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Role of the oviduct and oviduct-derived products in ruminant embryo development

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Abstract

The fact that embryos can be obtained *in vitro* undermines the role of the oviduct. However, it has been demonstrated that when *in vitro* produced bovine zygotes are cultured in the oviduct of sheep, cattle or mice the embryo quality is improved compared to the embryos produced *in vitro*. Thus the oviduct is not simply a passive organ required only for transporting the embryo to the uterus but also provides a suitable microenvironment for the early embryo. The study of physiological mechanisms and interactions between the embryo and the oviductal environment is essential to understand the correct processes of early embryo developmental. This knowledge can be used to improve current *in vitro* procedures providing high quality embryos capable of continued development and implantation, and resulting in viable births.

Keywords: bovine, embryo development, *in vitro,* oviduct.

Introduction

In vivo, oocytes and embryos develop in a complex and dynamic environment. First, in the ovarian follicle, the oocyte grows and matures, achieving full developmental competence just prior to ovulation. Subsequently, in the oviduct, the oocyte undergoes fertilization and early embryonic development. Finally in the uterus, the blastocyst forms, hatches from the zona pellucida, elongates, and progressively attaches to the uterine wall (Spencer *et al*., 2007). Therefore, the environment where the early embryo develops has a significant impact on the subsequent embryonic development in the short and long term.

In vitro embryo production seeks to mimic the physiological conditions in which embryos normally develop to produce embryos at the appropriate stage and optimal quality. These characteristics are necessary to establish a pregnancy and to produce a healthy offspring after transfer.

In the last 20 years, researches on *in vitro* embryo production in ruminants have focused on two crucial questions: how to maximize embryo development and optimize quality of the blastocysts produced. Although a certain amount of progress has been made in both areas, the quality of *in vitro* produced blastocysts continues to lag behind those obtained *in vivo*. This inferiority of *in vitro* produced embryos is manifested in terms of morphology, cryotolerance, gene expression and pregnancy rate after embryo transfer (Lonergan and Fair, 2008).

It has been demonstrated that the oviductal environment supports embryonic growth up to the blastocyst stage after trans-species transfer across a wide range of species (Fair *et al*., 2001; Lazzari *et al*., 2002; Rizos *et al*., 2007). Using the sheep oviduct *in situ* for culturing *in vitro* produced zygotes, it was clearly shown that the key part of the process responsible for suboptimal embryo quality is the post-fertilization period (Galli and Lazzari 1996; Enright *et al*., 2000; Rizos *et al*., 2002a, b). Thus, studying the oviductal environment and the signals exchanged between the oviduct and/or the early embryo is crucial to improve our understanding of the underlying regulatory mechanisms controlling embryo development (Aviles *et al*., 2015). Furthermore, this knowledge would allow the development of *in vitro* models capable to produce embryos of better quality and also to study embryo-maternal interactions. In this review we will discuss the role of the oviductal environment on early embryo development and embryo quality based on evidence from both *in vivo* and *in vitro* studies in ruminants.

Role of the oviduct during early embryo development

The oviduct is a tubular structure, sustained by the mesosalpinx, that connects the ovary to the uterine horn. The oviduct is divided in five morphological and functional parts: (i) the infundibulum, (ii) the ampulla, (iii) the ampullary-isthmic junction, (iv) the isthmus and (v) the utero-tubal junction (Maillo *et al*., 2016b). The infundibulum is the most proximal structure to the ovary and is funnel-shaped, and its fimbriae receive the oocyte after ovulation. The ampulla is the wider part of the tubal structure. The ampullary-isthmic junction is the place where fertilization takes place (Hunter, 2012). The isthmus presents a narrow lumen and is the place where the sperm reservoir is established prior to fertilization; and also where the early stages of embryo development take place. The utero-tubal junction connects the isthmus to the uterus (Yániz *et al*., 2000).

The oviduct is an active organ that maintains and modulates the fluidic milieu for sperm capacitation, transport and fertilization of the mature oocyte and early embryonic development (Rodriguez-Martinez, 2007; Leese *et al*., 2008; Lloyd *et al*., 2009). After fertilization, the developing embryo passes through the isthmus, through ciliary movements and muscular contractions, until it reaches the uterus about 16-cell stage on day 4 (Ellington, 1991). Therefore, the first

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stages of bovine embryo development occur in the oviduct (Hackett *et al*., 1993). All these events generate an interest in better understanding the role of the oviduct as a multifunctional and specialized reproductive organ (Rodriguez-Martinez, 2007; Leese *et al*., 2008).

Oviductal epithelium

Oviductal epithelium is composed of two different cell types, ciliated and secretory. During gamete and embryo transport, the ciliary cells exhibit a synchronized movement leading to a directed flow of fluids (Abe and Hoshi, 1997). Secretory cells have microvilli on their apical side and secrete substances and growth factors, usually by exocytosis, associated with the first days of the oestrous cycle, which contribute to the development of the early embryo (Abe, 1996; Murray and Smith, 1997).

Sperms transiently adhere to the epithelial cells lining the caudal isthmus, constituting the sperm reservoir. This interaction is important because it lengthens the fertile lifespan of sperm, regulates capacitation and also controls the number of sperm present at the site of fertilization to limit the opportunity for polyspermy (for review, see Miller, 2015)

Populations of the different epithelial cells are dynamic during the phases of the oestrous cycle. The proportion of ciliated cells decreases in the infundibulum and the ampulla during the luteal phase compared with the follicular phase (Yániz *et al*., 2000). Moreover, cell morphology is modified as a function of embryo development and cycle stage (Suuroia *et al*., 2002). The height of ciliated cells decreases in the infundibulum and ampulla during the luteal phase and in the isthmus the height of secretory cells also diminishes (Abe *et al*., 1999). Furthermore, transcriptome approaches have identified different functional groups of genes involved in the regulation of the oviduct during the oestrous cycle (Bauersachs *et al*., 2004). Recently, Cerny *et al.* (2015) identified, in bovine oviductal epithelial cells (BOEC), a large number of differentially expressed genes (DEGs) between the follicular (1563 DEGs) and luteal (1758 DEGs) phases, with 616 DEGs exclusive to the ampulla and 811 DEGs exclusive to the isthmus. Similarly, we identified DEGs between the oviductal epithelial cells from the ampulla and isthmus of pregnant heifers collected on day 3 after oestrus. This may reflect morphological and functional differences for those regions (Maillo *et al*., 2016a).

Oviductal fluid

Oviductal environment are reflected in the composition of the oviductal fluid (OF). The OF is generated by (i) transudation from plasma into the oviductal lumen together with (ii) the secretion of substances synthesized by the secretory cells (Menezo and Guerin, 1997). OF composition is very complex, containing simple and complex carbohydrates, ions, lipids, phospholipids and proteins (Leese *et al*., 2001; Avilés *et al*., 2010). Some of these components are metabolic substrates, such as lactate, pyruvate, amino acids, and glucose, whose concentrations differ from those present in the uterine fluid and serum (Hugentobler *et al*., 2007, 2008).

Secretions present in the OF affect oocyte and sperm function (Killian, 2011; Mondejar *et al*., 2013) with proteins such as glycodelins, and lactoferrin involved on gamete interaction (Ghersevich *et al*., 2015) and oviductin, osteopontins and the complement protein C3 involved in early embryo development (Tse *et al*., 2008). In addition, these proteins together with others present in the OF have been previously reported to play direct roles in sperm motility, viability (Kouba *et al*., 2000), sperm-ZP binding (Banerjee and Chowdhury 1994), ZP hardening (Kratz *et al*., 2003), embryo-maternal interactions (Reed *et al*., 1998), oocyte (Hess *et al*., 1999), early embryo development (Lim and Hansel 1998), cell proliferation (Hulboy *et al*., 1997), differentiation and apoptosis, fertilization rates (Dinara *et al*., 2001), and pH (Ekstedt *et al*., 2004).

The oviduct-specific glycoprotein (OVGP1) is a component of the OF identified in many species in a highly conserved form. It is one of the most studied proteins in the OF. OVPG1 synthesis and secretion is dynamic and related to oestrogen (Buhi, 2002; Killian, 2004) and luteinizing hormone stimulation (Sun *et al*., 1997). OVGP1 binds to the zona pellucida (ZP) of the oocyte and early embryo suggesting a role in early embryo development (Buhi, 2002). It has been shown that embryo culture in the presence of oviductin increased embryo development *in vivo* in pigs (McCauley *et al*., 2003) and sheep (Pradeep *et al*., 2011). Coy *et al.* (2008, 2012) demonstrated that OVGP1 and heparin-like glycosaminoglycans from the oviductal fluid of sows and cows participate in the functional modification of the ZP, affecting the sperm-oocyte interaction and contributing to the control of polyspermy. Besides, OVGP1 and sperm interactions increased rates of fertilization and embryonic development (Killian, 2004). In addition, it is suggested that OVGP1 stabilizes the microenvironment surrounding by gametes and embryo, preventing dispersal of essential nutrients and ions, particularly during ciliary beating or muscular contraction, increasing the viscosity of luminal fluid (Hunter, 1994; Mondejar *et al*., 2012).

Proteomic studies of the OF have demonstrated that gametes modulate the oviductal environment in a favourable way to prepare the oviduct milieu for the arrival of the embryo (Georgiou *et al*., 2005). Sperm regulated twenty proteins, while the oocyte regulated only one protein (Ig kappa light chain). Three proteins were commonly regulated by both gametes (Complement Component C3, Ig kappa variable region, and haemoglobin beta chain), and one protein showed regulation by sperm and oocytes in opposing directions (Complement Component C3; Georgiou *et al*., 2007).

Embryo-maternal communication in the oviduct

As mentioned before, after fertilization the first few mitotic cleavage divisions take place in the isthmus

(Hunter, 1998). On day 3.5 to 4 after fertilization, the early embryo, at the 8- to 16-cell stage, moves from the oviduct to the uterus (Hackett *et al*., 1993) continuing the mitotic divisions forming first a compact agglomerated of cells called morula and, by day 7 to 8, a blastocyst.

For a successful pregnancy establishment, a complex signal exchange between the newly formed embryo and the mother is essential. In ruminants, the principal pregnancy-recognition signal produced by the embryo is interferon-tau, secreted by the trophoblast from day 10 up to day 21-25 (Spencer and Bazer, 2004). Alterations in the environment of the early embryo could have consequences in the subsequent development. Thus, a high proportion of embryonic losses occur between days 8 and 17 of pregnancy (Humblot, 2001; Thatcher *et al*., 2001).

The oviduct, as the first site of embryo development, is considered a starting point to examine putative signals between the embryo and the reproductive tract (Wolf *et al*., 2003). The embryo in the oviduct undergoes epigenetic changes responsible for further development, implantation and postnatal phenotype (Wrenzycki *et al*., 2005). However, the mechanisms involved in this embryo-maternal communication currently are mostly unknown (Fazeli, 2008).

Evidence *in vivo* in mice, by RT-qPCR showed changes in the oviductal gene expression depending on the presence or absence of embryos (Lee *et al*., 2002). Recently, new transcriptomic technologies (e.g., microarrays) have been used to elucidate the complex molecular dialogue between maternal tract and the embryo. Thus, in pigs Almiñana *et al.* (2012) showed that embryo-maternal communication exists at earliest stages of pregnancy, before the well-known embryonic signal of maternal recognition. In contrast, Maillo *et al*. (2015) did not find differences in the bovine oviduct transcriptome in the presence or absence of an 8- to 16-cell embryo *in vivo*. Obviously, multi-ovulatory species like mice and pigs cannot be directly compared with mono-ovulatory species such as cattle. Thus, the bovine model would provide new information on early embryo maternal communication that may be important for humans.

In this communication, the embryo might play a role as a modulator of the immune system in the maternal tract, inducing the down-regulation of immune related genes to allow the refractory uterus to tolerate the embryo and support its development (Almiñana *et al*., 2012). In a recent study from our group, it was necessary to transfer multiple embryos (up to 50) into the oviducts of heifers to detect differences in the transcriptome. When a single embryo was transferd into the oviduct (pregnant vs cyclic heifers) no differences were found, suggesting a local effect of the embryo (Maillo *et al*., 2015). More recently, Smits *et al*. (2016) reported a local influence of the embryo on the transcriptome of the equine oviduct epithelium.

Oviductal environment and *in vitro* models

In vitro systems are a valuable tool to study pathways and mechanisms, which are difficult to study *in vivo,* and cell cultures provide valuable aspects of physiologic or pathologic mechanisms. Studying the oviductal environment is crucial to understand the underlying regulatory mechanisms controlling embryo development (Aviles *et al*., 2015). The advantages of the oviductal environment have been demonstrated in different models; many physiological aspects have been clarified; however, many others still remain unknown (Hunter, 2012).

The culture of bovine oviductal epithelial cells (BOEC) as a monolayer may provide useful information on early embryo maternal interaction signals. Recently, Schmaltz-Panneau *et al*. (2014) described transcriptome changes in BOEC related to the presence of bovine embryos. BOEC are usually obtained from oviducts of slaughtered heifers or cows. When a BOEC line is established for *in vitro* embryo co-culture, it is essential to determine the stage of the oestrous cycle of the oviducts used. BOEC at oestrus have been successfully used as *in vitro* model simulating embryo maternal interactions (Rief *et al*., 2002). Recently, Cordova *et al*. (2014) used oestrus-metoestrus (day 0-3) BOEC for early (day 1-4) or late (day 4-7) embryo co-culture showing that the presence of the cells during the first four days of development, which correspond to the presence of embryos in the oviduct *in vivo*, accelerated the kinetics of blastocyst development and induced changes in genes involved in epigenetic control. The positive effects of these cells on the embryos are attributed to embryotrophic substances, such as growth factors secreted by the cells (Nancarrow and Hill, 1994; Vanroose *et al*., 2001). Besides, BOEC modulates the surrounding environmental conditions, decreasing the oxygen levels in the culture medium, preventing the formation of deleterious radicals as reactive oxygen species (ROS; Thompson *et al*., 2000; Vanroose *et al*., 2001), removing toxic substances from the medium (e.g., ammonia; Nancarrow and Hill, 1994) and decreasing the glucose and ion levels that could have detrimental effects on the embryos (Vanroose *et al*., 2001). The drawback of co-culture systems is that they have been associated with methodological complexity, lack of repeatability and biosanitary risk (Menezo and Guerin, 1997). To avoid the use of primary cultures that have a risk of contamination, the use of established cell lines allows standardized culture conditions and better control (Pegoraro *et al*., 2000). We recently reported that an established BOEC line can be used successfully after freezing and thawing as an *in vitro* embryo co-culture system, avoiding the lack of reproducibility between replicates, and did not differ from BOEC in suspension in terms of embryo development (Lopera-Vasquez *et al*., 2016a).

An alternative to co-culture, avoiding a direct contact between BOEC and embryos, is the use of conditioned media from BOEC which has a positive effect on embryo development and percentage live calves after transfer (Lim *et al*., 1997). The BOEC conditioned media is able to support embryo development to the blastocyst stage (Mermillod *et al*., 1993) through identified secreted embryotrophic

components such as OVGP1 (Briton-Jones *et al*., 2004), ET-1 (Reinhart *et al*., 2003), IGF (Xia *et al*., 1996; Winger *et al*., 1997), VEGF, EGF, IGF1, TGFβ2, and IL4 (Okada *et al*., 2005). However, many other secretions still remain unknown. Therefore, BOEC co-culture and/or their secretions must be a key for studying embryo maternal interactions and improve *in vitro* current systems.

As mentioned before, the OF is responsible for nurturing the embryo during the early stages of development. Therefore, using OF as a supplement during the *in vitro* embryo culture may affect embryo development and quality. Coy *et al*. (2008) evaluated the effect of oviductal fluid (30 min incubation) on the ZP of pig and cow oocytes and demonstrated an increase in the proteolytic resistance of the ZP reflected in a prolonged pronase digestion periods (3-8 h), and a modulation of sperm-ZP interaction through an increase in monospermy rate. Lloyd *et al*. (2009) exposed *in vitro* matured porcine oocytes to bovine OF for 30 min before fertilization, thereby increasing the blastocyst rate and quality in terms of morphology, cell number, as well as gene expression patterns of apoptotic and developmentally-related genes. Similarly, in cattle, Cebrian-Serrano *et al*. (2013) evaluated the effect of short-term incubation of matured oocytes with bovine OF; no effect on embryo development was observed but abundance of genes transcripts including *G6PD* and *SOD32* was reported (Cebrian-Serrano *et al*., 2013). In a recent study, we showed that only low concentrations of OF (<5%) in embryo culture media, in the absence of serum, had a positive effect on development and quality in terms of cryotolerance, cell number and expression of qualitatively related genes (Lopera-Vasquez *et al*., 2015).

The extracellular environment contains a large number of mobile membrane-limited vesicles called ‘‘extracellular vesicles’’ (EVs). EVs contain microvesicles (MVs), apoptotic bodies and exosomes. Originally, the EVs were associated with removal process of receptors and with cellular waste function (Thery, 2011). Subsequently, they were found to have immune effects (Raposo *et al*., 1996). These data opened the possibility that EVs could play a role in intercellular communication (Thery, 2011). EVs have been found in many biological fluids, including plasma (Caby *et al*., 2005), serum (Taylor and Gercel-Taylor, 2008), urine (Pisitkun *et al*., 2004) epididymal fluid (Gatti *et al*., 2005), amniotic fluid (Asea *et al*., 2008), follicular fluid (da Silveira *et al*., 2012), and milk (Admyre *et al*., 2007). A major discovery was that the content of EVs included both mRNA and miRNA and that EV-associated mRNAs could be translated into proteins by target cells (Valadi *et al*., 2007). EVs with features of exosomes released by immune cells have been demonstrated to selectively incorporate miRNA that can be functionally transferred as a consequence of fusion with recipient cells (Mittelbrunn *et al*., 2011).

The possible role of EVs in reproduction has been reported recently. Da Silveira *et al.* (2012) isolated MVs and exosomes of equine ovarian follicular fluid and, by proteomics and real-time PCR analysis, demonstrated the presence of proteins and miRNAs. The miRNAs were present in surrounding follicular cells, suggesting that MVs and exosomes play a role in mediating cell communication within the mammalian ovarian follicle (da Silveira *et al*., 2012). In addition, Sohel *et al*. (2013) demonstrated the exosome-mediated transport of miRNAs in the bovine follicular microenvironment. Similarly, Ng *et al*. (2013) identified and examined the presence and potential role of MVs and exosomes in the uterine cavity. MVs and exosomes miRNA has enabled bioinformatic identification of pathways that could be influenced if the exosomes are taken up by trophectoderm or epithelium at the time of implantation, or transferred to sperm as they transit the uterine cavity (Ng *et al*., 2013). The results from Burns *et al*. (2014) support the hypothesis that exosomes and MVs present in uterine luminal fluid of pregnant and cyclic ewes contain specific proteins, miRNAs, and mRNAs, that are capable of delivering their contents *in vitro*. Recently, the same group found EVs emanating from both the conceptus trophectoderm and uterine epithelia supporting the notion that MVs in uterine fluid have a biological role in conceptus-endometrial interactions which may be important for the establishment and maintenance of pregnancy (Burns *et al*., 2016). Al-Dossary *et al*. (2013) revealed the expression and secretion via oviductal exosomes of PMCA4a (Ca2+ homeostasis) in the female reproductive tissues and luminal fluids during oestrus, and their sperm-uptake, with possible roles in sperm viability during their storage in the oviduct and during capacitation and the acrosome reaction. Recently, the same group have identified oviductosomes (exosomes and microvesicles present in the oviductal fluid) in murine and bovine species although further studies are needed to determine their interaction with gametes/early embryo(s; Al-Dossary and Martin-Deleon, 2016). Furthermore, we provided evidence that extracellular vesicles derived from BOEC-conditioned media improved blastocyst quality and induced cryoprotection in *in vitro* culture to the same extent as classical co-culture with fresh BOEC monolayers (Lopera-Vasquez *et al*., 2016a). In addition, when extracellular vesicles were obtained from bovine isthmic oviductal fluid and added during the *in vitro* embryo culture, they had a positive effect on gene expression patterns of developmental-related genes compared with serum supplementation, suggesting an association between the oviductal environment and the developing embryo (Lopera-Vasquez *et al*., 2016b).

Concluding remarks

The content of the oviductal environment and its short and long term effects on early embryo development are extremly relevant and may provide new insights on embryo-maternal communication, improving assisted reproductive tecnologies. The challenge today is to develop *in vitro* culture conditions that will allow growth of the embryo based on the physiological components to which it is exposed *in vivo* to enhance the development of high/better quality embryos.

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