute carrier family 2 (facilitated glucose transporter) member 8 transcripts was decreased in all *in vitro*-produced blastocysts. It has been reported that murine blastocysts that lack *SLC2A8* expression showed increased apoptosis levels, a finding that suggested a critical role in embryonic development and survival (Pinto *et al.*, 2002). In contrast with the other genes evaluated in the present study, the mRNA level of the *PCK2* gene, which is involved in glucose metabolism, tended to be up-regulated in embryos produced *in vitro* over their *in vivo*-produced counterparts.

Epigenetic processes such as DNA methylation and histone modifications have been shown to be heavily affected by ARTs (Niemann *et al.*, 2010). It has been stated that alterations in methylation patterns can lead to severe imprinting errors (Katari *et al.*, 2009; Wilkins-Haug, 2009), such as Beckwith–Wiedeman syndrome (Paoloni-Giacobino, 2007) in humans or the large offspring syndrome in cattle (Farin *et al.*, 2006). In oocytes and early embryos, the effects of *in vitro* procedures are also reflected in distinct mRNA expression profiles (Wrenzycki *et al.*, 2005a; Farin *et al.*, 2010; Heinzmann *et al.*, 2011; Diederich *et al.*, 2012). Accordingly, many studies have argued that ARTs can induce aberrant DNA methylation patterns in preimplantation embryos (Wrenzycki *et al.*, 2005a; Paoloni-Giacobino, 2007; Sawai *et al.*, 2011). In the current study, two satellite regions, the bovine testis satellite I (BTS) and the *Bos taurus* alpha satellite I (BTαS), were studied to evaluate global methylation profiles that are representative of the bovine genome (Kang *et al.*, 2005). The current results revealed significant hypermethylation for both satellite sequences in embryos derived from treatments that lack cAMP modulators in comparison with their *in vivo*-derived counterparts. Increased DNA methylation levels of BTS have been reported previously for embryos produced *in vitro* (Sawai *et al.*, 2011; Yamanaka *et al.*, 2011). In contrast, in the current study, similar profiles of DNA methylation for both satellite regions were observed in blastocysts produced *in vivo* or *in vitro* with cAMP modulators. These results could suggest that the embryos produced using the cAMP modulators forskolin, IBMX and cilostamide along with an extended IVM are similar to their *in vivo*-produced counterparts that usually serve as the physiological standard. This situation indicates that the global methylation profile of blastocysts can be influenced significantly by *in vitro* maturation conditions.

In conclusion, the present results suggest that epigenetic processes during embryo development are influenced by IVM protocols and oocyte retrieval system and that the addition of cAMP modulators in conjunction with an extended IVM may have a positive influence on embryo quality, probably associated with sustained levels of cAMP in oocytes and the surrounding cells. This situation is important to gain a better understanding of the SPOM system, which has been shown to be compatible with high blastocyst rates. The current findings highlight the need to continue the search for media and protocols that provide a physiological environment for the oocyte under *in vitro* conditions. In this context, the bovine species can serve as a useful model for human IVM due to the high degree of similarity in human and bovine embryo development.

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Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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