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Effect of the Bovine Oviductal Fluid on *In Vitro* Fertilization, Development and Gene Expression of *In Vitro*-Produced Bovine Blastocysts

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Contents

Oviductal microenvironment generally provides better conditions for early embryo development than the conventional *in vitro* system. In an attempt to simulate the oviduct conditions or the main potentially influencing factors, the effect was studied of a bovine oviductal fluid (bOF) treatment applied prior to IVF on (i) IVF parameters, (ii) cleavage rate, (iii) blastocyst yield and (iv) blastocyst quality. Embryo quality was assessed by morphological embryo quality and relative transcript abundance of several developmental genes in bovine blastocysts. Furthermore, to study the effect of bOF without the male effect and zona–sperm interaction, artificially activated metaphase II oocytes were also treated with bOF. *In vitro*-matured bovine oocytes from abattoir ovaries were treated or untreated with bOF for 30 min and then washed prior to IVF or activation. Subsequently, *in vitro*-fertilized and parthenogenetic embryos were *in vitro* cultured for 7 to 8 days. The bOF treatment had no effect on fertilization parameters, cleavage, blastocyst rates both on parthenogenetic and IVF bovine embryos and neither on morphological quality of IVF blastocysts. *G6PD* and *SOD2* genes from IVF blastocysts showed significant changes in their expression after a bOF treatment. Significant differences were found for the expression of *SCL2A1, GPX1*, *BAX*, *AKR1B1* and *PLAC8* genes between excellent or good blastocysts (Grade 1) and fair blastocysts (Grade 2). To our knowledge, this is the first study that evaluates the effect of bOF oocyte treatment on fertilization parameters, development and quality of bovine embryos.

Introduction

The approach of the *in vitro* embryo production (IVEP) system to the physiological conditions should result in an improvement in the blastocyst quality and development rates. In particular, the oviduct, where a part of oocyte maturation, capacitation of spermatozoa, fertilization and the first stages of embryonic development take place, has been described as decisive for proper physiological gamete and early embryo development (Hunter 1998). The oviductal microenvironment, even using oviducts from different mammal species, generally provides better conditions for early embryo development than conventional *in vitro* systems (reviewed by Rizos et al. 2010). Nevertheless, *in vivo* temporary embryo culture in oviduct is not practical for welfare reasons as two laparotomies are required, along with the technical complexities that it entails.

Oviductal fluid (OF) composition is very complex. It is composed by numerous metabolites such as growth factors, hormones, proteases and inhibitors, antioxidants, defence agents, glycosidases and glycosyltransferases, glycosaminoglycans and proteoglycans, chaperones, and heat shock proteins (reviewed by Aviles et al. 2010). It is known that OF growth factors could contribute to a more efficient embryo development (reviewed by Diaz-Cueto and Gerton 2001; Hardy and Spanos 2002) and that OF antioxidants could protect the gametes and embryos against reactive oxygen species (ROS) present in the microenvironment (reviewed by Guerin et al. 2001).

Regarding embryo culture, with the attempt to simulate the oviductal conditions, *in vitro* coculture with oviductal cells has been reported as beneficial for the embryo development (Xu et al. 2004). Nevertheless, coculture with cells has several problems, such as the methodological complexity, lack of repeatability and biosanitary risk. The effects of the different OF components have been studied previously. For example, iC3b, derived from the complement protein C3, has been shown to stimulate embryo development (Lee et al. 2004). Oviduct-specific glycoprotein (OVGP1) increased monospermy rates in *in vitro*-fertilized porcine oocytes while maintaining high penetration rates (Coy et al. 2008a) and exerted an embryotrophic effect on porcine embryos (Kouba et al. 2000; McCauley et al. 2003). Moreover, OVGP1 induced hardening of the zona pellucida (ZP), suggesting that OVGP1 modulates the interaction with spermatozoa and contributes to the regulation of polyspermy (Coy et al. 2008a). In the same way, Lloyd et al. (2009) observed an improvement in the cleavage rate after short exposure to bovine OF (bOF) prior to fertilization in porcine oocytes. Furthermore, in the latter study, production and quality of the IVP blastocysts were also improved, showing that bOF contained factors that could act positively on porcine oocytes, promoting embryo development and quality.

Analysis of gene expression is a useful tool to assess the normality of embryos and optimize the assisted reproduction technology (Wrenzycki et al. 2005). Lloyd et al. (2009) also showed that short treatment of porcine oocytes with bOF affected the gene expression of the blastocysts produced, promoting a better quality of embryos. Nevertheless, in cattle, no information was found about the effect of the OF on fertilization parameters and embryo development at once.

Therefore, the aim of this study was to examine the effect of the presence or absence of a bovine oviductal fluid (bOF) treatment applied prior to IVF on (i) IVF parameters, (ii) cleavage rate, (iii) blastocyst yield and

(iv) blastocyst quality, as assessed by morphological embryo quality and relative transcript abundance of several developmental genes in bovine blastocysts.

Materials and Methods

Culture media

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich Quimica SA (Madrid, Spain). Handling medium (HM199) consisted of Hepes Medium 199 (M7528) supplemented with 7.4% foetal bovine serum (FBS; Invitrogen-10108-157; Alcobendas, Madrid, Spain). To avoid the formation of blood clots during oocyte collection, 2.2 mg/ml heparin (H9399) was added to HM199. Maturation medium (MM199) consisted of medium 199 (M4530) supplemented with 10% FBS, 10 ng/ml EGF (E1257), 0.1 UI/ml FSH and 0.1 UI/ml LH. The IVF medium was Fert-TALP (Parrish et al. 1988) with 10 μg/ml heparin and without epinephrine, hypotaurine and glucose. Culture medium (CM) used was modified synthetic OF amino acids supplemented following Holm et al. recommendations (Holm et al. 1999) with some modifications (mSOFaaci): 4.2 mM sodium lactate (L4263), 0.73 mM sodium pyruvate (P4562), 30 μl/ml BME amino acids (B6766), 10 μl/ml MEM amino acids (M7145), 1 μg/ml phenol-red (P0290) and 5% FBS. On day 5 of culture (day 0 = IVF), the medium was supplemented with FBS until a concentration of 10% was reached. Of 75 μg/ml potassium penicillin G (P3032) and 50 μg/ml streptomycin sulphate (S6051) were added in all culture media as antibiotics. All culture media containing bicarbonate were covered with mineral oil (M8410) and equilibrated overnight in culture conditions. Further *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) were performed in four-well dishes with 500 μl of medium.

In vitro maturation

Cumulus–oocyte complexes (COCs) were aspirated from 2- to 8-mm-diameter follicles of slaughterhouse ovaries from heifers younger than 1 year old. Oocytes with several layers of cumulus cells, morphologically bright and uniform cytoplasm were washed thrice in HM199 and *in vitro* matured for 22–24 h in MM199 in humidified atmosphere at 38.5°C and 5% CO_2 in air. Groups of approximately 50 COCs were matured per well.

Obtaining and processing bOF

Genital tracts from 14- to 20-month-old heifers (Charolais, Limousine and Simmental) were obtained at the abattoir and transported to the laboratory on ice. Once in the laboratory, the oestrous cycle stage (early follicular, late follicular, early luteal and late luteal) was assessed on the basis of ovarian morphology (Carrasco et al. 2008). Once classified, 25 bovine oviducts were separated from the tracts and quickly washed once with 0.4% v/v cetrimide solution, twice in saline and transferred to Petri dishes on ice and dissected. Once dissected, the bOF from the whole oviduct was collected by aspiration with an automatic pipette using a tip for a maximum 200 μl volume as previously described (Carrasco et al. 2008). bOF was centrifuged (7000 × ***g***, 10 min, 4°C) to remove cellular debris and the supernatant immediately stored at −80°C until use. Frozen samples were lyophilized by freeze-drying (ALPHA 1–2 LD plus, Christ, 6.59 kPa).

Bovine oviductal fluid treatment of oocytes

Lyophilized bOF was reconstituted with 800 μl of water (W3500). After IVM and before IVF or artificial activation, oocytes were incubated in reconstituted bOF for 30 min, in microdrops of 10 μl (10 oocytes/microdrop) covered with mineral oil in humidified atmosphere at 38.5°C and 5% CO_2 in air. Control group oocytes (without bOF treatment) were incubated in HM199 medium under the same conditions as aforementioned.

Evaluation of zona pellucida hardening after bOF treatment

To assess the hardening of the oocyte ZP after bOF treatment, a pronase test was performed using some oocytes per group in each experimental session. Briefly, IVM-decumulated oocytes were transferred into PBS, washed by pipetting and introduced into 50 μl of 0.5% (w/v) pronase solution in PBS (Coy et al. 2002). ZP was continuously observed for dissolution under a stereomicroscope equipped with a warm plate at 37°C. The dissolution time of the ZP in control oocytes was always approximately 3 min, whereas for treated oocytes, the dissolution time was approximately longer than 1 h.

Artificial activation of oocytes

Furthermore, to study the effect of bOF without the male effect and zona–sperm interaction, parthenogenetic embryos were produced as follows. *In vitro*-matured oocytes were denuded of cumulus cells with a hyaluronidase (H4272) treatment (1 mg/ml in HM199, exposure of no longer than 1 min) and gently pipetted with a stretched glass Pasteur pipette. Oocytes showing one visible polar body under the stereomicroscope were considered as metaphase II (MII) oocytes. Then, these MII oocytes were artificially activated by exposure to 5 μM ionomycin (I0634) for 5 min, washed twice and incubated in 2 mM 6-dimethylaminopurine (D2629) for 3.5 h. After that, oocytes were washed and *in vitro* cultured for 8 days as detailed below in the IVC embryos conditions.

Sperm preparation and IVF

Two frozen semen straws of 0.25 ml from two bulls were thawed at 37°C in a water bath for 1 min and centrifuged for 20 min at 700 × ***g*** through a gradient of 2 ml of 45% Percoll and 2 ml of 90% Percoll, in 15-ml centrifuge tubes. The Percoll 90% was made according to the protocol described by Parrish et al. (1995). To prepare the Percoll 45%, the Percoll 90% was mixed 1 : 1 with HM199. The sperm pellet was isolated and

washed in 5 ml of HM199 by centrifugation at $350 \times g$ for 5 min. The remaining pellet was diluted with approximately 100 μl of HM199. Then, the sperm concentration was determined, and a final concentration of 1×10^6 sperm/ml was adjusted for IVF. Oocytes were washed thrice in fertilization medium and after that were incubated with spermatozoa for 18–20 h in 5% CO_2 in air at 38.5°C.

Assessment of nuclear status of presumptive zygotes

After IVF, putative zygotes (see Experimental design) were fixed and stained for 15 min with ethanol and Hoechst 33342 (25 μg/ml) (B2261) and mounted on glass slides. Mounted slides were examined under an epifluorescence microscope at ×400 and ×600 magnification and classified into four groups according to the morphological criteria to evaluate the fertilization process: (i) no fertilized oocytes (no pronuclear formation), (ii) fertilized oocytes (formation of two pronuclei), (iii) polyspermic-fertilized oocytes (with more than two pronuclei) and (iv) oocytes with other abnormal fertilization (oocytes with other nuclear structures).

In vitro culture of embryos

Presumptive *in vitro*-fertilized zygotes were denuded from surrounding expanded cumulus cells in HM199 by repeated pipetting using a pulled glass Pasteur pipette, washed thrice in CM and incubated at 38.5°C in a humidified atmosphere of 5% CO_2 and 5% O_2 in air. Culture was performed in groups from 20 to 30 and 30 to 40 for *in vitro*-fertilized and parthenogenetic embryos, respectively. Cleavage was recorded at day 2, and blastocyst formation rate was recorded at day 7 and day 8, both under a stereomicroscope. *In vitro*-fertilized blastocysts were classified according to their morphological quality as follows: grade 1 (G1), excellent or good blastocysts [code 1 on the International Embryo Transfer Society (IETS) morphological classification (Robertson and Nelson 1998)] and grade 2 (G2), fair blastocysts [code 2 on the IETS morphological classification (Robertson and Nelson 1998)]. For the later gene expression analysis, four blastocysts groups were considered: G1 and G2 blastocysts untreated with bOF (CG1 and CG2, respectively) and G1 and G2 blastocysts treated with bOF (bOFG1 and bOFG2, respectively). Finally, blastocysts were washed with PBS, placed in PCR Eppendorf tubes, frozen by immersion in liquid nitrogen, and kept at –80°C.

RNA extraction, reverse transcription and quantification of mRNA transcript abundance

We studied expression analysis of genes involved in glucose metabolism (*AKR1B1*), placenta formation (*PLAC8*), apoptosis (*BAX*), de novo methylation (*DNMT3A*), mitochondrial activity (*SOD2*), ROS detoxification (*GPX1*), metabolism (*SCL2A1*) and oxidative stress (OS) (*G6PD*). Molecular biology procedures were carried out as described previously (Bermejo-Alvarez et al. 2010b). Poly(A) RNA was extracted from pools of ten blastocysts following the manufacturer's instructions using the Dynabeads mRNA Direct Extraction Kit (Dynal Biotech, Oslo, Norway) with minor modifications: lysis was achieved in 100 μl lysis buffer for 10 min with occasional gently pipetting, and then, the fluid lysate was hybridized with 20 μl prewashed beads for 5 min with gently shaking. After hybridization, two washes in 100 μl washing buffer A and two more in washing buffer B were performed. Finally, beads were eluted in 30 μl Tris–HCl. Immediately after extraction, the RT reaction was carried out following the manufacturer's instructions (Bioline; Ecogen, Madrid, Spain) using poly(T) primer, random primers and Moloney murine leukaemia virus reverse transcriptase enzyme in a total volume of 40 μl to prime the RT reaction to produce cDNA. 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 100 units of reverse transcriptase. They were then incubated at 42°C for 60 min to allow the RT of RNA, followed by 70°C for 10 min to denature the RT enzyme. The quantification of all mRNA transcripts was carried by real-time quantitative RT (qRT)-polymerase chain reaction (PCR) in three independent experiments. Experiments were conducted to contrast relative levels of each transcript and histone *H2AFZ* (*H2AFZ*) in every sample (Table 1). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 1. For quantification, qRT-PCR was performed as described previously (Bermejo-Alvarez et al. 2010a). PCR conditions were tested to achieve efficiencies close to 1, and then, the comparative cycle threshold (C_t) method was used to quantify expression levels. Quantification was normalized to 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 in one cycle is equivalent to doubling of the amplified PCR product. According to the comparative C_t method, the DCT value was determined by subtracting the *H2AFZ* C_t value for each sample from each gene C_t value of the sample. Calculation of $\Delta\Delta C_t$ involved using the highest sample ΔC_t value (i.e. the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔC_t sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta C_t}$. The forward is the first primer in the Table 1, while the reverse is the second one.

Experimental design

To evaluate the effect of a short oocyte treatment with bOF prior to fertilization, several IVF and parthenogenetic activation sessions were carried out. After IVM, oocytes were treated for 30 min with bOF or non-treated, as described earlier, and divided into three groups:

In six replicates, 334 presumptive zygotes were fixed after IVF, and nuclear status was assessed as described previously.

In eight replicates, 744 presumptive zygotes were *in vitro* cultured. Cleavage and blastocyst rates were

Table 1. Details of primers used for real-time quantitative reverse transcription polymerase chain reaction

Gene	NCBI official name	Primer sequence (5′–3′)	Fragment size (bp)	GenBank accession number
H2AFZ	*H2A histone family, member Z*	AGGACGACTAGCCATGGACGTGTGCCACCACCAGCAATTGTAGCCTTG	209	NM_174809.2
AKR1B1	*Aldo-keto reductase family 1, member B1*	CGTGATCCCCAAGTCAGTGAAATCCCTGTGGGAGGCACA	152	M314631
PLAC8	*Placenta-specific 8*	CGGTGTTCCAGAGGTTTTTCCAAGATGCCAGTCTGCCAGTCA	166	NM_001025325.1
BAX	*BCL2-associated X protein*	CTACTTTGCCAGCAAACTGG TCCCAAAGTAGGAGAGGA	158	NM_173894.1
DNMT3A	*DNA methyltransferase 3A*	CTGGTGCTGAAGGACTTGGGCCAGAAGAAGGGGCGGTCATC	317	AY271299
SOD2	*Superoxide dismutase 2, mitochondrial*	GCTTACAGATTGCTGCTTGTAAGGTAATAAGCATGCTCCC	101	S67818.1
GPX1	*Glutathione peroxidase 1*	GCAACCAGTTTGGGCATCACTCGCACTTTTCGAAGAGCATA	116	NM_174076.3
SCL2A1	*Solute carrier family 2 (facilitated glucose transporter), member 1*	CTGATCCTGGGTCGCTTCATACGTACATGGGCACAAAACCA	168	NM_174602.2
G6PD	*Glucose-6-phosphate dehydrogenase*	CGCTGGGACGGGGTGCCCTTCATCCGCCAGGCCTCCCGCAGTTCATCA	347	XM_583628.4

recorded as embryo development parameters. Embryos reaching blastocyst stage were assessed with morphological quality parameters and used to analyse the relative mRNA expression of the genes aforementioned.

Two hundred and fifteen oocytes were artificially activated and *in vitro* cultured in three replicates. Cleavage and blastocyst rates were recorded as embryo development parameters.

Statistical analysis

Results of pronuclear formation, cleavage, blastocyst and morphological embryo quality rates were analysed using the chi-square test. $p < 0.05$ was considered statistically different. Results of gene transcriptions were analysed using the SigmaStat (Jandel Scientific, San Rafael, CA, USA) software package. Relative mRNA abundance differences among groups were analysed by one-way ANOVA.

Results

Results of pronuclear formation after IVF are shown in Table 2. Oocytes treated with bOF did not show significant differences in normal fertilization, non-fertilization, polyspermic fertilization or other abnormal fertilization rates in comparison with the control group.

Regarding embryo development (Table 3), no differences were found between control and bOF groups in cleavage or blastocyst rates. The effect of bOF on the morphological classification of the blastocysts produced by IVF is shown in Table 4. No significant differences were found between control and bOF group in the morphological classification. Table 5 shows the effects of bOF on parthenogenetic embryo development. Neither group, without (control) or with a bOF treatment (bOF), showed any differences in cleavage or blastocyst rates.

Figure 1 shows the relative mRNA expression of the genes aforementioned. No significant effect of bOF was found on gene expression in the G1 blastocysts, except for *G6PD* gene ($p < 0.05$). Similarly, no significant effect of bOF was found on gene expression in the G2 blastocysts, except for *SOD2* gene ($p < 0.05$). The genes *AKR1B1*, *PLAC8* and *GPX1* showed higher ($p < 0.05$) abundance of transcript in G1 blastocysts than in G2 ones regardless of bOF treatment. *BAX* and *SCL2A1* showed a higher transcription ($p < 0.05$) in the groups CG1 and bOFG1 than CG2, but not higher than bOFG2.

Discussion

The hardening of ZP subsequent to sperm fertilization is a widely studied process and has been considered an important event to avoid the penetration of additional spermatozoa (Ducibella et al. 1990, 1993; Vincent et al. 1990). However, between ovulation and fertilization, ZP of oocytes also undergoes hardening during its journey inside the oviduct (Coy et al. 2008b), and this fact has been less studied. The pre-fertilization ZP hardening, as described by Coy et al. (2008a), is produced by OVGP1 presents in the OF, but it is absent during IVF in conventional *in vitro* systems (Coy et al. 2002, 2005). It

Table 2. Effect of bovine oviductal fluid (bOF) on IVF parameters of bovine oocytes

Treatment	Sessions	Total number of oocytes	Percentage of non-fertilization[a]	Percentage of normal fertilization[b]	Percentage of polyspermic fertilization[c]	Percentage of other abnormal fertilization[d]
Control	6	167	9.58	70.06	14.37	5.99
bOF[e]	6	167	9.58	75.45	11.98	2.99

[a]Oocytes without pronuclear formation
[b]Oocytes with two pronuclei formation.
[c]Oocytes with more than two pronuclei formation.
[d]Oocytes with other nuclear structures.
[e]Treatment prior to fertilization, 30 min of incubation.

Table 3. Effect of bovine oviductal fluid (bOF) on the development rates of *in vitro* bovine embryos

Treatment	Sessions	Total number of oocytes	Percentage of cleavage	Percentage of blastocyst from cleavage embryos
Control	8	369	80.49	34.68
bOF[a]	8	375	81.07	31.58

[a]Treatment prior to fertilization, 30 min of incubation.

Table 4. Effect of bovine oviductal fluid (bOF) on the morphological quality of the *in vitro* bovine blastocysts produced by IVF

Treatment	Sessions	n	Percentage of grade 1 blastocyst[a]	Percentage of grade 2 blastocyst[b]
Control	7	77	61.04	38.96
bOF[c]	7	74	60.81	39.19

[a]Code 1 on the International Embryo Transfer Society (IETS) morphological classification, excellent or good blastocysts.
[b]Code 2 on the IETS morphological classification, fair blastocysts. Robertson and Nelson (1998).
[c]Treatment prior to fertilization, 30 min of incubation.

Table 5. Effect of bovine oviductal fluid (bOF) on the development rates of *in vitro* bovine parthenogenetic embryos

Treatment	Sessions	Total number of oocytes	Percentage of cleavage	Percentage of blastocyst from cleavage embryos
Control	3	93	88.17	23.17
bOF[a]	3	122	84.43	19.42

[a]Treatment prior to activation, 30 min of incubation.

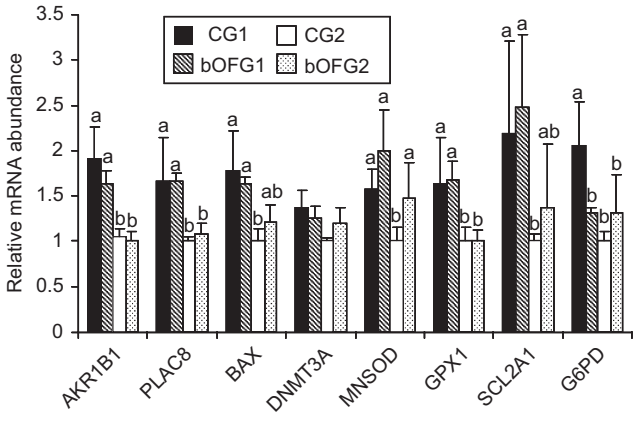

Fig. 1. Effect of bovine oviductal fluid (bOF) on relative mRNA expression of eight genes in grade 1 and grade 2 *in vitro* bovine blastocysts produced by IVF. CG1, control Grade 1 blastocysts. CG2, control Grade 2 blastocysts. bOFG1, bOF treatment Grade 1 blastocysts. bOFG2, bOF treatment Grade 2 blastocysts. Different letters (a, b) in each gene denote significant differences ($p < 0.05$) among groups. Error bars represent the SEM for the average $2^{-\Delta\Delta C_t}$ values

has been suggested that the ZP hardening has a direct correlation with the polyspermy block (Coy et al. 2008b). To date, the environmental conditions of the oviduct have not been completely reproduced in *in vitro* systems. In an attempt to simulate these conditions or the main factors that could be influencing them, we proposed the study of the effect of bOF on bovine oocytes in IVF parameters, embryo development, blastocyst quality and gene expression.

The bOF effect on the hardening of the oocyte ZP was verified through a pronase test (Coy et al. 2005) before IVF. So, the dissolution time of the ZP in control oocytes was approximately 3 min, whereas for treated oocytes, the dissolution time was usually longer than 1 h. Despite this modification of the ZP characteristics in the present work, the bOF oocyte treatment did not affect fertilization. Also in bovine oocytes and after a bOF treatment similar to our study (for 30 min prior to IVF), Coy et al. (2008a) observed that the mean number of spermatozoa per oocyte as well as the number of spermatozoa bound to the ZP remained invariable. In contrast, in the same study, oocytes treated with bOF showed an increase in the percentage of monospermy, but it was reduced the percentage of penetration. Also, no significant effect was found for ampullary or isthmic fluids on IVF rate of bovine oocytes (Suriyasathaporn et al. 1997). In other study, but in pig oocytes, the percentages of penetration and monospermy in bOF-treated and untreated groups of oocytes were similar between them (bOF treatment for 30 min prior to IVF) (Lloyd et al. 2009). The method to collect the bOF in this study as well as in the Coy et al. (2008a) work was similar to ours.

It is well known that polyspermy in bovine zygotes after IVF is not as high as in pigs (Coy et al. 2008b; Canovas et al. 2009). Therefore, the role of the OF in IVF and sperm–oocyte interaction as well as the pre-fertilization hardening of ZP could become more important in porcine than in bovine species, probably due to different ways of blocking the polyspermy, apparently more dependent on the cortical granule exudates in bovine species (Coy et al. 2008b).

Concerning embryo development, the bOF oocyte pre-treatment did not affect pre-implantational embryo development in our work. Incubation of bovine embryos in oviductal glycoproteins had no effect on blastocyst development rates (Hill et al. 1997; Vansteenbrugge et al. 1997) or only partially (at day 6, but not at day 7 of *in vitro* development) (Martus et al. 1997). However, other authors observed that the percentage of cleaved porcine embryos and blastocysts obtained from bOF-treated oocytes was higher than from untreated oocytes (Lloyd et al. 2009).

On the other hand, we studied the effect of bOF oocyte treatment, but without the male effect and zona–sperm interaction, using artificially activated MII oocytes (Gupta et al. 2008; Alfonso et al. 2009; Gomez et al. 2009). In our case, bovine OF treatment on bovine parthenogenetic embryos showed no effect on cleavage or blastocyst rate. To our knowledge, no other work in the literature has studied this effect on parthenogenetic oocytes to compare these results.

Regarding embryo quality, the treatment of bOF did not affect the morphological quality of the IVF blastocysts in our work. Anyway, it is convenient to note that a visual assessment of embryos continues being a subjective evaluation, and it is not an exact

science as IETS indicated. In agreement with us, Lloyd et al. (2009) did not find differences with the bOF treatment in the morphological quality of the porcine blastocysts.

Concerning gene expression, no difference between G1 blastocysts (from oocytes treated or not with bOF) was found, except for *G6PD* gene, in which the bOF-treated group presented lower gene expression than control group and at similar levels to G2 blastocysts. The gene *G6PD* is a potent indicator of the pentose phosphate pathway activity (Guerin et al. 2001) so consequently is a sentinel for ROS, leading rapidly to the generation of NADPH for maintenance of the cellular redox state (Lonergan et al. 2003). Oviductal proteins such as superoxide dismutase, glutathione reductase and thioredoxin families act for controlling ROS generation and are regulated by gametes in the oviduct (Georgiou et al. 2005); therefore, bOF could play an antioxidant role. Lopes et al. (2007) observed that IVF cattle embryos with poor quality showed a low expression of *G6PD* gene, although only the joint effect of embryo morphological quality and stage of development statistically significantly affected the gene expression. However, this link between embryo quality and gene expression is not evident, because other authors observed a significantly higher *G6PD* expression in *in vitro*-produced blastocysts than in others cultured *in vivo* in ewe oviduct (Lonergan et al. 2003) or obtained *in vivo* (Wrenzycki et al. 2002; Balasubramanian et al. 2007). Moreover, the expression of this gene can also be influenced *in vitro* by other factors such as sex of embryo, origin of embryo (nuclear transfer, parthenogenetic or IVF) or respiration rate (Gutierrez-Adan et al. 2000; Wrenzycki et al. 2002; Lopes et al. 2007).

In G2 blastocysts, there was no difference in gene expression between groups treated or not with bOF, except for *SOD2* gene, in which the embryos previously treated with bOF showed a higher expression than non-bOF-treated blastocysts, reaching levels similar to G1 embryos. The transcription of *SOD2* is performed by cells to neutralize the ROS and is located in the matrix of the mitochondria (Holley et al. 2010). Therefore, expression of *SOD2* in embryos could be indicative of mitochondrial activity (Rizos et al. 2002) and subsequently to good-quality embryos (Ramalho-Santos et al. 2009). This would be consistent with the results of Lloyd et al. (2009), who suggested lower mitochondrial activity in the untreated oocytes in comparison with bOF-treated oocytes. Furthermore, the expression of this gene in bovine embryos obtained *in vivo* was higher than *in vitro*-produced embryos (Rizos et al. 2002). In this way, Lonergan et al. (2003) observed that bovine blastocysts at day 7 *in vivo* cultured in ewe oviduct showed a higher level expression of this gene than those *in vitro* cultured. Moreover, in a study of embryo culture media, bovine blastocysts cultured in medium with the highest embryo survival rates after vitrification (higher quality) showed a significant increase in the gene expression of *SOD2* (Rizos et al. 2004). However, it seems that the relationship between gene expression and embryo quality changes with respect to the embryo stage, because several authors observed a higher gene expression of *SOD2* at day 3 in bovine embryos *in vitro* cultured than in *in vivo* (ewe oviduct) cultured (Lonergan et al. 2003; Gutierrez-Adan et al. 2004).

Observing the genetic expression of embryos with different morphological grades, in the control group blastocysts from G1 and G2 showed different genetic expression in the majority of the studied genes (seven of the eight genes). In contrast, in the bOF group blastocysts from G1 and G2 were only different in the expression of three genes. In the work of Lloyd et al. (2009), differences in gene expression between good-quality and poor-quality bOF-treated embryos were found in six of the eight genes analysed. However, in the control groups, all the gene expression was different between good and poor embryos. Lloyd et al. (2009) hypothesized that the ZP modifications, subsequent to bOF treatment, lead to select the better sperm to fertilized oocytes in comparison with the untreated oocytes. In this regard, Suriyasathaporn et al. (1997) observed that the proportion of abnormally fertilized bovine oocytes was lower in the presence of isthmic fluid than in the controls. In our experiment, it is possible that bOF mitigates some lacks on the poor-quality blastocysts. Lloyd et al. (2009) suggested that low-quality blastocysts would be more susceptible to being improved by the bOF effect because they are more sensitive to the stresses imposed by the IVC conditions.

On the other hand, also it could occur that bOF effects were shown after blastocyst stage. In this regard, Libik et al. (2002) did not obtain differences neither in penetration nor in monospermy rates in IVM sheep oocytes after a long exposure to ovine OF (during the final period of the oocyte maturation process) but obtained a higher pregnancy rate those embryos previously treated. In this study, OF was collected with permanent canullae inserted to the oviduct of adult sheep during the breeding season. We have not evaluated the pregnancy rates in the present study.

To our knowledge, this is the first study that evaluates the effect of bOF oocyte treatment on fertilization parameters, development and quality of bovine embryos. In conclusion, we showed here that a short bOF oocyte treatment had no effect on fertilization parameters, cleavage, blastocyst rates both on parthenogenetic and IVF bovine embryos and neither on morphological quality of IVF blastocysts. However, *G6PD* and *SOD2* genes from IVF blastocysts showed significant changes in their expression after a bOF treatment. Significant differences were also found for the expression of *SCL2A1, GPX1*, *BAX*, *AKR1B1* and *PLAC8* genes between excellent or good blastocysts and fair blastocysts.

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Conflict of interest

None of the authors of this study have any financial and personal relationships with other people or organizations that could inappropriately influence the research presented herein.

Author contributions

The work presented here was carried out in collaboration between all authors. EGR, AGA, PC and MAS designed the experiment. ACS, IS, AGA, EP, SPC, P.C and MAS carried out the laboratory experiments. ACS drafted the article and IS, EGR, EP, SPC, AGA, PC and MAS revised it critically. All authors have approved the final version of the manuscript.

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