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Effect of bovine oviductal fluid on development and quality of bovine embryos produced *in vitro*

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Abstract. To evaluate the effect of bovine oviductal fluid (OF) supplementation during *in vitro* culture of bovine embryos on their development and quality, *in vitro*-produced zygotes were cultured in synthetic oviductal fluid (SOF; negative control; C^-) supplemented with OF or 5% fetal calf serum (positive control; C^+). Embryo development was recorded on Days 7–9 after insemination and blastocyst quality was assessed through cryotolerance, differential cell counting of the inner cell mass and trophectoderm, and gene expression. OF was added to the culture medium at concentrations ranging from 0.625% to 25%. The higher OF concentrations (5%, 10% and 25%) had a detrimental effect on embryo development. Lower OF concentrations (1.25% and 0.625%) supported embryo development until Day 9 (27.5%) and produced higher-quality blastocysts, as reflected by their cryotolerance (53.6% and 57.7% survival at 72 h, respectively, vs 25.9% in C^+) and total cell number (mean (± s.e.m.) 165.1 ± 4.7 and 156.2 ± 4.2, respectively, vs 127.7 ± 4.9 in C^- and 143.1 ± 4.9 in C^+). Consistent with these data, upregulation of the water channel aquaporin 3 (*AQP3*) mRNA was observed in blastocysts supplemented with 1.25% OF compared with C^- and C^+. Serum supplementation resulted in a reduction in the expression of glucose and lipid metabolism-related genes and down-regulation of the epigenetic-related genes DNA methyltransferase 3A (*DNMT3A*) and insulin-like growth factor 2 receptor (*IGF2R*). In conclusion, *in vitro* culture with low concentrations of OF has a positive effect on the development and quality of bovine embryos.

Additional keywords: embryo culture, gene expression.

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Introduction

According to the annual statistics collated by the International Embryo Transfer Society, approximately 390 000 *in vitro*-produced bovine embryos were transferred worldwide in 2013 (Perry 2014). Of these embryos, >90% were transferred fresh, reflecting the fact that *in vitro*-produced (IVP) embryos have lower cryotolerance than those derived *in vivo*.

It has been well demonstrated that the post-fertilisation culture environment determines embryo quality and different strategies have been developed to improve culture conditions. Biological supplements, such as serum or bovine serum albumin (BSA), are commonly used in culture media to improve the suboptimal conditions. However, a paradoxical effect is evident, with serum increasing the number of blastocysts developing but reducing their quality (Rizos *et al.* 2003) and being associated with long-term effects, such as large offspring syndrome (Lazzari *et al.* 2002).

When IVP bovine zygotes are cultured *in vivo*, for example in the ewe oviduct, blastocysts can be produced of a quality similar to those derived *in vivo* in terms of cryotolerance and gene expression (Rizos *et al.* 2008). Furthermore, the significant effect of culture conditions, *in vitro* or *in vivo* in the homologous bovine oviduct, on the transcriptome of the embryos in relation to embryonic genome activation has been well demonstrated (Gad *et al.* 2012).

The oviduct is a dynamic organ in which fertilisation and early embryo development occur. Studying the oviductal environment is crucial to further our understanding of the underlying regulatory mechanisms controlling embryo development (Aviles *et al.* 2015). The advantages of the oviductal environment have been demonstrated in different models, through the substances isolated and the effect of environment *per se*. Many physiological aspects have been clarified; however, many others remain unknown (Hunter 2012).

The oviductal environment is primarily composed of secretions of oviduct epithelial cells and plasma components (Leese *et al.* 2001). The use of bovine oviduct epithelial cells (BOEC) was a major breakthrough in the *in vitro* culture (IVC) of bovine embryos (Gandolfi and Moor 1987), with positive effects on embryo development and quality (Cordova *et al.* 2014). Recently, Schmaltz-Panneau *et al.* (2014) demonstrated that oviductal cells modify their transcription in the presence of developing embryos, concluding that BOEC coculture may be a suitable model for studying the complex embryo–maternal cross-talk in bovine species.

The composition of oviductal fluid (OF) is very complex, containing simple and complex carbohydrates, ions, lipids, phospholipids and proteins (Avilés *et al.* 2010). Some of these components are metabolic substrates, such as lactate, pyruvate, amino acids and glucose, the concentrations of which differ from those present in the uterine fluid and serum (Leese 1988; Hugentobler *et al.* 2007; Leese *et al.* 2008). Individual oviductal secretions have an effect on oocyte and sperm function (Killian 2011; Mondéjar *et al.* 2013) and the possible role of oviductins, osteopontin, glycodelins and lactoferrin on gamete interaction has been described (Coy and Yanagimachi 2015; Ghersevich *et al.* 2015). Coy *et al.* (2008, 2012) demonstrated that oviduct-specific glycoprotein (OVGP) 1 and heparin-like glycosaminoglycans (GAGs) from the OF of sows and cows participate in the functional modification of the zona pellucida (ZP), affecting sperm–oocyte interaction and contributing to the control of polyspermy. In addition, a significant increase in cleavage rate and blastocyst yield has been reported from OF-treated porcine oocytes compared with untreated oocytes, suggesting OF protection of the embryo against adverse effects on mitochondrial (mt) DNA transcription or replication and apoptosis (Lloyd *et al.* 2009). In contrast, in cattle, oocyte exposure to OF before fertilisation had no effect on embryo development and morphological characteristics of the resulting blastocysts (Cebrian-Serrano *et al.* 2013). However, differences appear in the transcriptome of the embryos produced from oocytes previouslytreated with OF compared with controls (Cebrian-Serrano *et al.* 2013).

It is clear then that there is a need to improve IVC conditions and adapt strategies based on conditions *in vivo* to enhance embryo development. In addition, *in vitro* techniques permit the study of physiological processes under specific conditions, which are difficult to assess *in vivo*. To our knowledge, no evidence of any developmental consequences of bovine OF supplementation during post-fertilisation embryo culture *in vitro* exists. Thus, the aim of the present study was to evaluate the effect of OF supplementation during IVC on bovine embryo development and embryo quality.

Materials and methods

Unless stated otherwise, all chemicals were purchased from Sigma Aldrich Química (Madrid, Spain).

Oocyte collection and IVM

Immature cumulus–oocyte complexes (COCs) were obtained by aspirating follicles (2–8 mm diameter) from the ovaries of mature heifers and cows collected at slaughter. COCs were matured for 24 h in 500 µL maturation medium (TCM 199–M4530 supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng mL^{-1} epidermal growth factor (E4127)) in a four-well dish, in groups of 50 COCs per well at 38.5°C under an atmosphere of 5% CO_2 in air, with maximum humidity.

Sperm preparation and IVF

Frozen semen straws (0.25 mL) from a single Asturian Valley bull were thawed at 37°C in a water bath for 1 min and centrifuged for 5 min at 280*g* through a gradient of 1 mL of 40% Bovipure (Nidacon Laboratories AB, Göthenborg, Sweden) and 1 mL of 80% Bovipure according to the manufacturer's instructions. The sperm pellet was isolated and washed in 3 mL Boviwash (Nidacon Laboratories AB) by centrifugation at 280*g* for 5 min. The pellet was resuspended in the remaining 300 µL Boviwash. Sperm concentration was determined and adjusted to a final concentration of 1×10^6 sperm mL^{-1} for IVF. Gametes were coincubated for 18–22 h in 500 µL fertilisation medium (Tyrode's medium with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate and 6 mg mL^{-1} fatty acid-free BSA supplemented with 10 mg mL^{-1} heparin sodium salt; Calbiochem, San Diego, CA, USA) in a four-well dish, in groups of 50 COCs per well under an atmosphere of 5% CO_2 in air, with maximum humidity at 38.5°C.

Collection of OF

OF samples (NaturArts BOF-EL) were purchased from Embryocloud, Murcia, Spain (http://www.embryocloud.com). Specifically, NaturArts BOF-EL was used in the experiments. As described on the company website, oviducts ipsilateral to the corpus luteum were collected from ovaries of slaughtered heifers at the early luteal phase of the oestrous cycle (Ireland *et al.* 1980). Oviducts were transported on ice, washed twice in cold phosphate-buffered saline (PBS) and then transferred to a stainless steel tray on a bed of ice and straightened by trimming accessory ligaments. OF was collected according to the protocol of Carrasco *et al.* (2008). Each oviduct was squeezed gently from the uterotubal junction towards the ampulla and the contents were collected by aspiration from the infundibulum using a 200-µL automatic pipette. A mean volume of approximately 10–30 µL per oviduct was collected and a pool of OF from five oviducts was centrifuged at 7000*g* for 10 min at 4°C to remove cellular debris. The supernatant was aliquoted and stored at −80°C until use.

IVC of presumptive zygotes

At approximately 20 h post insemination (h.p.i.), presumptive zygotes were denuded of cumulus cells by vortexing for 3 min and then cultured in groups of 25 in 25-µL droplets of culture medium (synthetic oviductal fluid (SOF); negative control (C^-); Holm *et al.* 1999) with 4.2 mM sodium lactate (L4263), 0.73 mM sodium pyruvate (P4562), 30 µL mL^{-1} BME amino acids (B6766), 10 µL mL^{-1} minimum essential medium (MEM) amino acids (M7145) and 1 µg mL^{-1} phenol red (P0290) under mineral oil at 38.5°C under an atmosphere of 5% CO_2, 5% O_2

and 90% N_2. Depending on the experiment (see below), SOF was supplemented with 5% FCS (positive control; C^+) or with different concentrations of bovine OF.

Assessment of embryo development and quality

Embryo development

Cleavage rate was recorded at Day 2 (48 h.p.i.) and cumulative blastocyst yield was recorded at Days 7, 8, and 9 after insemination.

Embryo quality: blastocyst vitrification

The ability of the blastocyst to withstand cryopreservation was used as a quality indicator. Day 7 and 8 blastocysts and expanding blastocysts were vitrified in holding medium (HM; TCM 199 supplemented with 20% (v/v) FCS) and cryoprotectants, following the procedures of Rizos *et al.* (2002*b*), in a two-step protocol using the Cryoloop device (Hampton Research, Aliso Viejo, CA, USA). The first step consisted of HM with 7.5% ethylene glycol and 7.5% dimethyl sulfoxide (DMSO), whereas the second step (final solution) was consisted of HM with 16.5% ethylene glycol, 16.5% DMSO and 0.5 M sucrose. The blastocysts were warmed in two steps in HM with 0.25 and 0.15 M sucrose and then cultured in 25-μL droplets of SOF with 5% FCS. Survival was defined as re-expansion of the blastocoel and its maintenance for 24, 48 and 72 h.

Differential staining of blastocysts

Differential staining of inner cell mass (ICM) and trophectoderm (TE) cells was performed according to the procedures of Thouas *et al.* (2001). Day 8 expanded blastocysts were permeabilised and TE cells were stained by incubation in 500 μL PBS with 0.2% Triton X-100 and 100 μg mL^{-1} propidium iodide (PI) in the dark for 60 s at 37°C. For fixation and ICM staining, blastocysts were transferred to 500 μL absolute ethanol with 25 μg mL^{-1} bisbenzimide (Hoechst 33342) for 3 min. Fixed and stained blastocysts were transferred to glycerol and mounted on glass microscope slides, gently flattened with a coverslip and visualised for cell counting under a fluorescent microscope.

Gene expression analysis

Gene expression analysis was performed using four groups of 10 Day 8 expanding blastocysts per treatment group, namely C^- (SOF), OF (SOF supplemented with 1.25% oviductal fluid; selected based on developmental and cryotolerance data) and C^+ (SOF supplemented with 5% FCS). Poly(A) RNA was extracted using the Dynabeads mRNA Direct Extraction Kit (Dynal Biotech, Oslo, Norway) with minor modifications (Bermejo-Álvarez *et al.* 2008). Immediately after extraction, the reverse transcription (RT) reaction was performed according to the manufacturer's instructions (Bioline, Ecogen, Madrid, Spain) using poly(T) primer, random primers and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 50 units reverse transcriptase. Samples were incubated at 42°C for 60 min to allow the RT of RNA, followed by incubation at 70°C for 10 min to denature the RT enzyme. All mRNA transcripts were quantified using quantitative polymerase chain reaction (qPCR) with two replicates for all genes of interest. The qPCR was performed by adding a 2-μL aliquot of each cDNA sample to the PCR mix containing the specific primers to amplify transcripts for histone H2A histone family, member Z (*H2AFZ*), solute carrier family 2 (facilitated glucose transporter) member 1 (*SCL2A1*; previously known as *GLUT1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), lactate dehydrogenase A (*LDHA*), low-density lipoprotein receptor (*LDLR*), cytochrome P450 family 51 (*CYP51*), fatty acid desaturase 1 (*FADS1*), DNA methyltransferase 3A (*DNMT3A*), insulin-like growth factor 2 receptor (*IGF2R*), ubiquitin-conjugating enzyme E2A (*UBE2A*) and aquaporin 3 (*AQP3*). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are given in Table 1. For quantification, qPCR was performed as described previously (Bermejo-Alvarez *et al.* 2010*a*). The PCR conditions were tested to achieve efficiencies close to 1. The comparative cycle threshold (CT) method was used to quantify expression levels. Values were normalised against the endogenous control *H2AFZ*. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to a doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the *H2AFZ* CT value for each sample from each gene CT value of the sample. The calculation of ΔΔCT involved using the highest treatment ΔCT value (i.e. the treatment with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$.

Experimental design

The developmental capacity and quality of bovine zygotes cultured *in vitro* with SOF without FCS supplemented with different concentrations of OF was assessed. In a preliminary experiment, embryo culture with concentrations of 5%, 10% and 25% OF was found to be detrimental for embryo development, with blastocysts yields of 11%, 10% and 1% on Day 9, respectively. Therefore, these concentrations were not used in subsequent experiments. Thus, at 20 h.p.i., presumptive zygotes were cultured in SOF with (C^+; $n = 872$) or without (C^-; $n = 927$) 5% FCS or in SOF without FCS supplemented with 2.5% ($n = 855$), 1.25% ($n = 964$) or 0.625% OF ($n = 1011$). Overall cleavage rate was recorded at 48 h.p.i. and blastocyst development was recorded on Days 7, 8 and 9 after insemination. To assess blastocyst quality, a representative number of Day 7–8 blastocysts from each group were either: (1) vitrified–warmed ($n = 78$–91 per group), with survival rate recorded every 24 h up to 72 h after warming, and fixed for differential cell counts ($n = 40$ per group); or (2) frozen in liquid nitrogen (LN_2; $n = 40$ per group) in groups of 10 and stored at −80°C for gene expression analysis. Eleven replicates were performed.

Table 1. Primers used for reverse transcription–quantitative polymerase chain reaction

H2AFZ, H2A histone family, member Z; *SCL2A1*, solute carrier family 2 (facilitated glucose transporter) member 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *LDHA*, lactate dehydrogenase A; *LDLR*, low-density lipoprotein receptor; *CYP51*, cytochrome P450 family 51; *FADS1*, fatty acid desaturase 1; *DNMT3A*, DNA methyltransferase 3A; *IGF2R*, insulin-like growth factor 2 receptor; *UBE2A*, ubiquitin-conjugating enzyme E2A; *AQP3*, aquaporin 3

Gene	Primer sequence (5′–3′)	Fragment size (bp)	GenBank Accession no.
H2A.Z	Forward: AGGACGACTAGCCATGGACGTGTG Reverse: CCACCACCAGCAATTGTAGCCTTG	212	NM_016750
SCL2A1	Forward: CTGATCCTGGGTCGCTTCAT Reverse: ACGTACATGGGCACAAAACCA	68	NM_174602.2
GAPDH	Forward: ACCCAGAAGACTGTGGATGG Reverse: ATGCCTGCTTCACCACCTTC	247	BC102589
LDHA	Forward: TTCTTAAGGAAGAACATGTC Reverse: TTCACGTTACGCTGGACCAA	310	NM_174099.2
LDLR	Forward: CAAAACCCCGATCATCCCCA Reverse: TCGACCCTGAACTGGAAACG	194	NM_001166530
CYP51	Forward: GGCCCAADDTDATTTCCATTTC Reverse: CTCCCAAGAAACCCTGCACTGG	167	BC149346.1
FADS1	Forward: GCTGCCCAATCTGAGCAAAGC Reverse: TCCTGTCATGGTGTGGGTCCTG	208	Bt.4959.1.A1_at
DNMT3A	Forward: CTGGTGCTGAAGGACTTGGGC Reverse: CAGAAGAAGGGGCGGTCATC	318	AY271299
IGF2R	Forward: GCTGCGGTGTGCCAAGTGAAAAAG Reverse: AGCCCCTCTGCCGTTGTTACCT	201	NM_174352.2
UBE2A	Forward: GGGCTCCGTCTGAGAACAACATC Reverse: CATACTCCCGCTTGTTCTCCTGG	336	XM_864331
AQP3	Forward: CGGTGGTTTCCTCACCATCA Reverse: CAGAGGGGTAGGTGGCAAAG	299	NM_001079794.1

Table 2. Effect of *in vitro* embryo culture with low concentrations of bovine oviductal fluid (OF) on development *in vitro*

Within columns, values with different superscript letters differ significantly ($P < 0.05$). No. PZ, total number of presumptive zygotes placed in culture; C^-, negative control, cultured in the presence of synthetic oviductal fluid (SOF); C^+, positive control, cultured in the presence of SOF + 5% fetal calf serum

	No. PZ	Cleavage		Blastocyst yield					
		n	Mean ± s.e.m. (%)	Day 7		Day 8		Day 9	
				n	Mean ± s.e.m. (%)	*n*	Mean ± s.e.m. (%)	*n*	Mean ± s.e.m. (%)
C^+	872	747	86.7 ± 1.5	198	22.9 ± 1.2^{a}	226	26.6 ± 1.2^{a}	236	27.7 ± 1.9^{a}
C^-	927	823	88.8 ± 1.2	96	12.0 ± 1.7^{c}	164	18.3 ± 1.2^{c}	195	21.5 ± 1.4^{b}
OF									
2.5%	855	748	87.3 ± 1.1	110	13.9 ± 1.4^{bc}	180	21.2 ± 1.4^{bc}	192	22.7 ± 1.5^{b}
1.25%	964	855	89.1 ± 1.5	163	17.4 ± 1.5^{b}	236	24.4 ± 1.7^{ab}	266	27.5 ± 1.7^{a}
0.625%	1011	898	89.1 ± 1.3	160	16.0 ± 1.2^{b}	230	22.3 ± 1.0^{b}	279	27.5 ± 1.2^{a}

Statistical analysis

Data relating to cleavage rate, blastocyst yield and survival after vitrification–warming and relative mRNA abundance were analysed using one-way analysis of variance (ANOVA), with two-sided $P < 0.05$ considered significant. Embryo cell number (ICM, TE and the ICM : TE ratio) was analysed by multiple pairwise comparisons using *t*-tests. All analyses were performed using SigmaStat (Jandel Scientific, San Rafael, CA, USA).

Results

As noted above, supplementation of the culture medium with 5%, 10% and 25% OF was detrimental for embryo development, with blastocyst yields of 11%, 10% and 1% on Day 9, respectively, all of which were significantly lower than in the control groups. Therefore, these concentrations of OF were not used in subsequent experiments.

No differences were observed in cleavage rate between both control groups and the OF groups (range 86.7%–89.1%). Blastocyst yield on Day 7 was higher for the C^+ group compared with all other groups, whereas that in the 1.25% and 0.625% OF groups was significantly higher than in the C^- group (Table 2). On Day 8, no differences were observed in blastocyst yield between the C^+ and 1.25% OF groups. However, culture with 1.25% or 0.625% OF resulted in more blastocysts than in the C^- group (Table 2). On Day 9, the blastocyst yield for the 1.25%

Table 3. Survival rates after vitrification and warming of Day 7–8 blastocysts cultured with low concentrations of bovine oviductal fluid (OF)
Within columns, values with different superscript letters differ significantly ($P < 0.05$). C^-, negative control, cultured in the presence of synthetic oviductal fluid (SOF); C^+, positive control, cultured in the presence of SOF + 5% fetal calf serum

	No. blastocysts vitrified	Blastocyst survival after vitrification–warming							
		4 h		24 h		48 h		72 h	
		n	Mean ± s.e.m. (%)	*n*	Mean ± s.e.m. (%)	*n*	Mean ± s.e.m. (%)	*n*	Mean ± s.e.m. (%)
C^+	91	75	84.6 ± 2.6^{a}	35	41.3 ± 4.5^{a}	26	30.3 ± 2.5^{a}	22	25.9 ± 2.3^{a}
C^-	84	80	95.8 ± 1.8^{c}	63	74.1 ± 4.1^{c}	51	59.3 ± 3.2^{b}	48	56.1 ± 2.9^{b}
OF									
2.5%	85	75	88.4 ± 3.7^{abc}	49	58.7 ± 4.9^{b}	32	39.2 ± 4.9^{a}	30	36.1 ± 4.7^{a}
1.25%	78	68	86.9 ± 2.0^{ab}	50	63.1 ± 3.8^{bc}	47	61.3 ± 2.1^{b}	41	53.6 ± 1.7^{b}
0.625%	82	79	95.5 ± 2.4^{c}	62	71.7 ± 6.4^{c}	51	61.6 ± 4.1^{b}	48	57.7 ± 3.8^{b}

Table 4. Effect of *in vitro* embryo culture with low concentrations of bovine oviductal fluid (OF) on blastocyst cell number
Data are the mean ± s.e.m. Within columns, values with different superscript letters differ significantly ($P < 0.05$). C^-, negative control, cultured in the presence of synthetic oviductal fluid (SOF); C^+, positive control, cultured in the presence of SOF + 5% fetal calf serum; ICM, inner cell mass; TE, trophectoderm

	No. blastocysts processed	Total nuclei	No. ICM nuclei	ICM (%)	No. TE nuclei	TE (%)	Ratio ICM : TE
C^+	40	143.1 ± 4.9^{a}	36.0 ± 1.4^{a}	25.6 ± 0.9	107.1 ± 4.3^{a}	74.4 ± 0.9	0.35 ± 0.02
C^-	40	127.7 ± 4.9^{b}	31.1 ± 1.4^{b}	24.9 ± 1.1	96.6 ± 4.6^{a}	75.1 ± 1.1	0.34 ± 0.02
OF							
2.5%	40	150.2 ± 7.1^{ac}	37.1 ± 1.6^{a}	25.8 ± 1.1	113.1 ± 6.8^{ab}	74.2 ± 1.1	0.36 ± 0.02
1.25%	40	165.1 ± 4.7^{d}	38.0 ± 1.5^{a}	23.5 ± 0.9	127.1 ± 4.5^{c}	76.5 ± 0.9	0.31 ± 0.02
0.625%	40	156.2 ± 4.2^{cd}	36.2 ± 1.3^{a}	23.4 ± 0.8	119.9 ± 3.7^{bc}	76.6 ± 0.8	0.31 ± 0.01

and 0.625% OF groups was similar to that in the C^+ group, but significantly higher than in the C^- and 2.5% OF groups, as indicated in Table 2.

The survival rate of vitrified–warmed blastocysts produced in the C^- and 0.625% OF groups was significantly higher than in the 2.5% OF and C^+ groups at 24 h (Table 3), but was not different from that in the 1.25% OF group. At 48 and 72 h, the survival rate of blastocysts cultured in 1.25% and 0.625% OF and in the C^- group was higher than that of blastocysts in the 2.5% OF and C^+ groups (Table 3).

Total cell number in the C^- group was significantly lower than in all other groups (Table 4), whereas for total cell number in the 1.25% and 0.625% OF groups was significantly higher than in the C^+ and C^- groups (Table 4). The number of TE cells in embryos cultured with 1.25% and 0.625% OF was significantly higher than in the C^+ and C^- groups (Table 4). Blastocysts from the C^- group had a significantly lower number of cells in the ICM compared with all other groups (Table 4).

The expression of three genes related to glucose metabolism (*SCL2A1*, *GAPDH* and *LDHA*), three genes related to lipid metabolism (*LDLR*, *CYP51* and *FADS1*), three genes related to epigenetics (*DNMT3A*, *IGF2R* and *UBE2A*) and *AQP3* was determined in blastocysts cultured in C^+, C^- or C^- + 1.25% OF (Fig. 1). Overall, the C^- and OF groups showed no differences in the expression levels of the 10 genes analysed, except for *AQP3* expression, which was significantly upregulated ($P < 0.05$) in the OF compared with the other two groups. Expression of *SCL2A1* was significantly upregulated ($P < 0.05$) in both groups cultured without serum, whereas *GAPDH* expression levels were significantly higher ($P < 0.05$) in the C^- than C^+ group, with no differences observed for *LDHA*. Of the genes related to lipid metabolism, *LDLR* was significantly upregulated ($P < 0.05$) in the OF compared with C^+ group, and the expression of *CYP51* and *FADS1* was significantly higher ($P < 0.05$) in both groups cultured without serum (C^- and OF). Finally, both *DNMT3A* and *IGF2R* were significantly upregulated ($P < 0.05$) in both groups cultured without serum compared with the C^+ group, whereas the expression of *UBE2A* did not differ among groups.

Discussion

Over the past two decades, a considerable amount of research has focused on the quality improvement of IVP blastocysts, which lags behind that of their *in vivo* counterparts. Studies using the 'oviduct' as an intermediate host for embryo culture highlighted that the fundamental part of the process responsible for suboptimal embryo quality is the period after fertilisation (Rizos *et al.* 2010). Moreover, any modification of the IVC conditions can have a significant effect on the normality of the embryo. Thus, in an attempt to mimic conditions *in vivo*, we studied the effect of the presence of bovine OF during *in vitro* embryo culture on the developmental competence and quality of the blastocysts produced. Our findings indicate a positive effect of OF supplementation at low concentrations (1.25% and 0.625%) as a replacement for serum during embryo culture, with

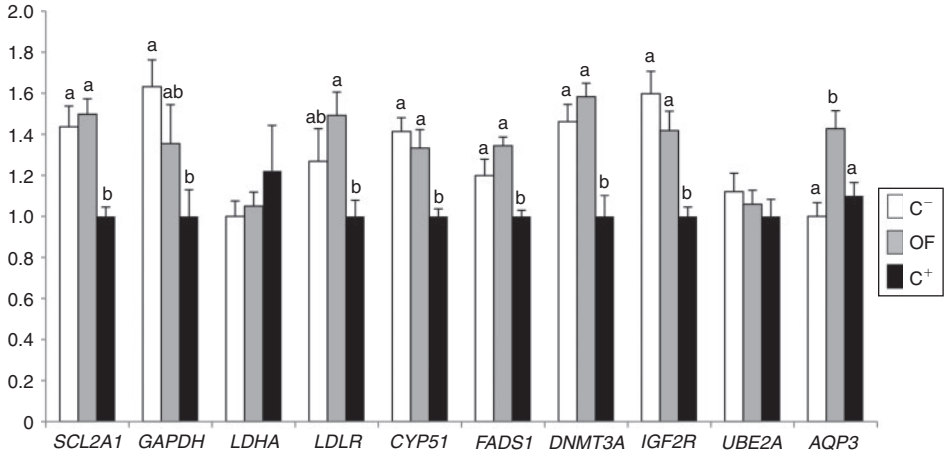

Fig. 1. Relative mRNA abundance (normalised against that of the housekeeping gene H2A histone family, member Z (*H2AFZ*)) of genes related with glucose metabolism (*SCL2A1*, solute carrier family 2 (facilitated glucose transporter) member 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *LDHA*, lactate dehydrogenase A), lipid metabolism (*LDLR*, low-density lipoprotein receptor; *CYP51*, cytochrome P450 family 51; *FADS1*, fatty acid desaturase 1), epigenetics (*DNMT3A*, DNA methyltransferase 3A; *IGF2R*, insulin-like growth factor 2 receptor; *UBE2A*, ubiquitin-conjugating enzyme E2A) and water channels (*AQP3*, aquaporin 3) in bovine *in vitro* blastocysts (Day 7) cultured with a low concentration (1.25%) of bovine oviductal fluid (OF). C^-, negative control, cultured in the presence of synthetic oviductal fluid (SOF); C^+, positive control, cultured in the presence of SOF + 5% fetal calf serum. Data are the mean ± s.e.m. Columns with different letters differ significantly within each gene group ($P < 0.05$).

an increase in developmental rates and better quality embryos in terms of survival after vitrification and warming, cell number and gene expression patterns.

The oviduct is the anatomical part of the reproductive tract where fertilisation and early embryo development take place, and it provides an optimal environment for gametes and early embryos to develop (Hunter 2012). Critical events, such as sperm storage, capacitation, sperm release and transport, final oocyte maturation, fertilisation, early embryo development and embryo transport to the uterus, occur in the oviduct. These sequential events require a dynamic and synchronised support system by the oviduct in order to occur successfully (Aguilar and Reyley 2005). In the oviductal milieu, molecular mechanisms and pathways represent the first signal exchange between the maternal environment and the embryo (Besenfelder *et al.* 2012). This milieu is comprised of the secretions of oviduct epithelial cells and from blood plasma, and is represented in the OF (Ellington 1991).

Most media used for *in vitro* embryo culture (e.g. SOF; Holm *et al.* 1999) have been designed based on the ion, energy substrate and amino acid composition of OF (Leese *et al.* 2008). However, the optimisation of an IVC environment that contains all the substances present in the oviduct regions may resolve part of the major limitations present during embryo culture, allowing the production of embryos at comparable rates and of comparable quality to those that occur *in vivo*. Thus, by mimicking *in vivo* conditions using animal models, we could improve ARTs applied to both domestic species and humans.

Exposure of mature pig and cow oocytes to pure OF for a short period of time (30 min) before fertilisation has been used to improve zona hardening, reduce polyspermy and improve embryo quality (Coy *et al.* 2008; Cebrian-Serrano *et al.* 2013). To our knowledge, the present study is the first to assess the effect of OF during the embryo culture period in a medium without serum on embryo development and embryo quality. When the medium was supplemented with a high concentration of OF, a negative effect on blastocyst development was evident (7% at Day 7). This may reflect the continuous renewal of OF in the oviduct *in vivo* as the reproductive tract modifies its activity in order to provide the optimal environment for the development of the embryo (Buhi 2002), whereas *in vitro* it degraded with a negative effect on the embryo. Gradual decreases in OF concentration (2.5%, 1.25% and 0.625%) diminish the detrimental effects and improve embryo development rates.

Consistent with our previous reports (Rizos *et al.* 2003), we observed that the presence of serum had a stimulatory effect on the speed of embryo development and on blastocyst yield. However, the effect of serum is detrimental to embryo cryotolerance and gene expression (Rizos *et al.* 2008) and has been linked to postnatal consequences for the offspring, such as large offspring syndrome (Lazzari *et al.* 2002). Replacement of serum with OF in the culture medium had a stimulatory effect on blastocyst yield, as observed with serum, but had the added benefit of improving embryo quality.

As mentioned before, the culture environment during embryo development has an effect on embryo quality in terms of cryotolerance (Rizos *et al.* 2002*b*, 2008), ultrastructural morphology (Fair *et al.* 2001), embryo cell number (Trigal *et al.* 2011) and gene expression (Rizos *et al.* 2002*a*; Wrenzycki *et al.* 2007). In the present study, we found that replacing serum with 1.25% or 0.625% OF doubled embryo cryotolerance compared with culture with serum. Conversely, embryos

cultured with 1.25% OF had a higher number of total cells and TE cells compared with embryos cultured with serum and the no-serum control group. ICM cell number did not differ between OF groups and serum control, but was higher than in the no-serum control. It has been shown that embryo quality determined by blastocyst total cell number, ICM and the ICM : TE ratio is an important index of embryo survival and pregnancy rate after embryo transfer (Fleming *et al.* 2004). Moreover, the trophoblast cells play a crucial role in pregnancy establishment when intense trophoblast proliferation begins together with increased trophoblast secretion of the pregnancy recognition factor interferon-τ (Ealy and Yang 2009). Therefore, trophoblast cells have an essential role in implantation and placentation. The increased proliferation of these cells is likely to have a positive effect on pregnancy rate. The positive effect of OF on embryo development and quality could be attributed to the oviductal embryotrophic secretions that are absent in defined and serum-supplemented media. Ballester *et al.* (2014) proposed OF and/or uterine fluid supplementation as a challenge for improving the IVC environment. However, it is important to note that many aspects of the oviductal environment remain unclear.

Of the OF fluid components that may play a beneficial role in embryo development, oviduct-specific glycoproteins, specifically OVGP1, have been shown to play a critical role during porcine fertilisation in sperm–ZP binding (Coy *et al.* 2008) and ZP hardening (Mondéjar *et al.* 2013). Martus *et al.* (1997) also demonstrated a positive effect on fertilisation rates when OVGP1 was used before or during the fertilisation process. Thus, OVGPs may have a specific function on early embryo developmental events.

Recently, Cebrian-Serrano *et al.* (2013) evaluated the effect of a short incubation of mature oocytes with OF before fertilisation on early embryo development and quality. No effect on embryo development was found; however, embryos derived from OF preincubation showed modification of glucose-6-phosphate dehydrogenase (*G6PD*) and superoxide dismutase 32 (*SOD32*) transcripts. Similarly, IVM porcine oocytes exposed to bovine OF for 30 min before fertilisation had increased blastocyst rates and embryo cell numbers, and the gene expression patterns of apoptotic and developmentally related genes were modified (Lloyd *et al.* 2009).

Gene expression analysis in the present study suggested that glucose and lipid metabolism is affected by the addition of serum to the culture medium. In particular, expression of the glucose transporter *SCL2A1* and the enzyme *GAPDH*, whose expression levels are related to anaerobic glycolysis during preimplantation development (Bermejo-Álvarez *et al.* 2010*b*), was lower in the C^+ compared with C^- group, suggesting reduced glucose metabolism in embryos cultured in the presence of serum. *LDLR*, downregulated in the C^+ compared with OF group, has been reported to be downregulated in embryos derived from obese mice, where it may act as a regulator of lipid uptake (Bermejo-Alvarez *et al.* 2012). Thus, the downregulation of *LDLR* in the C^+ group may be a response to an excessive amount of lipids in the C^+ medium. Another two genes related to lipid metabolism (*CYP51* and *FADS1*) were upregulated in the C^- and OF groups compared with the C^+ group. Both genes were reported to be upregulated in *in vitro*-derived blastocysts compared with their *in vivo* counterparts (Clemente *et al.* 2011), which may indicate that C^+ conditions are closer to the *in vivo* situation than C^- or OF, and therefore of better quality. However, the differences in metabolite use between *in vivo* and *in vitro* embryos should preclude direct extrapolation from the comparison of *in vivo* versus *in vitro* to good- versus poor-quality embryos (Sturmey *et al.* 2010). Both *CYP51* and *FADS1* are involved in the synthesis of molecules required for membrane formation: *CYP51* is required for sterol biosynthesis (Lepesheva and Waterman 2004), whereas *FADS1* is involved in the unsaturation of fatty acids (de Antueno *et al.* 2001), key molecules in the regulation of membrane fluidity and thereby related to survival after vitrification, which was higher in the C^- and OF groups than in the C^+ group. Similarly, *AQP3* expression was higher in the presence of OF compared with the C^+ and C^- groups. The AQPs are water channel proteins that have been suggested to play an important role in cryopreservation. The artificial expression of *AQP3* in mouse oocytes (Edashige *et al.* 2003) improved survival after cryopreservation and *AQP3* has been proposed as a major water and cryoprotectant transporter in bovine morulae (Jin *et al.* 2011). Finally, the *de novo* DNA methyltransferase *DNMT3A* and the imprinted gene *IGF2R* were downregulated in the C^+ group compared with the other two groups. These transcriptional changes are compatible with previous molecular observations of the large offspring syndrome caused by suboptimal IVC conditions. In particular, loss of methylation in the differentially methylated region 2 of *IGF2R* was associated with an increase in *IGF2R* transcription and fetal overgrowth (Young *et al.* 2001).

In conclusion, the results of the present study indicate that the presence of low concentrations of OF in serum-free culture medium of bovine embryos has a positive effect on embryo development and the quality of the resulting blastocysts, increasing their cryotolerance and the number of TE cells and modifying the relative abundance of developmentally important gene transcripts, including imprinted genes.

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