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Effects of oviductal fluid on the development, quality, and gene expression of porcine blastocysts produced *in vitro*

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Abstract

In mammals, fertilization and early pre-implantation development occur in the oviduct. Previous results obtained in our laboratory have identified specific molecules in the oviduct that affect porcine sperm–egg interactions. The aim of the present study was to determine whether the contact between oocytes and oviductal fluid also affect embryo development, quality, and gene expression. *In vitro* matured porcine oocytes were exposed to bovine oviductal fluid (bOF) for 30 min prior to fertilization. Cleavage and blastocyst development rates were significantly higher from bOF-treated oocytes than from untreated oocytes. Blastocysts obtained from bOF-treated oocytes had significantly greater total cell numbers than those obtained from untreated oocytes. Using real-time PCR, grade 1 (very good morphological quality) and grade 2 (good morphological quality) blastocysts were analyzed for gene transcripts related to apoptosis (*BAX*, *BCL2L1*), mitochondrial DNA (mtDNA) transcription/replication (*POLG*, *POLG2*, and *TFAM*), blastomere connection and morula compaction (*GJA1*), and blastocyst formation and pluripotency (*POU5F1*). We found that the entire set of genes analyzed was differentially expressed between grade 1 and 2 blastocysts. Furthermore, bOF treatment reduced the ratio of *BAX* to *BCL2L1* transcripts and enhanced the abundance of *TFAM* transcripts in grade 2 blastocysts. Not only do these findings demonstrate that factors within the bOF act on porcine oocytes both quickly and positively, but they also suggest that such factors could promote embryo development and quality by protecting them against adverse impacts on mtDNA transcription/replication and apoptosis induced by the culture environment.

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Introduction

Recent studies have shown that the oviductal fluid (OF), the physiological milieu in which gametes meet, affects the biology of the oocyte by changing the resistance of the zona pellucida (ZP) to proteolysis. This change is mediated by the oviduct-specific glycoprotein (OVGP1), which modifies the sperm–egg interaction by binding to the ZP and, as a result, increases the levels of monospermy. Moreover, bovine oviductal fluid (bOF) produces a similar, but steadier, effect to the homologous fluid in pig (Coy *et al*. 2008*a*). Additionally, it has been shown that co-culturing embryos with oviductal cells or *in vivo* culture of embryos in oviducts of recipient animals increase the success of development following IVF (Rizos *et al*. 2002, Xu *et al*. 2004). These two observations indicate that the oviductal environment, and probably the OF, contains important molecules that affect the sperm–oocyte interaction and the subsequent developmental competence of zygotes.

Interestingly, other studies in pigs, mice, and humans have shown that supplementing culture media with oviductal proteins, such as OVGP1 (Kouba *et al*. 2000, McCauley *et al*. 2003) and a derivative of the complement component 3 (C3), iC3b, increases the number of *in vitro* embryos generated (Lee *et al*. 2004). However, the mechanism by which these proteins affect embryo development or whether other factors in the OF, apart from OVGP1 or iC3b, have an embryotrophic effect remain under study. Using such proteins or factors in a culture system might be a less complex, and therefore a more attractive alternative to embryo co-culture with somatic cells.

Previous experiments in our laboratory have shown that co-incubating porcine oocytes with bOF for a short period (30 min), after *in vitro* maturation (IVM) but prior to IVF, produces beneficial effects on fertilization outcomes by reducing polyspermy (Coy *et al*. 2008*a*). In these experiments, sperm and oocytes were co-incubated for a period of 18 h following bOF treatment

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of the oocytes. The aim of the present study was to determine whether the same short exposure of oocytes to bOF prior to fertilization, followed by a shorter (15 min) sperm–oocyte co-incubation, would also affect subsequent pre-implantation embryo development rates. It was hoped that a shorter sperm–oocyte co-incubation period would allow the effect of bOF on embryo development to be ascertained in the absence of any effect on polyspermy, as the percentage of polyspermic embryos is known to increase with increasing sperm–oocyte co-incubation time (Coy *et al*. 1993).

IVF and embryo culture have a profound effect on the pattern of gene expression in mouse blastocysts (Giritharan *et al*. 2007). IVF embryos differ from *in vitro* cultured (IVC) embryos in the expression of 3058 genes and the principal pathways concerned are cell proliferation, apoptosis, and morphogenetics. Also, the changes in gene expression observed in IVC embryos are thought to predispose them to certain diseases (Lonergan *et al*. 2003, Fernandez-Gonzalez *et al*. 2004, Lee & Yeung 2006). As a second objective, we also wanted to determine whether the bOF treatment of porcine oocytes influences blastocyst quality. Total cell number and the transcript abundance of seven genes were assessed. Specifically, we wanted to determine the transcript abundance of four genes that have previously been shown to be differentially expressed between *in vivo* and *in vitro* blastocysts, and are therefore considered as markers of blastocyst quality in the bovine: BCL2-associated X protein (*BAX*; pro-apoptotic; Rizos *et al*. 2002, Gutierrez-Adan *et al*. 2004, Rho *et al*. 2007), BCL2-like 1 (*BCL21*; anti-apoptotic; Rho *et al*. 2007), gap junction protein, alpha 1, 43 kDa (*GJA1*; involved in the formation of gap junctions for cell–cell communication during pre-implantation development; De Sousa *et al*. 1997, Rizos *et al*. 2002, Gutierrez-Adan *et al*. 2004), and POU class 5 homeobox 1 (*POU5F1*; essential for peri-implantation development and embryonic cell pluripotency; Boiani *et al*. 2003, Rizos *et al*. 2007). The transcript abundance of three additional genes was also compared between blastocysts obtained from bOF-treated and untreated oocytes, namely mitochondrial polymerase gamma (*POLG*), mitochondria polymerase gamma 2 accessory subunit (*POLG2*), and mitochondrial transcription factor A (*TFAM*). These nuclear genes encode factors essential for the transcription and replication of mitochondrial DNA (mtDNA) and when either *Polg* or *Tfam* gene expression is disrupted, e.g. in mouse embryos where these genes have been knocked out, embryonic lethality ensues soon after implantation (Gray & Wong 1992, Larsson *et al*. 1998, Hance *et al*. 2005). As a result of these observations, we also wondered whether the expression of such mtDNA transcription/replication factor genes could also serve as markers of quality in the blastocysts and be differentially expressed between blastocysts obtained from bOF-treated and untreated oocytes.

Our observations show that exposing porcine oocytes to bOF for a short time (30 min), in the absence of any effect on polyspermy, not only increases the percentage of cleaved embryos and the percentage of blastocysts formed, but also enhances the total cell number in blastocysts. Moreover, grade 2 blastocysts (classified according to the IETS manual, Robertson & Nelson 1998) obtained from bOF-treated oocytes had greater *TFAM* transcript abundance and a clear anti-apoptotic gene expression profile, as defined by a reduced ratio of *BAX* to *BCL2L1* transcripts, compared with the grade 2 blastocysts obtained from untreated oocytes.

Results

Labeling of porcine ZP with the bovine anti-OVGP1

After bOF treatment, immunocytochemical analysis revealed that bovine OVGP1 was found associated with the ZP of the porcine oocytes (Fig. 1).

bOF increases the output for in vitro *production of porcine blastocysts*

The percentages of penetration and monospermy in both groups of oocytes (pre-incubated with or without bOF) were similar. These results differ to those obtained in this laboratory previously under different experimental conditions, which showed that bOF-treated oocytes, when co-incubated with sperm for 18 h post-insemination, had a higher percentage of monospermy compared with untreated oocytes (Coy *et al*. 2008*a*; Supplementary Tables 1 and 2, which can be viewed online at www.reproduction-online.org/supplemental/). This result demonstrates that, in the present study, the experimental design we chose (15 min of co-incubation time) was effective to guarantee that any effects of bOF treatment on subsequent embryo development rates were not a result of differences in penetration and monospermy. From Supplementary Table 2, it can be observed that the final efficiency of the IVF system (calculated approximately as the percentage of penetrated oocytes

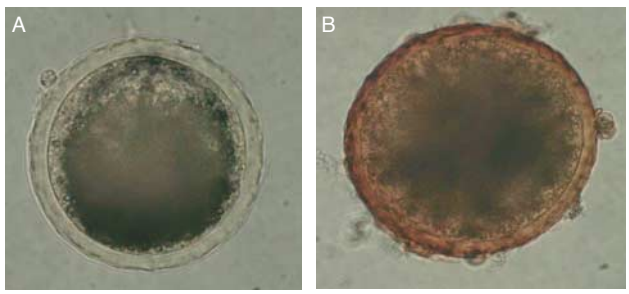

Figure 1 Binding of bovine OVGP1 to porcine ZP was detected immunocytochemically. (A) Porcine oocyte incubated with rabbit anti-OVGP1+ anti-rabbit HRP showed a transparent non-stained ZP. (B) Porcine oocyte incubated 30 min in bovine oviductal fluid+rabbit anti-OVGP1+ anti-rabbit HRP showed a labelled ZP.

multiplied by the percentage of monospermic oocytes divided by 100) was 39 and 35.5% respectively, for control and bOF-treated groups.

The percentage of cleaved embryos at 48 hpi obtained from oocytes pre-incubated for 30 min with bOF was around 10% higher than from untreated oocytes ($P<0.05$; Table 1). The percentage of blastocysts obtained from bOF-treated oocytes at day 7 was also 3% higher than from untreated oocytes ($P<0.05$; Table 1).

bOF increases the quality of in vitro *produced blastocysts*

The morphological classification of the blastocysts produced *in vitro* yielded similar proportions of grade 1, grade 2, and 'other' (grade 3 and 4) blastocysts from bOF-treated and untreated oocytes (Table 2). Furthermore, in a preliminary experiment, it was shown that the stereomicroscopic appearance of the blastocysts, used to classify them as either grade 1, 2, 3 or 4, corresponded with their total cell number; e.g. grade 1 blastocysts, observed using an epifluorescence microscope, had a greater total cell number than grade 3 blastocysts (Fig. 2). Interestingly, the mean total cell number of grade 3 and 4 blastocysts obtained from the bOF-treated oocytes was higher ($P<0.05$) than from the untreated oocytes group (31.4 ± 7.9 vs 25.3 ± 4.2 respectively).

Oviductal fluid affects the mtDNA transcription/ replication factor and apoptotic gene expression of porcine blastocysts produced in vitro

In this study, we analyzed the transcript abundance of several developmentally important genes related to apoptosis (*BAX* and *BCL2L1*), blastomere connection and morula compaction (*GJA1*), blastocyst formation and pluripotency (*POU5F1*), and mtDNA transcription and replication (*POLG*, *POLG2*, and *TFAM*). Grade 1 blastocysts had a greater abundance of the anti-apoptotic *BCL2L1* transcripts and also *POLG*, *POLG2*, *TFAM*, *GJA1*, and *POU5F1* transcripts, and a lower abundance of the pre-apoptotic *BAX* transcripts than grade 2 embryos (Fig. 3). Pre-incubating oocytes with bOF had no effect on the transcript abundance of any of the genes analyzed in grade 1 embryos. However, the ratio between *BAX* and *BCL2L1* transcripts was lower ($P<0.05$), and *TFAM* transcript abundance was higher ($P<0.05$) in grade 2 embryos obtained from oocytes pre-incubated with bOF than from oocytes incubated without bOF (Fig. 3).

Table 1 Effect of incubating porcine oocytes in bovine oviductal fluid (bOF) on the percentage (mean±S.E.M.) of cleaved embryos and blastocysts formed.

Group	*N*	Percentage of 2–4 cells	Percentage of blastocysts[a] (*N*)
Control	381	$53.2\pm2.3^{*}$	$12.8\pm1.3^{*}$ (26)
bOF[b]	338	$63.6\pm2.6^{\dagger}$	$15.8\pm1.5^{\dagger}$ (34)

*,†Different superscripts in the same column denote significant differences ($P<0.05$).
[a]From the cleaved embryos. [b]bOF, oocytes were incubated for 30 min in bOF after IVM but before IVF.

Table 2 Effect of incubating porcine oocytes in bovine oviductal fluid (bOF) on the morphological classification of *in vitro* produced blastocysts.

Group (*N*)	Grade 1 (%)	Grade 2 (%)	Others (%)
Control (50)	36.00	38.00	26.00
bOF[a] (47)	40.41	36.17	23.42
ANOVA	$P=0.66$	$P=0.85$	$P=0.77$

[a]bOF, oocytes were incubated for 30 min in bOF after IVM but before IVF.

Discussion

Previous experiments in our laboratory have shown that pre-incubating porcine oocytes for a very short period (30 min) with bOF and then co-incubating the sperm and oocytes for 18 h modifies sperm–egg interactions (Coy *et al.* 2008*a*). In the present study, we show that a similar methodological approach but with a much shorter (15 min) sperm–oocyte co-incubation period following bOF treatment, to avoid any effects of polyspermy, enhances embryo cleavage rates and the proportion of blastocysts produced. Not only this, it also improves the

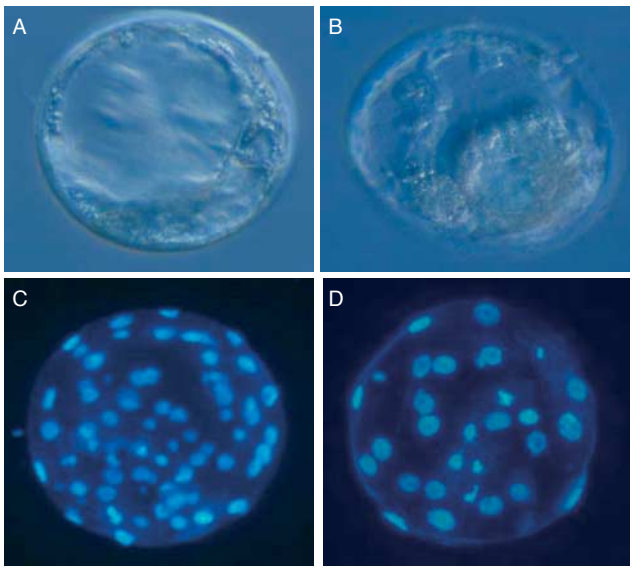

Figure 2 (A) Porcine blastocyst at day 7 observed under the stereomicroscope and classified as grade 1. (B) Porcine blastocyst at day 7 observed under the stereomicroscope and classified as grade 3. (C) Porcine blastocyst at day 7 stained with Hoechst 33342, fixed, observed under the epifluorescence microscope, and classified as grade 1. (D) Porcine blastocyst at day 7 stained with Hoechst 33342, fixed, observed under the epifluorescence microscope, and classified as grade 3.

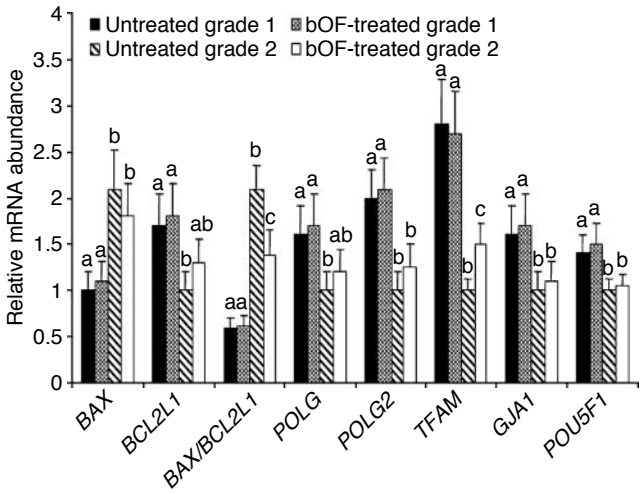

Figure 3 Relative mRNA expression of seven different genes in grade 1 and grade 2 porcine blastocysts produced from oocytes incubated for 30 min in bovine oviductal fluid (bOF) after IVM but before IVF, or not treated with bOF. Different letters (a, b) in each gene denote significant differences ($P<0.05$) among groups.

total number of cells observed in grade 3 and 4 blastocysts, and alters the expression of certain developmentally important genes involved with regulating mtDNA transcription/replication (*TFAM*) and apoptosis (*BAX* and *BCL2L1*) in grade 2 blastocysts.

Blastocyst development rates *in vitro* in the pig are variable, ranging from 6–7% to 30% (Nagai *et al*. 2006). Our final percentages of blastocysts produced for the two groups (bOF treated and untreated) were mid-range (~12–16% respectively), based on the estimates above. Nonetheless, our data demonstrate that the bOF treatment of oocytes has a beneficial effect on the final proportion of blastocysts produced because, even coming from a 3.5% difference of the final efficiency in the IVF system (Supplementary Table 2), the bOF-treated group reached a 3% of increase in the final number of blastocysts.

In a preliminary experiment, the stereomicroscopic appearance used to classify the porcine blastocysts into morphological grades corresponded to the total number of cells per blastocyst observed using an epifluorescence microscope. It has been recently shown that stereomicroscopic evaluation of porcine blastocysts is correlated with ultrastructural alterations and cell death (Cuello *et al*. 2007). This observation, along with previous observations that blastocysts with greater total cell numbers often result in higher pregnancy and birth rates than those with lower total cell numbers (Merton 2002), suggests that our grade 1 blastocysts were of better quality than our grade 3 blastocysts (because the former contained more cells than the latter), and that bOF treatment stimulated cleavage and the production of blastocysts with higher number of cells than in the control group for grade 3 and 4 blastocysts.

The transcript abundance of seven developmentally important genes was also differentially expressed between our grade 1 and 2 blastocysts. Specifically, our grade 1 blastocysts expressed greater levels of *GJA1*, *POU5F1*, *POLG*, *POLG2* and *TFAM* transcripts and a greater ratio of *BCL2L1* to *BAX* transcripts than our grade 2 blastocysts. Both *GJA1* and *POU5F1* transcripts are more abundantly expressed in *in vivo* than *in vitro* produced bovine embryos (Rizos *et al*. 2002, 2007, Gutierrez-Adan *et al*. 2004). Moreover, in mice, blastocysts with fewer cells exhibit incorrect expression of *Pou5f1* and have lower rates of foetal and postnatal development (Boiani *et al*. 2003).

POLG, *POLG2*, and *TFAM* are nuclear-encoded mtDNA-specific transcription and replication factors. *TFAM* is involved with the initiation of mtDNA replication and transcription (Fisher & Clayton 1985), whereas *POLG* is involved with the polymerization of new mtDNA, a process that is assisted by *POLG2* (Gray & Wong 1992). As already mentioned in the introduction, disrupting *Polg* and *Tfam* gene expression, e.g. by knocking out the genes in mouse embryos, results in embryonic lethality soon after implantation (Larsson *et al*. 1998, Hance *et al*. 2005).

BCL2L1 and *BAX* are anti- and pro-apoptotic regulatory genes respectively. Increased incidence of apoptosis could result in early embryonic death or early abortions (Brill *et al*. 1999). The ratio of *BCL2L1* to *BAX* is proposed to govern whether cells live or die (Oltvai *et al*. 1993). Characteristically, *BCL2L1* transcripts (Rho *et al*. 2007) are more abundantly expressed than *BAX* transcripts (Rizos *et al*. 2002, Gutierrez-Adan *et al*. 2004, Rho *et al*. 2007) in *in vivo* rather than *in vitro* produced embryos. The functional importance of these genes during embryo development in conjunction with the fact that several of the genes (*GJA1*, *POU5F1*, *BCL2L1* and *BAX*) are differentially expressed between *in vitro* and *in vivo* produced embryos (which are generally accepted to differ in their developmental potential) suggest strongly that our grade 1 are of better quality than our grade 2 embryos, which in turn further substantiates the suitability of the morphological classification system used in this study.

The most important and interesting findings in the present study were the beneficial effects of treating matured porcine oocytes with bOF for a short period (30 min) prior to fertilization on embryo cleavage and blastocyst development rates, on the total cell numbers (grade 3 and 4 blastocysts), as explained above, and on the gene expression (grade 2 blastocysts). In our opinion, it is possible that the higher quality embryos (grade 1) are more resistant to the stresses imposed by the IVC conditions than the lower quality embryos (grade 2). Thus, the protective effect of bOF only manifests in grade 2 embryos.

The embryo output in the porcine IVM/IVF/IVC system is still very low (Nagai *et al*. 2006), and this

simple and novel approach could be widely used in laboratories to increase the output of the system. The mediators responsible for positive effects of the oviductal environment on early embryonic development remain a fundamental question in reproductive biology. The OF has numerous components, including ions, nutrients, amino acids, hormones and proteins (Leese *et al*. 2001), and deciphering which of these is the active component within bOF responsible for the beneficial effects observed in the present study will not be an easy task. Supplementing culture media with proteins is known to play a crucial role in influencing the efficiency of *in vitro* embryo production systems (Warzych *et al*. 2007), but in such a complex milieu as the OF none of the components should be underestimated.

Although it was not the objective of this study to isolate and identify the factor(s) responsible of the beneficial effect, it has to be mentioned that the OF used in the present study was of bovine origin. Not only this, it exerted its effect on the porcine oocytes within a very short (30 min) period of time. Consequently, the active component within the bOF is most likely to be a conserved protein that is capable of binding to or traversing the ZP and ooplasm within 30 min. OVGP1 appears to fit both of these criteria; its nucleotide and amino acid sequences are highly conserved among mammalian species (Buhi 2002) and it is clearly capable of being transferred from the bOF to the ZP of the porcine oocytes within 30 min (Fig. 1). Moreover, it has been shown to increase embryo development rates in the porcine previously (Kouba *et al*. 2000, McCauley *et al*. 2003), and it is a component of OF (Leese *et al*. 2001). In this regard, perhaps C3 is also a likely candidate. C3 is also a component of OF, which when activated is cleaved to produce C3b, and then iC3b, which has known embryotrophic activity in the mouse (Lee *et al*. 2004). It is interesting to note that specific nuclear receptors for these factors (that act as transcription factors) could exist and be similar to those for the steroids. One attractive hypothesis might be that these factors traverse the oocyte membrane and interact with their receptors, which then elicit an effect on the expression of certain genes and consequently embryo development.

Alternatively, the active component within bOF could be a protein that protects the embryos produced against disrupted mtDNA transcription/replication and/or apoptosis induced by the culture environment. We infer this on the basis of *TFAM* transcripts being more abundant and the ratio of *BCL2L1* to *BAX* transcripts, being greater in our grade 2 blastocysts derived from bOF-treated oocytes rather than from untreated oocytes. As mentioned earlier, *TFAM* is essential for mtDNA transcription/replication, which in turn is essential for development. This is best illustrated by *Tfam* knockout mouse embryos that die soon after implantation due to severe mtDNA depletion, mitochondrial respiratory chain dysfunction, and morphologically abnormal mitochondria (Larsson *et al*. 1998). In light of this information, it is possible that our grade 2 blastocysts obtained from untreated oocytes have less developmental potential than those obtained bOF-treated oocytes because they have lower *TFAM* transcript abundance and, assuming the transcript levels mirror protein levels, this in turn could reflect lower rates of mtDNA transcription/replication, fewer mtDNA-encoded transcripts, and therefore fewer functional subunits for the mitochondrial respiratory chain. This hypothesis should be further investigated but it is not unusual for genes that are involved with maintaining the integrity of the mitochondrial respiratory chain (e.g. *ATP5B* and *COX7B*) to be downregulated in poor quality blastocysts compared with good quality blastocysts in the pig (Miles *et al*. 2008).

Interestingly, the fact that our grade 2 blastocysts obtained from untreated oocytes exhibit an apoptotic gene expression-like profile relative to our grade 2 blastocysts obtained from bOF-treated oocytes could in fact be related to the differences in *TFAM* transcript abundance observed between the two blastocyst groups. Apoptosis is incredibly prevalent in mouse *Tfam* knockout embryos, suggesting that a dysfunctional mitochondrial respiratory chain can cause apoptosis (Wang *et al*. 2001). It is unclear what factor within bOF could maintain the enhanced *TFAM* transcript abundance and the similarly enhanced ratio of *BCL2L1* to *BAX* transcripts observed in our grade 2 blastocyst obtained from bOF-treated oocytes relative to untreated oocytes.

However, embryos produce more reactive oxygen species (ROS) *in vitro* than *in vivo* (Guerin *et al*. 2001), and it is generally accepted that ROS are capable of inducing apoptosis, although it is not known how (Rho *et al*. 2007). It is possible that an enzyme like thioredoxin (TXN), shown to be upregulated in OF in response to the presence of gametes *in situ* and known to alleviate oxidative stress by breaking down ROS like H_2O_2 to harmless H_2O (Georgiou *et al*. 2005), is the active component within the bOF responsible for the enhanced ratio of *BCL2L1* to *BAX* transcripts observed in our grade 2 blastocyst obtained from bOF-treated oocytes relative to untreated oocytes.

Alternatively, the beneficial effect of the bOF treatment on our grade 2 embryos could be mediated by sperm selection. Previously, we have shown that bOF treatment increases the resistance of ZP to proteolysis (Coy *et al*. 2008*a*). Consequently, we hypothesized that only the 'stronger' sperm, possibly with 'better' genes, can cross the ZP. Thus, if our hypothesis is correct, our grade 2 embryos derived from bOF-treated oocytes would have been fertilized by better sperm than those derived from untreated oocytes. This in turn could also account for the differences in gene expression observed

between the two embryo groups. The fact that transcription, although very limited, which is more prominent in the male pronucleus than in the female pronucleus at the zygote stage (Beaujean *et al.* 2000), would tend to support this view.

In conclusion, the bOF is capable of providing porcine oocytes with components over a very short period of time between IVM and IVF, which benefit embryo development and embryo quality. The challenge is now to identify this (these) embryotrophic component(s), and then to add it (them) to porcine IVC systems to improve their output.

Materials and Methods

Culture media

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich Química S.A. The medium used for oocyte maturation was NCSU-37 and the basic medium used for IVF was TALP supplemented as described previously (Coy *et al.* 2008*b*). The embryo culture medium was NCSU-23 (Long *et al.* 1999).

Oocyte collection and IVM

Within 30 min of slaughter, ovaries from landrace×large white gilts were transported to the laboratory in saline containing 100 μg/ml kanamycin sulfate at 38 °C, washed once in 0.04% cetrimide solution and twice in saline. Oocyte–cumulus cell complexes (COCs) were collected from antral follicles (3–6 mm diameter), washed twice with Dulbecco's PBS supplemented with 1 mg/ml polyvinyl alcohol (PVA) and 0.005 mg/ml red phenol, and twice more in maturation medium previously equilibrated for a minimum of 3 h, at 38.5 °C under 5% CO_2 in air. Only COCs with complete and dense cumulus oophorus were used for the experiments. Groups of 50 COCs were cultured in 500 μl maturation medium for 22 h at 38.5 °C under 5% CO_2 in air. After culture, oocytes were washed twice with fresh maturation medium without dibutyryl cAMP, eCG, and hCG and cultured for an additional 20–22 h (Funahashi *et al.* 1997).

Collection of oviductal fluid

Oviducts were obtained at the abattoir and transported in saline to the laboratory at room temperature. All the oviducts came from heifers between 14 and 20 months old, and only those coming from animals at the late follicular phase were used because it has been demonstrated that this type of fluid consistently shows beneficial effects on porcine IVF (Coy *et al.* 2008*a*). Oviducts were washed twice, transferred to Petri dishes on an ice bath and dissected. bOF was collected by aspiration with an automatic pipette using a tip for a maximum 200 μl volume and centrifuged at 7000 ***g*** for 10 min at 4 °C to remove cellular debris (Carrasco *et al.* 2008). The supernatant was stored at −80 °C until use.

Treatment of pig IVM oocytes with bOF and demonstration of heterologous binding for bovine oviduct-specific glycoprotein to porcine ZP

After IVM but before IVF, porcine oocytes were gently stripped of cumulus cells and incubated in bOF (1 oocyte/μl fluid) in groups of 10–30 oocytes for 30 min at 38.5 °C under 5% CO_2 in air. Oocytes were washed three times and transferred to fresh TALP medium. A control group of oocytes incubated in TALP medium, instead of bOF, under the same conditions as above was used in all experiments.

A polyclonal antibody produced in rabbit (Sigma Genosys) against bovine OVGP1 was used to check the binding of bovine OVGP1 to porcine ZP. First, porcine oocytes were incubated in bOF as explained above, second, with a primary antibody anti-OVGP1 IgG, and finally, with a secondary goat peroxidase-labelled anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, peroxidase activity was detected using 3,3′-diaminobenzidine as chromogen with H_2O_2 substrate to observe the staining around ZP.

IVF and embryo production

After bOF treatment, groups of 30–35 partially denuded oocytes were transferred into each well of a four-well multidish containing 250 μl of TALP medium pre-equilibrated at 38.5 °C under 5% CO_2. Following this, a sperm-rich fraction of semen from a mature, fertility tested boar, was collected by the gloved hand method and rapidly brought to the laboratory diluted 1:1 in Beltsville thawing solution (Pursel & Johnson 1975). Aliquots of the semen samples (0.5 ml) were centrifuged (700 ***g***, 30 min) through a discontinuous Percoll (Pharmacia) gradient (45 and 90% v/v), and the resultant sperm pellets were diluted in TALP medium and centrifuged again for 10 min at 100 ***g***. Finally, the pellet was diluted in TALP, and 250 μl of the suspension was added to wells with oocytes to a final content of 10^5 cells/ml. At 15 min post-insemination, oocytes were washed twice with fresh TALP by gentle aspiration through a glass pipette and allowed to continue in culture. At 3 h post-insemination (hpi), putative zygotes were transferred to NCSU-23 medium and allowed to develop for 7 days.

Assessment of embryo quality

Embryo quality for both control and experimental groups was assessed by (i) morphological classification of the day 7 blastocysts under the stereomicroscope, (ii) Hoechst staining and counting of cell number in each blastocyst, and (iii) measuring the level of seven selected gene transcripts by real-time RT-PCR.

(i) All of the blastocysts produced were morphologically classified using a stereomicroscope as either grade 1, grade 2, grade 3, or grade 4 in accordance with the criteria set out in the IETS Manual (Robertson & Nelson 1998) for bovine blastocysts.

(ii) After morphological classification, all the grade 3 and grade 4 embryos were processed for cell counting. The total number of cells per blastocyst was recorded under an epifluorescence microscope for each individual sample after

dry air fixation on a slide and Hoechst staining (1% Hoechst 33342 in PBS).

(iii) Additionally, after morphological classification, all grade 1 and grade 2 embryos were processed for real-time RT-PCR. Indeed, individual blastocysts were collected into 5 µl of PBS supplemented with 0.1% PVA and stored at –80 °C for this purpose. It seemed logical to just include grade 1 and 2 blastocysts in the real-time RT-PCR assays because, typically, only these grade 1 and 2 blastocysts are used for embryo transfers commercially, at least in cattle. The quality of grade 3 and 4 embryos is too poor to be used for developmental purposes.

Furthermore, in a preliminary experiment, a sample of embryos was taken and the total numbers of cells in grade 1 and 2 blastocysts was compared with that of grade 3 and 4 blastocysts, as described above.

Real time RT-PCR analysis of embryos

Forty blastocysts from each treatment group (bOF-treated and untreated oocytes) were analyzed by real-time RT-PCR. RNA was extracted from blastocysts in pools of six to eight using an RNAqueous-Micro RNA Isolation kit (Ambion, Austin, TX, USA), according to the manufacturer's instructions. RNA was incubated with DNaseI to ensure the removal of contaminating DNA. RNA extracted from embryos was reverse transcribed to produce cDNA using the ABsolute MAX 2-Step QRT-PCR kit (Abgene, Epsom, UK). Briefly, each reaction contained 6.5 µl RNA, 4 µl RT buffer (5X), 2 µl dNTPs (5 mM each), 1 µl oligo-dT primer (500 ng/ml), 1 µl RT enhancer, 1 µl Reverse-iTTM MAX RTase Blend (which includes an RNase inhibitor; Abgene, Surrey, UK), and made up to a total volume of 20 µl using sterile ddH_20. Additionally, parallel reactions containing all the components above, with the exception of the enzyme (no enzyme control), were set up to screen each RNA sample for the presence of contaminating DNA. Furthermore, a reaction containing all the reagents above, but no RNA (no template control) was set up to screen the reagents for contaminants. The reactions were carried out at 42 °C for 1 h, followed by 75 °C for 10 min using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The resulting cDNA was then diluted 1:5 in sterile ddH_20 and either analyzed immediately or stored at −20 °C until use. Real time RT-PCR was performed on each cDNA sample using a Rotorgene 2000 Real Time CyclerTM (Corbett Research, Sydney, Australia) and SYBR Green (Molecular Probes, Eurogene, OR, USA). Each reaction contained: 2 µl cDNA, 1X PCR buffer, 3 mM $MgCl_2$, 2 U Taq Express (MWGAG Biotech, Ebersberg, Germany), 100 mM of each dNTPs, and 0.2 mM of each forward and reverse primer (see Table 3 for primer sequences) and SYBR Green I (diluted 1:3000, from a 10 000 X stock solution), and each reaction was made up to a total volume of 25 µl using sterile ddH_20. Each reaction had an initial denaturation step at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at the appropriate temperature for the primers (Table 3) for 30 s and elongation at 72 °C for 10 s. Fluorescence data were acquired at 72–83 °C after the elongation step. At the end of a reaction, the PCR products were melted by ramping from 50 to 94 °C, holding for 5 s and acquiring fluorescence data at each step. Each cDNA sample was analyzed by real-time RT-PCR in triplicate. Product identity was confirmed by ethidium bromide-stained 2% agarose gel electrophoresis (see Table 3 for expected product sizes from each primer pair). The comparative C_t method was used to determine the transcript levels of *BAX*, *BCL2L1*, *GJA1*, *POU5F1*, *POLG*, *POLG2* and *TFAM*, i.e., the so-called target genes, relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) transcript levels in each sample, as described previously (Bermejo-Alvarez *et al*. 2008). *GAPDH* was chosen as a normalizer gene as its transcript levels have been shown to be unchanged between IVF and *in vivo* derived blastocysts (Kameyama *et al*. 2007). Briefly, fluorescence data were 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 doubling of the amplified PCR product. According to the comparative C_t method, the ΔC_t value was determined by subtracting the *GAPDH* C_t value for each sample from the target gene C_t value of the sample.

Table 3 Details of primers used for quantitative real-time RT-PCR.

Gene symbol	NCBI official name	Primers sequence (5′–3′)	Fragment size	GenBank accession number
BAX	BCL2-associated X protein	5′-CTACTTTGCCAGTAAACTGG 5′-TCCCAAAGTAGGAGAGGA	158	AJ606301.1
BCL2L1	BCL2-like 1	5′-GGAGCTGGTGGTTGACTTTC 5′-CTAGGTGGTCATTCAGGTAAG	518	AF216205
POLG	Mitochondrial polymerase gamma	5′-ACTGGCTGGACATCAGCAGT 5′-GACAGTACTGCATCAGGTCC	194	AK291281.1
POLG2	Mitochondrial polymerase gamma 2	5′-GCCTTGGAGCATTATGTT 5′-GAAGCTTCAGTCTTTTCACCG	148	AK235352
TFAM	Mitochondrial transcription factor A	5′-GGCAGACTGGCAGGTGTA 5′-CGAGGTCTTTTTGGTTTTCCA	164	AF311909
GJA1	Gap junction protein, alpha 1, 43 kDa	5′-TGCCTTTCGTTGTAACACTCA 5′-AGAACACATGAGCCAGGTACA	142	AY382593.1
POU5F1	POU class 5 homeobox 1	5′-TTGGGCTAGAGAAGGATGTGGTT 5′-GGAAAAGGGACTGAGTAGAGTGTGG	364	NM001113060.1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	5′-ACCCAGAAGACTGTGGATGG 5′-ACGCCTGCTTCACCACCTTC	247	BC102589

Calculation of $\Delta\Delta C_t$ involved using the highest sample ΔC_t value (i.e., the sample with the lowest target gene expression) as an arbitrary constant to subtract from all other ΔC_t sample values. Fold changes in the transcript levels of the target genes aforementioned relative to *GAPDH* transcript levels were then determined using the formula $2^{-\Delta\Delta C_t}$.

Statistical analysis

The percentage of cleaved embryos, blastocysts formed and the mean number of cells per blastocyst are presented as the mean $\pm$ S.E.M. All percentages were modeled according to the binomial model of variables. The variables were analyzed by one-way ANOVA. A P value <0.05 was taken to denote statistical significance. One-way repeated-measures ANOVA (followed by multiple pair-wise comparisons using Student–Newman–Kleus method) was used for the analysis of differences in mRNA expression assayed by real-time RT-PCR.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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