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TESIS DOCTORAL

Embryo development *in vitro* in cattle: role of oviduct cells, oviductal fluid and extracellular vesicles

Desarrollo embrionario *in vitro* en bovino : función de las células del oviducto, fluído oviductal y vesículas extracelulares

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Directores

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Miguel Ángel Ramírez de Paz

Madrid, 2015

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Los doctores Dimitrios Rizos y Miguel Ángel Ramírez de Paz, Investigadores Titulares del Departamento de Reproducción Animal del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) hacen constar:

Que la memoria de Tesis Doctoral presentada por Ricaurte Lopera Vásquez con el título: **"Embryo development *in vitro* in cattle: role of oviduct cells, oviductal fluid and extracellular vesicles / Desarrollo embrionario *in vitro* en bovino: función de las células del oviducto, fluido oviductal y vesículas extracelulares"**, ha sido realizada bajo nuestra dirección y que tras su revisión consideramos que tiene la debida calidad para su presentación y defensa.

Madrid a 18 de mayo de 2015

Fdo. D. Dimitrios Rizos

Fdo. D. Miguel Ángel Ramírez de Paz

“The results you achieve will be in direct proportion to the effort you apply”

Denis Waitley

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List of Abbreviations

µm	Micrometres
µl	Microlitres
100k	10000 x*g*
10k	10000 x*g*
18S	18 s ribosomal rna
A	Ampulla
ACACA	Acetyl-coa carboxylase alpha
AQP11	Aquaporin 11
AQP3	Aquaporin 3
ATP1A1	Atpase, Na^+/K^+ transporting alpha 1
BME	Basal medium eagle
BOEC	Bovine oviduct epithelial cells
bOF	Bovine oviduct fluid
BP	Binding proteins
BSA	Bovine serum albumin
C-	Control group without foetal calf serum
C+	Control group with foetal calf serum
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary dna
CL	Corpus luteum
CM	Conditioned media
CO_2	Carbon dioxide
COCs	Cumulus oocyte complexes
CT	Cycle threshold
CX43	Connexin 43
CYP51	Cytochrome p450 family 51
D7	Day 7
D8	Day 8
D9	Day 9
DMEM	Dulbecco's modified eagle medium
DNMT3A	Dna methyltransferase 3a
EDTA	Ethylenediaminetetraacetic acid
EGA	Embryo genome activation
EGF	Epidermal growth factor
EM	Electron microscopy
ESCRT	Endosomal sorting complexes required for transport
ET-1	Endothelin 1
EV	Extracellular Vesicles
F	Fresh
FADS1	Fatty acid desaturase 1
FCS	Foetal calf serum
FrCM	Frozen conditioned media
FrM	Frozen monolayer
FSH	Follicle stimulating hormone

G6PD	Glucose 6 phospathe dehydrogenase
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
GLUT1	Solute carrier family 2 (*SCL2A1*)
GPR3	G protein-coupled receptor 3
GPX1	Gluthathione peroxidase 1
GRB10	Growth factor receptor-bound protein 10
GV	Germinal vesicle
h	Hours
H2AFZ	Histone *2AFZ*
HA	Hyaluronic acid
HK	Housekeeping
HM	Holding medium
I	Isthmus
ICM	Inner cell mass
IFNT	Interferon tau
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGF2R	Insulin-like growth factor 2 receptor
IL4	Interleukin 4
ILV	Intraluminal vesicle
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* embryo production
k	Kilo (10^3)
LDHA	Lactate dehydrogenase A
LDLR	Low-density lipoprotein receptor
LH	Luteinizing hormone
LIF	Leukaemia inhibitory factor
LN_2	Liquid nitrogen
M	Molar concentration
min	Minutes
miRNA	Micro ribonucleic acid
ml	Millilitres
mm	Millimetres
mM	Millimolar
MnSOD	Manganese superoxide dismutase
mRNA	Messenger ribonucleic acid
mtRNA	Mitochondrial rna
MV	Microvesicles
MVBs	Multi vesicular bodies
n	Number
N_2	Nitrogen
nm	Nanometres
NTA	Nanoparticle tracking analysis
O_2	Oxigen
ºC	Degrees celsius
OEC	Oviduct epithelial cells

OF	Oviductal fluid
OPU	Ovum pick up
OVGP	Oviduct-specific glycoprotein
p.i.	Post insemination
PAF	Platelets activating factor
PAG1	Pregnancy associated glycoprotein 1
PBS	Phosphate buffered saline
PI	Propidium iodide
PKA	Protein kinase A
PLAC8	Placenta specific 8
PLIN2	Periplin 2
PMCA4a	Plasma membrane Ca^{2+} atpase 4a
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcriptase
RT-qPCR	Quantitative real time polymerase chain reaction
S.D	Standard deviation
S.E	Standard error
SC	Suspension cells
SCL2A1	Solute carrier family 2 (*SCL2A1*)
Sec	Seconds
SNRPN	Small ribonucleoprotein-associated protein N
SOF	Synthetic oviduct fluid
TALP	*Tyrode's* albumin lactate pyruvate
TCM-199	Tissue culture medium 199
TE	Trophectoderm
TFAM	Transcription factor A, mitochondrial
TGF	Transforming growth factor
TGFβ2	Transforming growth factor beta 2
tRNA	Transfer ribonucleic acid
U	Units
UBE2A	Ubiquitin-conjugating enzyme e2a
UK	United Kingdom
v/v	Volume/volume
Vault RNA	Vault ribonucleic acid
VEGF	Vascular endothelial growth factor
xg	Centrifugal force
Y RNA	Small noncoding ribonucleic acid
ZP	Zona pellucida

SUMMARY

Summary

Early embryo development and its physiological environment have a high effect on the subsequent embryonic development in short and long term. The embryo losses before or after implantation are consequences from a number of factors, some of them still unknown.

The advances in studies on early embryo environmental conditions support the development of assisted reproductive techniques such as *in vitro* embryo production that seeks to mimic the physiological conditions in order to develop an embryo in a proper stage and quality for transfer into a recipient, or for cryopreservation. These advances solved fertility problems in humans and other mammals, like in cattle that they have increased the efficiency in production and breeding schemes. In research, these techniques are important tools to assess reproductive processes that under physiological *in vivo* conditions are difficult to study.

The study of physiological mechanisms and interactions of the oviductal environment is essential to understand the fertilization process and early embryo development. To date, the use of *in vitro* embryo culture and co-culture systems with bovine oviductal epithelial cells (BOEC) allowed the discovery of many secreted components, and their relation with the embryo, associating them as beneficial (embryotrophic) for embryo development.

The oviductal fluid (OF) as a main component of oviductal environment, and a secretions vehicle, constitutes an essential element for the study of the preimplantation development. There are strong evidences of its effect on fertilization; however, currently the effect of OF during *in vitro* culture is unknown. The extracellular vesicles (EV) are somatic cell exocytosis-mediated secretions that contain lipids, proteins, miRNA and mRNA, and acting as mediators of intercellular transport. The study of EV advanced rapidly in areas such as immunology, but very little is known about their role in reproductive sphere and even less about its implications on early embryo development.

Embryo co-culture systems offer advantages attempting to mimic the *in vivo* conditions. However, BOEC primary cultures are associated with a lack of uniformity between replicates, which could be avoided using standardized/established BOEC lines. Moreover, the ability of conditioned media (CM) to support cell secretions provides an alternative to co-culture in the study of unidentified secretions. Currently, the role of the EV as mediators of cell-cell communication is unknown in the oviductal environment.

In the first experiment of this thesis the conditions to obtain the embryotrophic factors secreted by the BOEC were standardized, through *in vitro* embryo culture, and then the EV secreted by BOEC were characterized and evaluated their effect on embryo culture environment. For this purpose, different BOEC co-culture systems, BOEC-CM, BOEC-EV at different concentrations (100, 50, 25%), as well as the effects of EV freezing and culture media used on the subsequent EV secretion were compared on bovine embryo development and quality. Embryo development was determined by the number of blastocysts obtained between days 7 and 9, and embryo quality was determined by blastocyst cell number, cryotolerance, and gene expression of implantation, epigenetics, metabolism and oxidative stress related genes. The conditions to obtain BOEC embryotrophic factors were standardized, and the BOEC-EV were characterized. The average size of the BOEC-EV was ≈ 220 nm using a nanoparticles tracking analysis system (NTA) and confirmed with electron microscopy images. The BOEC, BOEC-CM and BOEC-EV improved embryo development and quality, reflected in an increased survival rates after vitrification/warming, and higher embryo cell number. In addition, the BOEC-EV modified the expression of genes related to intercellular junctions (*CX43*), implantation (*PAG1, IFNT, PLAC8*) and embryo metabolism (*GAPDH, G6PD*).

The changes on embryo development and quality could be attributed to the BOEC secretion of embryotrophic factors to the culture environment, which are present in the CM where BOEC-EV are found. The EV could act as a transporter of embryotrophic substances constituting an interaction mechanism between the

BOEC and embryo. The results of this experiment showed that BOEC co-culture besides to improve embryo quality is a suitable *in vitro* model to study possible interaction mechanisms between the mother and embryo. This is the first study where the BOEC-EV are isolated and characterized, demonstrating their effect on *in vitro* embryo development and quality.

In order to reproduce *in vitro* the physiological conditions of the oviductal environment, and to assess the effects of oviductal fluid (OF) on bovine embryo development, the second experiment of the thesis was carried out.

After a preliminary experiment testing the effect of serial dilutions of OF (25 to 0.62%), on embryo development, the lower concentrations were chosen (2.5, 1.25 and 0.62%) based on their positive effect. The optimal concentration of OF was evaluated on embryo development and quality of the produced blastocysts. The OF secretions added at low concentrations (1.25 and 0.62%) during *in vitro* culture, increased the development and quality of embryos which was reflected in higher trophectoderm cell number, cryotolerance, and expression of glucose metabolism (*SCL2A1, GAPDH*), lipid metabolism (*LDLR, CYP51, FADS1*), epigenetic (*DNMT3A, IGF2R*) and water channels transmembrane (*AQP3*) related genes. To our knowledge this is the first study demonstrating that *in vitro* culture with low concentrations of OF has a positive effect on the development and quality of bovine embryos.

It is known that the oviductal epithelium activity between the oviduct regions is depended on the stage of the estrous cycle, which reflects a dynamic in the oviductal environment. Similarly, the EV could present different functional profiles based on population or cell type origin. Based on that and in our previous results in the third experiment we purify and characterize the EV fractions present in the OF from the ampulla and isthmus using two different centrifuge forces (10k and 100k x*g*) and evaluate their impact during *in vitro* embryo culture. Embryo development and quality in terms of cryotolerance and gene expression analysis were assessed. No differences were observed in embryo development between groups. However, the oviductal fluid EV from isthmus purified at 100k improve embryo

cryotolerance, and modify the gene expression patterns of metabolism (*LDLR*), epigenetic (*DNMT3A*, *SNRPN*) and water channels (*AQP3*). These changes demonstrate an essential association between the oviductal environment and the embryo given from the EV nature. This is the first study that characterizes the OF-EV and studies its effect on embryo development *in vitro*.

RESUMEN

Resumen

El desarrollo embrionario preimplantacional y su ambiente fisiológico tienen un enorme impacto en el desarrollo embrionario posterior. Las pérdidas embrionarias antes o después de la implantación son consecuencia de una serie de factores, algunos de ellos aún desconocidos.

Los avances en estudios sobre el ambiente preimplantacional han permitido el desarrollo de técnicas de reproducción asistida como la producción *in vitro* de embriones, que busca imitar las condiciones fisiológicas preimplantacionales, con el objeto de crear un embrión en un estadio de desarrollo y con calidad suficiente que o bien permita su implantación tras la transferencia a una hembra receptora, o bien permita su criopreservación. Estos avances han logrado, resolver problemas de fertilidad tanto en humanos como en otras especies de mamíferos, como el vacuno, en la cual han permitido incrementar la eficiencia en los esquemas productivos y de mejora genética. En investigación, estas técnicas suponen herramientas importantes, al permitir evaluar procesos reproductivos que en condiciones fisiológicas *in vivo* son difíciles de estudiar.

El estudio de los mecanismos fisiológicos que acontecen en el ambiente preimplantacional oviductal es de vital importancia, para comprender tanto los fenómenos de fecundación como de desarrollo embrionario temprano. Hasta la fecha se han empleado sistemas *in vitro* de cultivo y co-cultivo embrionario con células epiteliales del oviducto (BOEC) que permitieron descubrir muchos componentes secretados, así como los procesos del desarrollo embrionario en los que intervenían, atribuyéndoles la liberación de sustancias beneficiosas (embriotróficas) para el desarrollo del embrión.

El fluido oviductal (OF) constituye tanto el componente principal del ambiente oviductal, como el propio vehículo de las secreciones y lo convierte en una pieza clave en el estudio del desarrollo preimplantacional. Si bien existen evidencias importantes de su efecto sobre la fecundación, actualmente no se conoce su efecto en los sistemas de cultivo *in vitro*. A su vez, las Vesículas Extracelulares

(EV) son secreciones de las células somáticas mediadas por mecanismos de exocitosis que contienen lípidos, proteínas, ARNmi y ARNm, y actúan como mediadoras del transporte intercelular. Si bien su estudio avanza rápidamente en áreas como la inmunología, actualmente se sabe muy poco sobre el rol de las EV en el ámbito reproductivo y menos aún sobre sus implicaciones a nivel preimplantacional.

Los sistemas de co-cultivo embrionario ofrecen muchas ventajas al buscar imitar las condiciones *in vivo*, sin embargo, los cultivos primarios de BOEC presentan falta de homogeneidad entre réplicas, suceso que se podría evitar usando líneas estandarizadas / establecidas de BOEC. Por otra parte, la capacidad de los medios condicionados (CM) para vehiculizar las secreciones celulares, los constituye en una alternativa a los co-cultivos, y una herramienta de estudio para secreciones no identificadas. Las EV actualmente son secreciones que actúan como mecanismos de comunicación intercelular de los cuales se conoce poco en el ambiente oviductal.

En el primer experimento de esta tesis se estandarizaron las condiciones para la obtención de factores embriotróficos secretados por las BOEC, y se analizaron sus efectos sobre el cultivo *in vitro* de embriones en términos de desarrollo y calidad. Para este fin, se compararon diferentes sistemas de co-cultivo con BOEC, de sus CM y de diferentes concentraciones de EV (100, 50, 25%). También se evaluó el efecto de la congelación de EV, así como el efecto de distintos medios de cultivo de las BOEC sobre la secreción de EV. El desarrollo embrionario se determinó con el número de blastocistos obtenidos entre los días 7 y 9, y su calidad mediante el recuento de las células embrionarias, la tolerancia a la vitrificación, y la expresión de genes relacionados con implantación, epigenética, metabolismo y estrés oxidativo entre otros. Además de lograr estandarizar las condiciones para la obtención de sustancias secretadas a través de líneas de BOEC, se caracterizaron las EV presentes en los CM de las BOEC. El tamaño medio de las EV fue ≈ 220 μm, calculado mediante un sistema de análisis de nanopartículas (NTA) y confirmado con imágenes de microscopía electrónica. Las BOEC, los CM y las EV favorecieron

el desarrollo embrionario *in vitro,* así como la calidad embrionaria al reflejar una mayor supervivencia tras la vitrificación y un mayor número de células embrionarias. Además, las EV modificaron la expresión de genes relacionados con uniones intercelulares (*CX43*), implantación (*PAG1, IFNT, PLAC8*) y metabolismo embrionario (*GAPDH, G6PD*).

Como posibles responsables de los efectos en el desarrollo y calidad del embrión bovino, se encuentran los factores embriotróficos secretados por las BOEC al sistema de cultivo y presentes en los CM, donde también se encuentran las VE, cuya naturaleza además de constituirlas en un medio de transporte para sustancias embriotróficas, puede perfilarlas como un mecanismo de interacción entre las BOEC y el embrión. Los resultados de este capítulo evidencian cómo el co-cultivo con BOEC además de modificar la calidad embrionaria, es un modelo *in vitro* adecuado para el estudio de posibles mecanismos de interacción entre la madre y el embrión. Este es el primer estudio de aislamiento y caracterización de EV provenientes de BOEC, demostrando además su implicación en desarrollo y calidad embrionaria *in vitro.*

Con el objeto de reproducir *in vitro* las condiciones fisiológicas del ambiente oviductal, para evaluar los efectos del fluido oviductal (OF) sobre el desarrollo embrionario bovino, se llevó a cabo el segundo experimento de la tesis.

Tras realizar un experimento preliminar testando el efecto de diluciones seriadas de OF (25 a 0,62%) sobre el desarrollo embrionario, se eligieron aquellas concentraciones de OF que tuvieron un efecto positivo (2.5, 1.25 y 0.62%). Se evaluó la concentración óptima sobre el desarrollo y la calidad de los blastocistos producidos *in vitro.* Las secreciones presentes en el OF, adicionadas a bajas concentraciones (1.25 y 0.62%) durante el cultivo *in vitro,* incrementaron el desarrollo y la calidad de los embriones, reflejado en un incremento de su criotolerancia y del número de células de trofoectodermo, y en la expresión de genes relacionados con metabolismo de glucosa (*SCL2A1, GAPDH*), metabolismo de lípidos (*LDLR, CYP51, FADS1*), epigenética (*DNMT3A, IGF2R*) y canales de agua transmembrana (*AQP3*). Este es el primer estudio que demuestra que el cultivo

in vitro con bajas concentraciones de OF presenta un efecto positivo sobre el desarrollo y la calidad de los embriones de bovino.

Existen diferencias en la actividad del epitelio oviductal entre las distintas zonas del oviducto en función de la fase del ciclo estral, que demuestran una dinámica en el ambiente oviductal. Igualmente, las EV pueden presentar perfiles funcionales diferentes en función de la población o tipo celular del que proceden. En el tercer experimento, y en base a los resultados de los capítulos anteriores, se aislaron y caracterizaron las fracciones de EV presentes en el OF de las dos regiones anatómicas del oviducto (ampolla e istmo) usando dos velocidades de centrifugación diferentes (10k y 100k x*g*), y se evaluó su repercusión en los sistemas de cultivo embrionario *in vitro,* en términos de desarrollo y calidad embrionaria, a través de los análisis de criotolerancia y de los perfiles de expresión génica. Las EV procedentes del istmo purificadas a 100k x*g*, mejoran significativamente la criotolerancia de los embriones, y los patrones de expresión de genes relacionados con metabolismo (*LDLR*), epigenética (*DNMT3A*, *SNRPN*) y canales de agua (*AQP3*). Estas modificaciones evidencian una asociación esencial entre el ambiente oviductal y el embrión dada la naturaleza de las EV. Este es el primer estudio que caracteriza las EV presentes en el OF y estudia su efecto sobre el desarrollo embrionario *in vitro*.

1. LITERATURE REVIEW

1. Literature Review

1.1. Physiological aspects of gametes and early embryo development *in vivo*

Previous to fusion, the gametes experiment different events. In cattle, the oogenesis starts in the fetal development (day 75-80 of pregnancy) but after that remains inactive until puberty. The oogonias experiment mitotic divisions and produce primary oocytes (Gondos *et al.*, 1986). During oogenesis, the meiotic prophase begins in prepleptotene and ends in arrested metaphase II (Baker and Franchi, 1967). The primary oocytes are surrounded by mesenchymal cells constituting the primordial follicles (Rüsse, 1983). The follicular growth begins when the shape of the granulosa cells change from flattened to cuboidal constituting the primary follicle (Braw-Tal and Yossefi, 1997). Then, the increase of granulosa cells in layers in the follicles, originates the secondary follicles. Granulosa cell proliferation finish in the tertiary follicles, also called antral follicles (Lussier *et al.*, 1987).

The follicular development is regulated by gonadotropins, the luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These hormones are secreted in the pituitary gland. The elevation of FSH during the follicular phase has a stimulatory factor to recruit a new batch of primary follicles to begin the development. When a follicle reaches 9-10 mm of diameter (dominant follicle) (Ginther *et al.*, 1996) the LH receptors increase on its granulosa cells (Adams *et al.*, 2008) and change its dependence from FSH to LH to continue growing. In addition, the dominant follicle secretes oestrogens and inhibin that block the secretion of FSH, necessary for the growth of the subordinate follicles (Ginther *et al.*, 1996) and therefore they become atretic. The oestrogen secretion is achieved by a coordinated mechanism between theca cells that produce androgens and granulosa cells, which aromatize androgens to estradiol. The dominant follicle can secrete more estradiol because it has more theca and granulosa cells (Fortune, 1994). The increase in estradiol levels initiates the oestrus behavior, prepares the reproductive tract for fertilization and initiates the ovulatory peak of LH (Ball and Peters, 2008).

It is proposed that LH surge stimulates the process of ovulation by activating an inflammatory reaction which: (1) thins and ruptures the follicle wall (Espey, 1980); and (2) initiates the luteinisation of the granulosa and theca cells of the follicle, in preparation for the development of the CL.

The oocyte maturation begins in the meiotic arrest (prophase I - first meiotic division) and characterized by a large nucleus called germinal vesicle (GV) (Voronina and Wessel, 2003). Within the possible mechanism of oocyte arrest resumption, are proposed the GV interaction with stimulant factors as hormones and paracrine factors (cAMP, PKA, GPR3, ions) (Freudzon *et al.*, 2005) as well as the cAMP mechanism regulated by FSH (Sutton *et al.*, 2003). During maturation the oocyte secreted factors as TGF has an effect in the expansion of the surrounding cumulus cells (Hussein *et al.*, 2006).

During fertilization process many events occur, which end with male and female gametes fusion. At the time of ejaculation sperm is not able to fertilize. The sperm require a cascade of changes to acquire the fertilizing ability. These events take place during sperm transit through the different sections of female reproductive tract (Machaty *et al.*, 2012). These events are known as capacitation, and are specie-specific (Florman and First, 1988).

Sperm capacitation begins with seminal plasma proteins removal by glycosaminoglycans present in the uterus and finishes in a characteristic sperm motility called hyperactivation (Lin *et al.*, 1994). Hyperactivation makes the spermatozoa capable of crossing the surrounding cumulus cells to arrive to the zona pellucida (ZP). Zona pellucida is composed of three types of glycoproteins, ZP1, ZP2 and ZP3. The contact between sperm and ZP induces sperm acrosome reaction. During the acrosome reaction the acrosome content are released outwardly (proteolytic enzymes) (Bleil and Wassarman, 1980). Sperm binding to ZP is mediated by receptors to stimulate the acrosome reaction and to permit the ZP penetration (Breitbart *et al.*, 1997; O'Toole *et al.*, 2000). After that, sperm and oocyte membranes fuse due to associated unclear mechanisms (Primakoff and Myles, 2000).

After gamete fusion, oocyte cytoplasm begins to divide in smaller compartments due to mitotic events called "cleavage". The mRNA and proteins synthesized and stored in the oocyte during oogenesis, support the first stages of embryo development (Memili and First, 1999). The conditions which the first mitotic division take place (cleavage) are critical for the subsequent development of the embryo (Betts and King, 2001). In cattle, when the embryo reached the 8-16 cell stage (blastomeres) maternal RNA is degraded and the genome of the embryo is activated (Embryo Genome Activation - EGA) (Barnes and First, 1991) as shown in figure 1. The oocyte has the ability to support several mitotic divisions without substantial new mRNA transcription, surviving with maternal mRNA until the EGA. The mechanism behind this mRNA stability in the oocyte is not yet understood (Holt and Bullock, 2009). It is believed that the RNA stability is due to the RNA configuration of oocyte, and may result in interaction with ribonucleoproteins to suppress translation (Sirard, 2012). Of the maternal transcriptomes, the majority was not influenced by environmental factors up to a certain stage (Mamo *et al.*, 2012). Upon EGA, many genes are expressed differentially due to embryo dynamic and environmental effects (Vallée *et al.*, 2009). Studies over EGA in *in vivo* bovine embryos through microarrays, detected the expression of an average of 15,000 genes or variants in oocytes and early embryos (Robert *et al.*, 2011). Before EGA, some of the maternal mRNA is recruited for translation and some is degraded (Tadros and Lipshitz, 2009). Degradation of maternal transcriptome is not synchronic with the EGA. However some genes may start to be transcribed at earlier stages (Plante *et al.*, 1994; Memili and First, 1999); this is based on the presence of novel RNA or proteins in the early stages (two to four-cell stages) (Memili and First, 1999). EGA constitutes a crucial event in the early embryo development. If this activation fails, differentiation and embryo implantation will not occur (Memili and First, 1999; Gad *et al.*, 2012; Graf *et al.*, 2014).

When the embryo reaches the 32-64 cell stage (morula), the adjacent blastomeres are tightly bind by gap junctions and desmosomes becoming nearly

indistinguishable in a process called compaction (Van Soom *et al.*, 1997). This will result in the formation of a communicating polarized epithelium (Schultz *et al.*, 1999). Then, the activation of an ion transport favors the formation of a cavity inside the morula called blastocoel. During this process the cells start to differentiate, originating the inner cell mass (ICM) that will give rise to the embryo and the trophectoderm cells (TE), which will give rise to extra-embryonic tissue (Van Soom *et al.*, 1997). This embryonic stage is known as blastocyst. In cattle, the embryo transits in the oviduct until day 3-4 when passes into the uterus during the morula stage.

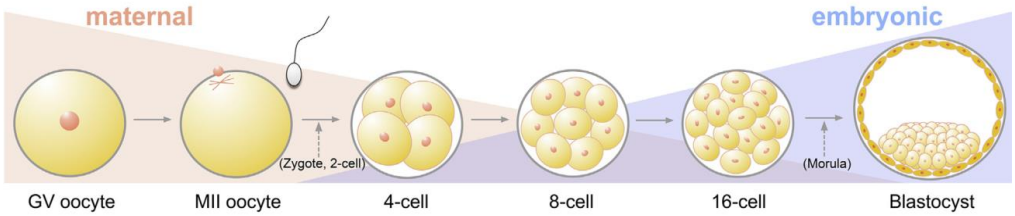

Figure 1. Embryo Genome Activation Scheme.
Adapted from Graf *et al.* (2014)

1.2. Embryo development *in vitro*

Cattle production plays an important role in food and dairy industry around the world. To increase the efficiency of breeding schemes in cattle, *in vitro* embryo production (IVP) is a powerful tool (Galli *et al.*, 2003). Moreover, the IVP in different mammals allows us to enhance our understanding of early embryo development, above all during the preimplantational stages.

In cattle, IVP allows to obtain embryos from: (i) oocytes derived from slaughtered heifers (Galli *et al.*, 2003); or (ii) live donors by ultrasonography follicular aspiration (Ovum pick up - OPU) (van Wagtendonk-de Leeuw, 2006). Besides, IVP offers the opportunity to recover and safe oocytes from high value animals when they die, as well as to continue the existence of valuable endangered breeds (Wu and Zan, 2012).

The advantages of IVP in cattle are evident; however its efficiency is still low. When cattle IVP are compared with other species (e.g: mice) is considerably less efficient. Lonergan and Fair showed that IVP in bovine presented significantly lower yield of blastocysts and competent embryos than the *in vivo* process (Lonergan and Fair, 2008). These deficiencies are subject of research to identify the mechanisms involved that will permit to improve the conditions of IVP systems and the subsequent blastocyst yield and quality.

The IVP is divided in three stages that try to mimic what take place *in vivo*: *in vitro* maturation, *in vitro* fertilization and embryo culture.

1.2.1. *In vitro* maturation (IVM)

The *in vitro* maturation is designed to support the development of the oocyte from the meiotic arrest to the metaphase II stage. During this event, the oocyte becomes matured (cytoplasmatically and nuclearly) which is determinant for the subsequent fertilization and further development. It has been demonstrated that factors related with the oocyte source like follicle size, age, breed and health of donors influence its competence (Gilchrist *et al.*, 2004). A better understanding of the *in vivo* mechanisms and pathways involved are required to improve IVM (Wrenzycki and Stinshoff, 2013). After 20-24 hours of incubation the oocyte complete maturation with the extrusion of the first polar body and is ready to be fertilized (Galli *et al.*, 2003).

1.2.2. *In vitro* fertilization (IVF)

The IVF is designed to facilitate the union of the gametes. The media used is designed with a specific ionic balance for oocyte and sperm requirements (TALP or SOF media) (Parrish *et al.*, 1988). To induce the sperm capacitation media is supplemented with capacitate substances as glycosaminoglycan (Heparin) (Parrish, 2014). For IVF, frozen sperm is usually used. Dead spermatozoa and diluents are separated from the motile sperm through a sperm selection method. Techniques like "swim up" (Parrish *et al.*, 1986) and a discontinuous density gradient (Percoll® or Bovipure®) are successfully used (Mendes Jr. *et al.*, 2003; Samardzija *et al.*, 2006)

(Figure 2). The time of co-incubation between matured oocytes and selected motile sperm is between 17 and 22 hours. This period spermatozoa cross all physiological barriers, fuse with the ooplasma, the oocyte is activated and the pronuclear formation begins (Schultz and Kopf, 1995; Galli *et al.*, 2003).

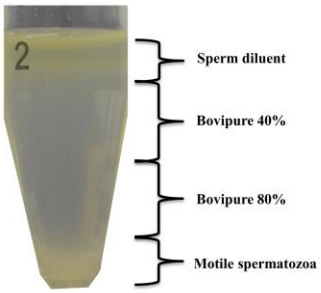

Figure 2. Discontinuos density gradient for motile sperm selection - Bovipure®.

1.2.3. *In vitro* embryo culture

There are different systems used to culture bovine zygotes. In cattle, IVC media is constituted by chemically defined elements (Defined media) based on the biochemical composition of the oviductal fluid (synthetic oviductal fluid - SOF) (Tervit *et al.*, 1972) with amino acids (Rosenkrans *et al.*, 1993; Holm *et al.*, 1999). This media can be supplemented with biological elements more (Bovine serum albumin-BSA) or less defined (fetal calf serum-FCS) to support successfully early embryo growth but with different results in terms of embryo yield and embryo quality (Rizos *et al.*, 2003). Other system are the co-culture *in vitro* with somatic cells (oviductal epithelial cells, granulosa cells, buffalo rat liver, and Vero cells) in chemically defined media with FCS or BSA, or in medium conditioned by somatic cells (Vansteenbrugge *et al.*, 1994). In the latter systems, most of the blastocysts will form 6-7 days later (i.e. 7-8 days post insemination). The embryos secrete factors that sustain their development and for this reason they grow better in groups than alone (Gardner *et al.*, 1994). Thus, in most of the *in vitro* culture systems the embryos are cultured in droplets under paraffin oil. The incubation is performed at 38.5ºC in 5% CO_2, and under 5% O_2 or not. Alternatively, *in vitro* produced zygotes

can be cultured *in vivo* after transfer to the oviduct of a temporary recipient as sheep (Galli and Lazzari, 1996) or cow (Wetscher *et al.*, 2005) or *ex vivo* in mouse isolated oviducts (Rizos *et al.*, 2010).

1.2.3.1. Culture conditions and embryo development

The oocyte source and the environment of the ovarian follicle could influence the transcriptome of the matured oocyte and the subsequent cleavage stage of the embryo (Sirard, 2012). A correctly programmed oocyte can support the subsequent embryo development, evidenced in the efficiency and quality of blastocyst produced *in vivo* (Graf *et al.*, 2014). The cleavage speed (mitotic divisions) has been correlated with the subsequent blastocyst rates in bovine (Lonergan *et al.*, 2000) and humans (Salumets *et al.*, 2003), and related factors as oocyte, spermatozoa and culture conditions affect the timing of this event (Lechniak *et al.*, 2008). Besides, it is known that the faster cleaved embryos have a higher chance to develop to the blastocyst stage (Lonergan *et al.*, 1999) and that IVC environment impacts the mRNA expression and quality of the embryo (Rizos *et al.*, 2002a). During the evolution of IVC associated problems like "zygote arrest" or "8-16 cell block" have been identified (Camous *et al.*, 1984) and resolved (Rosenkrans *et al.*, 1993). Under *in vitro* conditions the dynamic of embryo development previous to compaction, are related to the subsequent developmental stages (Gutiérrez-Adán *et al.*, 2004a). Crucial processes in early embryo development are compaction and cavitation of the morula. Compaction is a prerequisite to TE formation, where are involved cell-cell adhesions between blastomeres, mediated by molecules (E-cadherin) and tight junction proteins (Van Soom *et al.*, 1997; Gordon, 2003). The blastocoel formation is accompanied for the formation of TE and the ICM. This process is mediated by water influx of polarized epithelium were several molecules are involved as aquoporins (Offenberg *et al.*, 2000). Compaction and cavitation are dependent of adequate embryonic gene transcription. In IVP embryos the compaction occurs in lesser degree that in the *in vivo* counterparts (Van Soom *et al.*, 1997). *In vitro* culture systems have advanced on embryo production sacrificing the health of the embryo and the subsequent fetus and offspring. To obtain a competent embryo

under *in vitro* conditions requires accurately mimicking of most if not all constituents of the *in vivo* environment. The differences between *in vivo* and *in vitro* embryo culture systems (Eyestone *et al.*, 1987; Galli *et al.*, 2001) are evidenced in the pregnancy rates of *in vivo* derived embryos (Wetscher *et al.*, 2005). All the knowledge used to develop an optimal embryo culture environment has been generated from the results obtained with animal models (rodents and mammals) (Ménézo and Hérubel, 2002). However, we are still far away from the optimum *in vitro* culture system such as only 30-40% of the matured oocytes used develop to blastocyst stage (Rizos *et al.*, 2008).

1.2.3.2. Culture conditions and embryo quality

Despite the considerable improvements of assisted reproductive technologies in the last two decades, *in vitro* embryo production processes, designed in simulated environment to support early embryo development, are yet far to the physiological conditions (Lonergan and Fair, 2008). These conditions are suboptimal, as is evidenced by lower blastocyst yields (30 - 40%), lower cryotolerance (Rizos *et al.*, 2008), altered inner cell mass/trophectoderm cells ratio (Plourde *et al.*, 2012), altered gene expression patterns (Niemann and Wrenzycki, 2000), and lower pregnancy rates of transferable embryos (Pontes *et al.*, 2009).

The addition of foetal bovine serum to the culture media accelerates embryonic developmental kinetics and increases the number of embryonic cells (Van Langendonckt *et al.*, 1997). However, embryos cultured in the presence of serum have a lower level of compaction at the morula stage (Thompson, 1997); exhibit a greater accumulation of lipid droplets in the cytoplasm (Rizos *et al.*, 2002a); have lower cryotolerance (Rizos *et al.*, 2002a) and present alterations in gene expression (Rizos *et al.*, 2003) compared to *in vivo* produced embryos. In addition, serum has been linked to the "Large Offspring Syndrome" (LOS) (Farin *et al.*, 2001; Lazzari *et al.*, 2002), that causes the birth of large calves with musculoskeletal disorders, alterations in the development of the allantois and defects in vascularization and development of the placenta, showing a smaller area of maternal-fetal contact (Farin *et al.*, 2001, 2006).

It has been showed that the ligated sheep oviduct can provide an adequate environment, not only for sheep embryos but also for those from other farm species, including cattle (Rizos *et al.*, 2002a). While culture of *in vitro* produced zygotes in the ewe oviduct did not affect blastocyst yields, the oviductal environment of the intermediate recipient clearly improved the quality of the blastocysts, as measured by survival after cryopreservation (Rizos *et al.*, 2002b) and pregnancy rates (Lazzari *et al.*, 2002). Furthermore, the significance influence of culture conditions, *in vitro* or *in vivo* in the homologous bovine oviduct, on the transcriptome of the embryos in relation to embryonic genome activation has been well demonstrated (Gad *et al.*, 2012).

The best method of evaluation of each embryo produced is the ability to establish and maintain a pregnancy after transfer. However, for practical and economic reasons, it is only ever possible to transfer a subset of embryos. Therefore, alternative methods for evaluating embryo quality have been developed. These embryo quality assessments are considered valuable tools to understand or identify the *in vitro* culture conditions.

1.2.3.2.1. Morphology

The blastocyst morphology is the first parameter considered for the immediate transference or cryopreservation. Under light microscope, *in vivo* embryos present a lighter color when compared to their *in vitro* counterparts (Fair *et al.*, 2001; Rizos *et al.*, 2002a) (Figure 3). These morphological differences between *in vivo* and *in vitro* embryos have been studied at ultrastructural level. Fair *et al.* elucidated the effect of culture environment on the blastocyst morphology; showing that zygotes cultured *in vivo* (ewe oviduct) had similar morphology to the *in vivo* counterparts, and the blastocyst cultured in SOF with FCS present differences from these two groups, in terms of cell-to-cell contacts (Fair *et al.*, 2001). Rizos *et al.* evidenced that *in vivo* embryos present a higher plasma membrane attachment to ZP, a dense-continuous cover of microvilli, a closely connected TE an ICM cells surrounded by small intercellular spaces, a small number of lipid droplets and a healthy mitochondria (Rizos *et al.*, 2002a). The *in vitro* counterparts exhibited less

and discontinuous microvilli over the plasma membrane, and increased lipid content (Rizos *et al.*, 2002a). Higher lipid content in blastocyst is related to a lower cryotolerance (Abe *et al.*, 2002). The differences at ultrastructural level, between *in vivo* and *in vitro* embryos, even between embryos produced *in vitro* in different culture systems may in part explain the variation in cryotolerance observed (Fair *et al.*, 2001; Rizos *et al.*, 2002a).

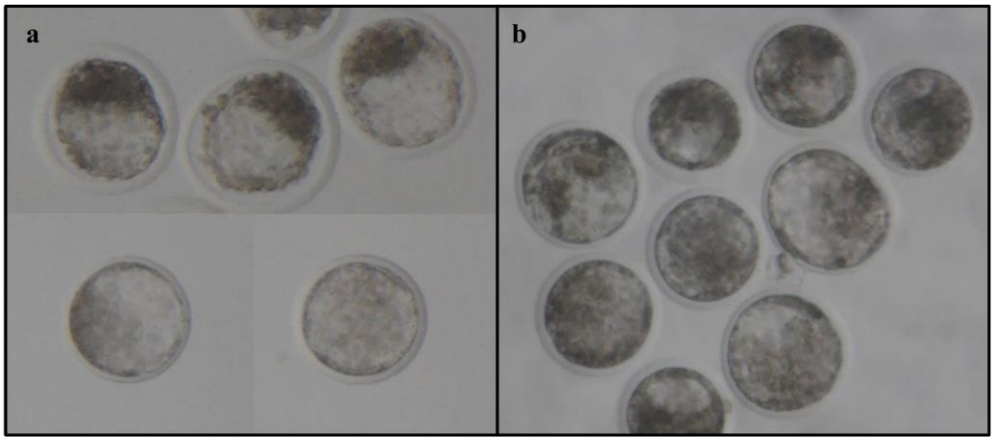

Figure 3. In vivo (a) and in vitro (b) derived D7 embryos

1.2.3.2.2. Cryotolerance

The ability of the embryo to tolerate the stress caused by cryopreservation and to continue the development is considered a predictor of embryo quality and viability (cryotolerance) (Rizos *et al.*, 2002b, 2003; Moore *et al.*, 2007). Culture conditions influence the survival and subsequent development of *in vitro* produced embryos after the cryopreservation. The *in vitro* embryos are more sensitive to cryoinjury than the *in vivo* embryos (Hasler *et al.*, 1995). Mucci *et al*. compared the supplementation of Charles Ronsenkrans + amino acids medium (CR1aa) with estrous cow serum and⁄or BSA, reporting similar blastocyst rates (30.9-33.1%), but higher post-thaw survival rates (24h) with CR1aa with BSA (51.9 vs. 25%) (Mucci *et al.*, 2006). Rizos *et al*. showed that SOF with FCS reduced the cryotolerance of vitrified bovine blastocysts. Moreover, the presence of FCS during *in vitro* culture increase the number and size of lipid droplets (2–6 and >6 mm) in the embryos when compared with the once produced in FCS free systems (2 mm) (Rizos *et al.*,

2003). This lipid accumulation plays an important role reducing embryo cryotolerance (Abe *et al.*, 2002). Embryo co-culture with granulosa cells monolayers reduced blastocyst yiels on day 7 (13 vs. 31%), but had a positive effect on survival rate after vitrification and warming when compared to SOF with FCS produced embryos (Rizos *et al.*, 2001). Similarly Mermillod *et al*. using a BOEC co-culture improved the blastocyst rates of zygotes cultured in SOF (41 vs. 27%) and TCM media (28 vs. 10%), and the survival rates after vitrification (69 vs. 22%) (Mermillod *et al.*, 2010). *In vivo* culture (ewe oviduct) of IVF zygotes, increase dramatically the embryo cryotolerance, to a similar levels to their *in vivo* counterparts (Galli and Lazzari, 1996; Rizos *et al.*, 2002b). This was confirmed by Rizos *et al* who showed that blastocysts cultured either in the ewe oviduct or produced totally *in vivo* presented higher cryotolerance than the *in vitro* once produced in SOF with FCS (88.0 vs. 5.6%) (Rizos *et al.*, 2002b).

1.2.3.2.3. Gene Expression

Analysis of expression patterns of developmentally important genes essential in early development provides valuable information about the effect of environmental factors on the early embryo. The products of these genes are involved in various biological processes including metabolism, growth factors - cytokine signaling, stress adaptation, transcription and translation, epigenetic regulation of transcription, apoptosis, compaction and blastocyst formation (Wrenzycki *et al.*, 2005).

It has been clearly demonstrated the effect of culture environment on gene expression in the developing embryo (Lonergan *et al.*, 2003; Wrenzycki *et al.*, 2005; Cagnone *et al.*, 2012) and this effect is evidenced under *in vitro* and *in vivo* culture systems, and between *in vitro* systems (Eckert and Niemann, 1998; Natale *et al.*, 2001; Rizos *et al.*, 2002c; Rinaudo and Schultz, 2004; Wrenzycki *et al.*, 2005). A very clear example is the presence of FCS in SOF media (Rizos *et al.*, 2003) modifying the expression level of qualitatively important genes related with metabolism, compaction and implantation. On the other side, *in vivo* cultured (e.g.:

ewe oviduct) bovine blastocyst had similar expression patterns with totally *in vivo* once (Lazzari *et al.*, 2002; Rizos *et al.*, 2002b).

In the same extend, Rizos *et al*. after culturing bovine zygotes *ex vivo* in isolated mouse oviducts showed that most of the gene expression transcripts studied on these embryos were similar to that of embryos derived either from zygotes cultured *in vivo* in the ewe oviduct or *in vivo* produced; however, the effect was related with the media used for culturing the oviducts, SOF or KSOM (Rizos *et al.*, 2007). In a microarray study between *in vitro* and *in vivo* produced embryos, (Corcoran *et al.*, 2006) found that 85% of the differentially expressed transcripts were downregulated in *in vitro* blastocysts, suggesting that the causal of lower developmental competence of *in vitro* embryos is a deficiency of the machinery associated with transcription and translation.

1.2.3.2.4. Embryo cell number

The evaluation of the embryo cell number is considered also as a valuable embryo quality test (Thouas *et al.*, 2001). A possible asynchrony in the mitotic division in the blastomeres could be reflected in the compaction stage of the morula, and in the subsequent inner cell mass and trophectoderm cell differentiation (Johnson and Ziomek, 1981). Differential staining can be used to obtain a better estimation of embryo quality and embryo differentiation (Figure 4). Therefore, this technique allows to compare ICM and TE development in different culture conditions to evidence deviations in embryo development (Van Soom *et al.*, 2001). Iwasaki *et al*. evaluated the percentage of ICM to TE cells in bovine embryos founding them cultured *in vitro* with fewer total cells as well as a significantly lower proportion of ICM compared to those cultured *in vivo* (Iwasaki *et al.*, 1990). Clemente *et al*. used differential cell count to evaluate the quality of *in vitro* bovine blastocysts in presence or absence of progesterone (Clemente *et al.*, 2009). Similarly, Rodriguez *et al*. demonstrated that retinoic acid and specific receptor agonist increases the number of cells in the ICM and TE of *in vitro* produced bovine embryos (Rodríguez *et al.*, 2006, 2007). Recently, Trigal *et al*. evaluated the use Activin A under *in vitro* conditions on bovine embryo development and quality,

finding a lower number of TE cells in embryos cultured in absence of Activin A (Trigal *et al.*, 2011).

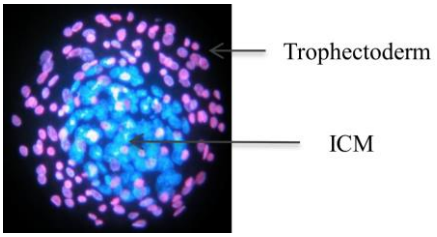

Figure 4. D8 p.i. bovine blastocyst stained with bisbenzimide and propidium iodide**.**

1.3. Role of the oviduct in early embryo development

1.3.1. Anatomophysiological characteristics of the bovine oviduct

The oviduct in bovine is a tubular structure, sustained by the mesosalpinx that connects the ovary with 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. The infundibulum is the most proximal structure to the ovary and is funnel shaped, and with its fimbriae receives 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. The isthmus presents a narrow lumen, is the sperm reservoir previous to fertilization and also where the early stages of embryo development take place. The utero-tubal junction, connect the isthmus with the uterus (Yániz *et al.*, 2000).

Histologically, the oviduct in their external surface it is composed by a serosa layer (*Tunica serosa*), followed by the Tela subserosa, constituted by muscle fibers and blood vessels. The subsequent muscle layer (*Tunica muscularis*) is different in each oviduct segments, in the infundibulum and the ampulla it is thinner than in the isthmus. The inner part of the oviduct (*Tunica mucosa*) is composed by fibrous and cellular connective tissue and epithelial cell layer (Ellington, 1991).

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). The first stages of bovine embryo development occur in the oviduct, where the embryo spends around 4 days (Hackett *et al.*, 1993). This, generate an interest to know its role as multifunctional and specialized reproductive organ (Rodriguez-Martinez, 2007; Leese *et al.*, 2008).

The infundibulum receives the oocyte in the ovulation with dynamic movements. The ciliated cells receive and guide the oocyte to the oviduct lumen. In the ampulla, the final events of oocyte maturation and the fertilization take place (Talbot *et al.*, 2003). At the other extreme, the sperm remains in the isthmus particularly in the distal part. The isthmus described as functioning as a sperm reservoir, sperm adhere transiently to the epithelium, and at the time of ovulation they are released (Hunter and Wilmut, 1984; Talevi and Gualtieri, 2010). In these phase, the oviductal environment and sperm-OEC interactions ensures their proper viability, motility and fertility (Talevi and Gualtieri, 2004). Sperm release from the OEC is attributed to a mechanism mediated by heparin-like glycosaminoglycans considered physiological components of the bovine oviductal fluid which concentrations and activity are dynamic, during the estrous cycle (Bosch *et al.*, 2001). Oviduct adhesion contact also contributes to sperm selection of higher quality sperm subpopulation (Gualtieri and Talevi, 2000, 2003) represented by the ability to bind the zona pellucida and fertilize the oocyte (Talevi and Gualtieri, 2004). After fertilization in the ampullary-isthmic junction, the developing embryo passes through the isthmus, supported by ciliary beat and muscular contractions, until it reaches the uterus at about the 16 cell stage on day 4 (Ellington, 1991). A schematic representation of the oviduct and processes involved are shown in the figure 5.

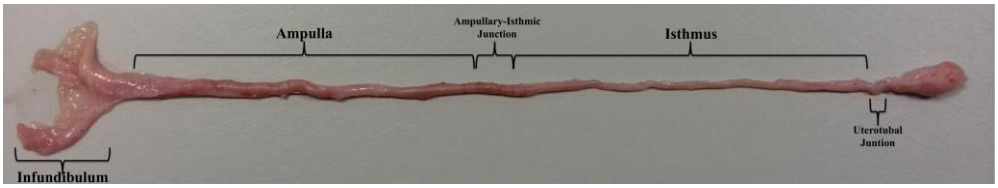

Figure 5. Schematic representation of the oviduct and its anatomical parts

1.3.1.1. Oviductal epithelium

The oviductal epithelium is the intimate part of the oviduct. Oviductal epithelium is composed of two different cell types, ciliated and secretory. During transport processes, the ciliary show a synchronized movement leading a directed flow of fluids (Abe and Hoshi, 1997). Secretory cells present microvilli on their apical side and secrete substances (oviduct-specific glycoprotein) and growth factors, usually by exocytosis, associated with the first days of the estrous cycle, which contribute on the development of the early embryo (Abe, 1996; Murray, 1997).

Populations of OEC types are dynamic during the phases of the estrous cycle. The percentage of ciliated cells decreases in the infundibulum and the ampulla during the luteal phase compared with the follicular phase (Yániz *et al.*, 2000). Interestingly, it has been suggested that cell morphology is modified in function of embryo development and cyclic changes (Suuroia *et al.*, 2002). Thus, the height of ciliated cells decreases in the infundibulum and ampulla in the luteal phase and in the isthmus the height of secretory cells also diminishes (Abe *et al.*, 1999). Studies concerning gene expression in bovine oviducts, evidenced the ovary and/or oocyte effect on the OEC function (Bauersachs *et al.*, 2003), as well as, the differences during the estrous cycle (Bauersachs *et al.*, 2004)

1.3.1.2. Oviductal fluid

The conditions of oviductal environment are reflected in 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 the serum (Leese, 1988; Leese *et al.*, 2007; Hugentobler *et al.*, 2008). In bovine, aspartate, glutamate, serine, glycine, tyrosine, phenylalanine, lysine and alanine of amino acids were present in higher concentrations in oviductal fluid than in blood plasma (Hugentobler *et al.*, 2007). Secretions present in the OF affect oocyte and sperm function (Killian, 2011; Mondéjar *et al.*, 2013) with proteins as glycodelins, and lactoferrin involved on gamete interaction (Ghersevich *et al.*, 2015), and oviductin, osteopontins and the complement protein C3 on early embryo development (Tse *et al.*, 2008).

During preimplantation development, bovine embryos obtain energy by oxidative phosphorylation (pyruvate and amino acids oxidation), while at compaction and blastulation switch to glycolysis (Kim *et al.*, 1993; Thompson, 2000). Hugentobler *et al*. show the association in glucose, lactate and pyruvate concentration between oviductal fluid and plasma, being OF composition mediated the composition determined by the BOEC (Hugentobler *et al.*, 2008). Georgieu *et al*. demonstrated the modulation of the oviductal environment (secretory proteomic profile) in presence of gametes in a favorable way, as well as prepare the oviduct milieu for the arrival of the embryo. Twenty proteins were regulated by sperm, and one protein was regulated by oocytes (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.*, 2005, 2007). These regulated proteins 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) and 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. Nowadays it is clear the interaction with oocytes and sperm (Buhi, 2002). OVPG1 synthesis and secretion is dynamic and related to estrogen (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 development (Buhi, 2002). Coy *et al.*, (2008, 2012). demonstrated that OVGP1 and heparin-like glycosaminoglycans (GAGs) 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 immediately surrounding gametes and embryo, preventing dispersal of essential nutrients and ions, particularly during ciliary beating or muscular contraction, by increasing the viscosity of luminal fluid (Hunter, 1994; Mondéjar *et al.*, 2012).

Coy *et al*. evaluate the oviductal fluid (30 min. incubation) influence on the ZP in pig and cow oocytes, and demonstrate an increasing in the proteolytic resistant of the ZP reflected in increased pronase digestion times (3-8h.), and a modulation of sperm-ZP interaction through increased monospermy (Coy *et al.*, 2008). Lloyd *et al*. exposed *in vitro* matured porcine oocytes to bovine OF during 30 min before fertilization increasing the blastocyst rate, and quality in terms of morphology, cell numbers, as well as gene expression patterns of apoptotic and developmentally related genes (Lloyd *et al.*, 2009). In the same line, in bovine, Cebrian Serrano *et al*. evaluated the effect of short incubation of matured oocytes with bovine OF with no effect on embryo development but with notable modification on genes transcripts like G6PD and SOD32 (Cebrian-Serrano *et al.*, 2013).

1.3.2. Embryo-maternal dialogue in the oviduct

For a successful pregnancy establishment, a complex signal exchange between the newly formed embryo and the maternal reproductive tract is essential. In ruminants, the principal pregnancy-recognition signal sent by the embryo is interferon tau, secreted by the trophoblast from day 10 up to Day 21-25 (Spencer and Bazer, 2004). While, a high proportion of embryonic losses occur between Day 8 and 17 of pregnancy (Humblot, 2001; Thatcher *et al.*, 2001). Alterations in the environment of the early embryo could have consequences in the subsequent development. The oviduct as first environment of the embryo development is considered the starting point to search any signal 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). Thus, the exchange of signals between the embryo and the oviduct are remarkable. However, the mechanisms involved on this embryo-maternal communication currently are mostly unknown (Fazeli, 2008).

Evidence *in vivo* in mice, by RT-qPCR showed changes in the oviduct gene expression depending on the presence or absence of embryos (Lee *et al.*, 2002). Recently, new sequencing technologies (microarrays) have used to elucidate this question of a complex molecular dialogue between the maternal tract and embryo in pigs (Almiñana and Fazeli, 2012). Using these technologies Almiñana *et al*. showed that embryo maternal communication exists at the very earliest stages of pregnancy, before that well-known embryonic signal of maternal recognition. 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 contrast, Maillo *et al.* (2015) in a microarray study, did not found differenced in the bovine oviduct transcriptome in the presence or absence of an 8-16 cell embryo *in vivo*, concluding that the small embryo may have a specific side effect which is probably lost in the long oviduct. Obviously, multi-ovulation species like mice and pigs cannot be directly compared with mono-

ovulated once like bovine. Thus, the bovine model would provide new information on early embryo maternal communications that may be important for humans.

In bovine, substances like growth factors (GH - IGF-system), the hyaluronic acid (HA) and the platelets activating factor (PAF) could be involved in very early embryo-maternal communication (Wolf *et al.*, 2003). Equally, OEC express the GH receptor (Hull and Harvey, 2001) enabling them to react with the GH produced by the embryo. It has been shown that embryos cultured *in vitro* in medium with GH showed similarly characteristics to their *in vivo* counterparts, in terms of morphology, cell numbers and apoptosis rates (Kölle *et al.*, 2001, 2002).

Insulin and insulin-like growth factors (IGFs) are family of structurally related polypeptides that regulate growth of many cell types. The activity of these peptides is mediated by IGF transport and its binding to membrane receptors. The function is modulated by interactions with six insulin-like growth factor binding proteins (IGFBPs) which are present in many extracellular fluids and in early embryos (Winger *et al.*, 1997; Pushpakumara *et al.*, 2002). Studies in bovine oviduct *in vivo* reveal different expression patterns of IGF types, their receptors and binding proteins (Pushpakumara *et al.*, 2002). Secreted IGFs, regulated by IGFBPs, may act directly on the embryo or via modulation of oviduct secretions and muscular activity to positively influence the success of early embryonic development (Pushpakumara *et al.*, 2002). Hyaluronic acid (HA) is a glucosaminoglycan and a component of the extracellular matrix that regulates cellular events such as signaling, gene expression, proliferation, differentiation, adhesion, migration and morphogenesis. HA is a normal component of mammalian follicular, oviductal and uterine fluid (Lee and Ax, 1984). The effect of HA in *in vitro* embryo culture has been reflected in increased blastocyst rates, cell number and gene expression of bovine embryos (Stojkovic *et al.*, 2002; Palasz *et al.*, 2006)).

1.3.3. 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 further our understanding of the underlying regulatory mechanisms controlling embryo development (Aviles *et al.*, 2015). The advantages of the oviductal environment have been demonstrated in different models, through the substances isolated and the effect of environment *per se*. Many physiological aspects have been clarified; however, many others are still unknown (Hunter, 2012).

Thus, interactions between oviductal epithelial cells and sperm observed under *in vitro* studies (Boquest and Summers, 1999; Boquest *et al.*, 1999; Boilard *et al.*, 2002; Gualtieri and Talevi, 2003; Hunter, 2003; Talevi and Gualtieri, 2004) find sperm selection mechanisms (Ellington *et al.*, 1999; Pauw *et al.*, 2002; Talevi and Gualtieri, 2004). Cell culture has been used for studying physiological processes and their regulatory mechanisms of embryotrophic function as complement C3b (Lee *et al.*, 2002). Besides, protein patterns were compared *in vivo* vs. *in vitro* using different culture systems (Woldesenbet and Newton, 2003) as well as the regulatory effects on single proteins (Eberhardt *et al.*, 1999).

Under *in vitro* conditions expression of relevant embryotrophic factors as OVGP1 (Briton-Jones *et al.*, 2004), endothelin-1 (ET-1) (Reinhart *et al.*, 2003), members of the insulin-like growth factor (IGF) system (Xia *et al.*, 1996; Winger *et al.*, 1997), or leukaemia inhibitory factor (LIF), vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), IGF1, tumor growth factor β 2 (TGFβ2), and interleukin-4 (IL4) (Okada *et al.*, 2005) were applied to obtain information about the differentiation state of cultured cells.

Gene expression patterns of *in vitro* cell cultures constitute an excellent tool for established cell lines characterization. For example, Schmaltz–Panneau *et al*. used BOEC monolayers as *in vitro* models to evidenced differences in the gene expression of OEC in presence of bovine embryos (Schmaltz-Panneau *et al.*, 2014). Thus, this model may provide useful information on early embryo maternal interaction signals.

1.3.3.1. Bovine oviductal epithelial cells (BOEC) and their conditioned media

Bovine oviduct epithelial cells (BOEC) as the intimal structure of the oviduct in contact with gametes and embryos, are considered as the most suitable *in vitro* model to study early embryonic events and embryo maternal interactions approaching to mimic phypsiological conditions (Rottmayer *et al.*, 2006; Ulbrich *et al.*, 2010). BOEC are usually obtained from oviducts of slaughtered heifers or cows. The BOEC, could be isolated by enzymatic procedures using trypsin and/or pancreatin (McNutt-Scott and Harris, 1998) or collagenase (Sun *et al.*, 1997; Bosch *et al.*, 2001; Mishra *et al.*, 2003) in different concentrations. Also BOEC could be isolated by mechanical procedures remaining in cell aggregates which are mostly cultured non-adhesively in a suspension culture system (Lim *et al.*, 1997; Pegoraro *et al.*, 1998; Yadav *et al.*, 1998; Boquest and Summers, 1999; Kamishita *et al.*, 1999; Gualtieri and Talevi, 2000; Pauw *et al.*, 2002; Ulbrich *et al.*, 2003). Isolated cells are seeded to attach to the culture dish and growth to a confluent monolayer (Figure 6).

Figure 6. BOEC confluent monolayer. (Day 7 of culture).

When a BOEC line is established for embryo culture *in vitro* (Figure 7) it is important to determine the stage of the estrous cycle of the animal of which oviduct is used. Thus, estrous cycle is estimated by ovarian morphology, ipsilateral to the oviduct, according criteria described in the literature (Ireland *et al.*, 1979).

The use of BOEC allowed the study of mechanisms involved in sperm storage in the oviduct (Abe and Hoshi, 1997) and was a major breakthrough in the *in vitro* culture of bovine embryos (Gandolfi and Moor, 1987). In co-culture experiments with sperm, BOEC have been obtained from a luteal phase (Boquest and Summers,

1999; Boquest *et al.*, 1999) and from follicular phase (Ellington *et al.*, 1998; Bosch *et al.*, 2001).

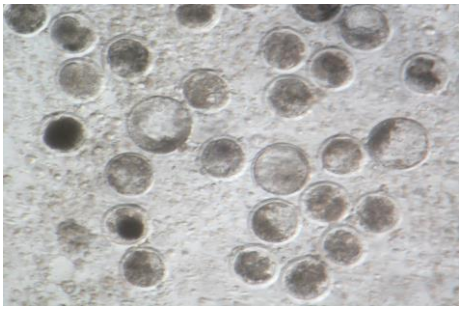

Figure 7. BOEC - Embryo co-culture after 7 days post insemination

In embryo co-culture, BOEC at estrous have been successfully used as *in vitro* model for intercept embryo maternal interactions (Rief *et al.*, 2002). Recently, Cordova *et al*. used oestrous-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 four first days of development, which correspond to the presence of embryos in the oviduct *in vivo*, accelerate the kinetics of blastocyst development and induce changes of genes involved in epigenetic control (Cordova *et al.*, 2014). Moreover, BOEC co-culture overcomes the early embryo developmental block occurring at the 8-16 cell stage in ruminant, and provide an optimized *in vitro* environment for embryo culture until blastocyst stage (Gandolfi *et al.*, 1989; Ellington *et al.*, 1990). 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; Tse *et al.*, 2008). 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, 2000; Vanroose *et al.*, 2001), removing toxic substances from the medium (ammonia) (Nancarrow and Hill, 1994), decreasing the glucose and ion levels, and change the culture metabolites (Edwards *et al.*, 1997) that could have detrimental effects on the embryos (Vanroose *et al.*, 2001). A successfully use of somatic cells could be attributed to the fact that not only few soluble factors are responsible for successful development but also the different signals and factors which act cooperatively (Killian, 2004).

Co-culture systems has been associated with methodological complexity, lack of repeatability and biosanitary risk (Guerin *et al.*, 1997). To avoid the use of primary cultures that may be a risk of contamination, the use of established cell lines will allow to have a standardized culture conditions and a better control (Pegoraro *et al.*, 1998). Established cell lines constitutes an attractive co-culture system for assisted reproductive technologies by providing a continuous supply of cells that are easily cultured *in vitro*, cryopreserved and screened for pathogens (Eyestone *et al.*, 1991; Ulbrich *et al.*, 2010). The use of co-culture systems offer the opportunity to do essential and functional studies of candidate genes in embryo-maternal dialogue (Schmaltz-Panneau *et al.*, 2014) that under *in vivo* conditions are difficult to study.

An alternative to co-culture avoiding a direct contact between BOEC and embryos is the use of conditioned media with positive effects on embryo development and live calves after transfer (Eyestone and First, 1989; McCaffrey *et al.*, 1991; Lim *et al.*, 1997). Conditioned media offer advantages over that monolayer co-culture in that no additional cells are required for the culture system and the media may be stored frozen, eliminating the need for preparing fresh cells for each subsequent embryo group (Eyestone *et al.*, 1991; Ramos-Ibeas *et al.*, 2014). The frozen-thawed conditioned media culture system allows the same batch of media to be used with sequential groups of embryos. This would potentially reduce the variation in embryo development due to variations often noted in monolayer culture systems. The BOEC conditioned media are able to support embryo development to blastocyst stage (Wang *et al.*, 1990; Mermillod *et al.*, 1993) through secreted embryotrophic components 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) that have been properly identified, however many other secretions are still unknown. Therefore, conditioned media can be used to investigate the mechanism(s) by which somatic cells support the development of embryos *in vitro* (Rieger *et al.*, 1995).

1.4. Extracellular Vesicles

Extracellular environment contains a large number of mobile membrane-limited vesicles called ‘‘extracellular vesicles’’ (EV). EVs or cell secreted vesicles contain microvesicles (MV), apoptotic bodies and exosomes. Originally the EV were associated with removal process of receptors and with cellular waste function, being unnoticed and remained without importance. Then, the concept changed and it was found to have immune effects (Raposo *et al.*, 1996). These evidences opened the possibility that EVs could play a role in intercellular communication (Théry, 2011) generating interest, and discovering different types and tissues *in vivo*. The EVs have been found in biological fluids (Simpson *et al.*, 2008) as plasma (Caby *et al.*, 2005), serum (Taylor *et al.*, 2006; Taylor and Gercel-Taylor, 2008), urine (Pisitkun *et al.*, 2004) epididymal fluid (Gatti *et al.*, 2005), amniotic fluid (Asea *et al.*, 2008), follicular fluid (Silveira *et al.*, 2012), and milk (Admyre *et al.*, 2007).

The EVs are classified according to: size, biological content, biogenesis and way of release from the cell. In relation to the size, EVs are in a range from 30 to 1000 nm and can be divided in:

- Exosomes, which size is between 30–90 nm (Théry, 2011), correspond to the internal vesicles of multivesicular bodies (MVBs) that are released in the extracellular environment upon fusion of MVBs with the plasma membrane (Théry *et al.*, 2002).
- The MV are over 100 nm (Raposo and Stoorvogel, 2013) and are shed by all types of cells by outward budding of the cellular plasma membrane and subsequent fission of the formed vesicle (György *et al.*, 2011a).
- The Apoptotic Bodies also are over 100 nm and are released from apoptotic cells which undergo several steps in their dying process including cell shrinkage, chromatin condensation, nuclear fragmentation, membrane blebbing and finally the breakdown of the cell into apoptotic bodies (Reed, 2000).

Nowadays, it is known that exosomes contain proteins secreted by epithelial, tumor and haematopoietic cells, denoting that EV could be released from several types of cells.

The commonly applied and basic method for separating exosomes, apoptotic bodies, and microvesicles (fractions) is the differential centrifugation (Théry *et al.*, 2006). The sedimentation of vesicles depends on their size, density and shape. Under centrifugation, minor size vesicles (exosomes) are typically isolated by sedimentation at 100.000-200.000x*g* (100-200k) (Théry *et al.*, 2009), and the major size vesicles (MV) are typically reported to be isolated at 10.000-20.000x*g* (10-20k) (Witwer *et al.*, 2013).

Quantitative and qualitative assessment of EV Electron microscopy (EM) techniques are well established and have proven very useful in EV research (Raposo *et al.*, 1996), providing direct evidence for the presence of vesicular structures. Heavy metal stains such as osmium tetroxide and uranyl acetate in transmission EM enables recognition of membrane-surrounded vesicles, and contrast is enhanced by embedding in methylcellulose. In most cases, concentrated EV suspensions are applied to grids and fixed, for example with paraformaldehyde. As an alternative method, EV pellets at the bottom of a centrifuge tube are fixed, and ultrathin cross sections are analyzed by transmission EM (György *et al.*, 2011b).

Nanoparticle tracking analysis (NTA) is the commercial name of an optical particle tracking method for obtaining concentration and size distribution of EV populations (Dragovic *et al.*, 2011). A laser beam is scattered by particles in the sample, and the mean velocity of each particle is calculated by the Stokes-Einstein equation on the basis of Brownian motion recorded by a CCD camera. For accurate quantification of number and size of heterogeneous populations of vesicles, the procedure requires accurate optimization of camera and analysis settings, and separate measurements with different settings may be needed to obtain accurate readings for EV subsets in heterogeneous mixtures.

1.4.1. Biogenesis

EV formation is carried out by endocytical pathways of proteins, and usually are clathrin dependent (Mayor and Riezman, 2004). Endocytic vesicles are released to early endosomes, and then recycled back to the plasma membrane or transported to late endosomes together with other proteins and lipids (Maxfield and McGraw, 2004). In late endosomes, two steps have been recognized for sorting protein during MVBs formation. The first step is the lateral sorting of proteins at the limiting membrane of endosomal lumen, and the second step is the formation of intraluminal vesicles (ILV), with the incorporation of proteins, lipids, mRNAs and miRNAs. The MVBs can either fuse with lysosomes for degradation of proteins, or interact with the plasma membrane, resulting in the release of the ILVs as exosomes (Lakkaraju and Rodriguez-Boulan, 2008). The mechanisms of ILVs formation and the sorting of proteins and lipids into these vesicles, as well as docking and fusion of the MVBs with the plasma membrane are poorly understood, although the endosomal sorting complexes required for transport (ESCRT) have been shown to be involved in this processes (Gassart *et al.*, 2003). The ESCRT machinery are composed by protein complexes (ESCRT-0, I, II, III) with a sequential recruiting and binding of proteins that ends in ILV formation (Babst, 2011). Other pathways of protein sorting are mediated by cholesterol, tetraspanins and lipids. Sphingomyelin, form specialized microdomains in the endosomal limiting membrane called lipid rafts can bend inwards and consequently form ILVs (Wubbolts *et al.*, 2003). Biogenesis mechanisms of EVs are showed in the figure 8. Proteins can also be co-sorted into ILVs by binding to membrane proteins or lipids (Gassart *et al.*, 2003). The engulfment of the cell cytoplasm during the vesicle formation can lead to random packaging of cytosolic proteins. The sorting of RNA into ILVs is a greater enigma, however the poor correlation between the mRNA content of exosomes and their donor cells (Mittelbrunn *et al.*, 2011) suggests that the RNA is specifically packed into the vesicles, rather than randomly engulfed from the cells cytoplasm (Valadi *et al.*, 2007).

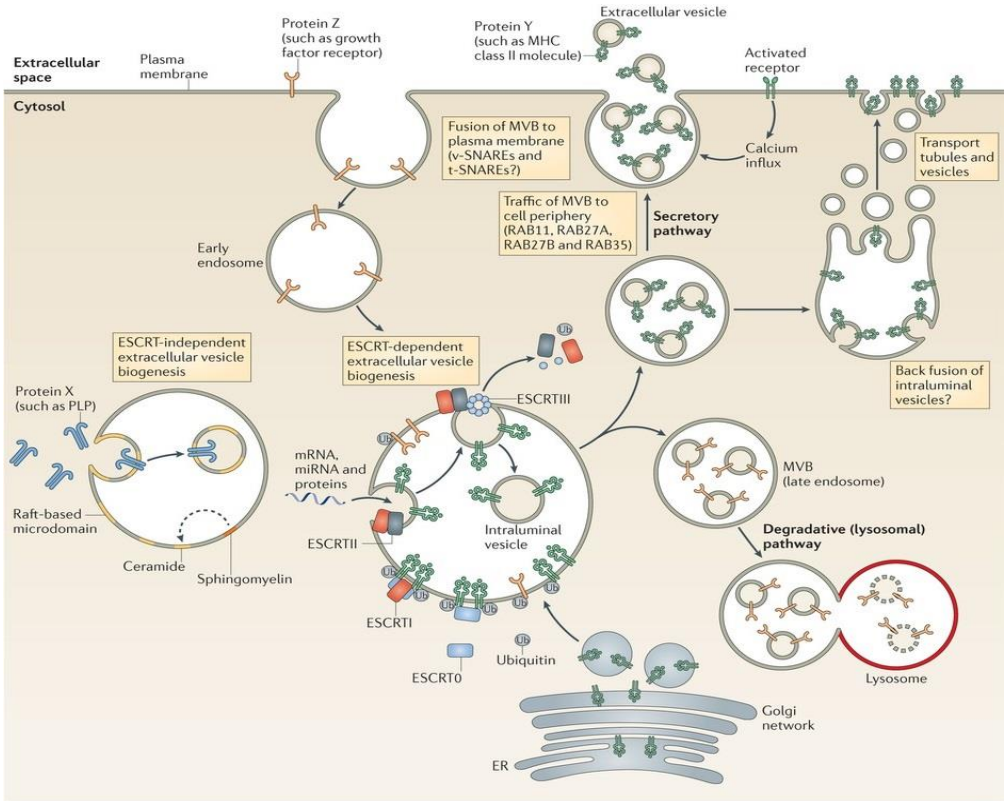

Figure 8. Exosome biogenesis.
Membrane proteins are internalized in early endosomes; the proteins can be recycled to the plasma membrane or delivered to late endosomes Intraluminal vesicles are formed by budding of the endosomal limiting membrane into the endosomal lumen, forming multivesicular bodies (MVBs). MVBs either can fuse with lysosomes for protein degradation or they can also dock to and fuse with the plasma membrane. This results in the release of the intraluminal vesicles to the extracellular compartment. The vesicles are now called exosomes. Exospores display the same topology as the plasma membrane, with extracellular domains exposed on the outside and cytoplasm on the inside enclosed by a lipid membrane. Adapted from Robbins and Morelli (2014)

1.4.2. Characteristics and composition of extracellular vesicles (EV)

It has been demonstrated that exosomes/microvesicles can horizontally transfer mRNAs to other cells, which can then be translated into functional proteins in the new location (Hergenreider *et al.*, 2012). The protein content of EVs from different sources has been analyzed. As a consequence of their origin, exosomes contain endosome-associated proteins coming from the different cell types, some of which are involved in MVB biogenesis (van Niel *et al.*, 2006). Membrane proteins

known to cluster into microdomains at the plasma membrane or at endosomes usually are also enriched on EVs. These include tetraspanins, a family of >30 proteins that are composed by four transmembrane domains (Hemler, 2003). Tetraspanins such as CD63, CD81, CD82, CD53, and CD37 were first identified in B cell exosomes in which they can be enriched with >100-fold relative to the transferrin receptor, which in this cell type can be considered as a genuine marker for both the plasma membrane and early endosomes (Escola *et al.*, 1998). EVs are also enriched with proteins associated with lipid rafts, including glycosylphosphatidylinositol- anchored proteins and flotillin (Wubbolts *et al.*, 2003).

Exosomes from a variety of cells (Wubbolts *et al.*, 2003; Brouwers *et al.*, 2013) are highly enriched with cholesterol, sphingomyelin, and hexosylceramides at the expense of phosphatidylcholine and phosphatidylethanolamine. The fatty acids in exosomes are mostly saturated or monounsaturated. Together with the high concentration of cholesterol, this may account for lateral segregation of these lipids into ILVs/exosomes during their formation at MVB. Less is known about the protein and lipid contents of MVs and whether particular components are enriched with MVs relative to their originating plasma membrane.

A major discovery was the demonstration 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). Later studies reported the RNA contents of EV isolated from other cell cultures (Skog *et al.*, 2008) and from body fluids (Hunter *et al.*, 2008; Rabinowits *et al.*, 2009; Michael *et al.*, 2010).

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). Recently, analysis of RNA from EVs by unbiased deep sequencing approaches demonstrated that EVs also contain a large variety of other small noncoding RNA species, including RNA transcripts overlapping with protein coding regions, repeat sequences, structural RNAs, tRNA fragments, vault RNA, Y RNA, and small

interfering RNAs (Bellingham *et al.*, 2012). Many RNAs that were isolated from EVs were found to be enriched relative to the RNA profiles of the originating cells (Valadi *et al.*, 2007; Skog *et al.*, 2008), indicating that RNA molecules are selectively incorporated into EV.

1.4.3. Role of EV in cell to cell communication

In the last years, it became increasingly accepted that EV represent additional novel means of intercellular communication (Raposo and Stoorvogel, 2013). The role as mediators of intercellular communication is supported in the transport of proteins, lipids, nucleic acids, ligands and receptors from their cell of origin to the recipient cell (Cocucci *et al.*, 2009). They cannot only influence neighboring cells, but are also transported to distant sites when they are secreted into lymphatic or blood vessels (Peinado *et al.*, 2012). A major advance was the finding that EV are often enriched in specific RNA molecules and can transport mRNA as well as miRNA to their recipient cells, where they are transcribed and modulate target gene expression (Mittelbrunn *et al.*, 2011). In few years should show whether RNA and miRNA in EV will prove to be useful biomarkers of various diseases, providing new diagnostic or prognostic tools. Although they were originally described in cells of the immune system, exosomes are now known to be secreted by many different cell types, and a large amount of recent data highlight their possible functions as messengers during development of tumors, in some functions of stem cells, in the nervous system, or during interactions of pathogens with their host, although the last 3 decades have expanded the knowledge of exosomes considerably, there is still no definite evidence for their physiological functions *in vivo*, and demonstrating such functions remains a major challenge of this field (Théry, 2011).

1.4.4. EV in reproduction

The possible role of EV in reproduction has been discovered recently. Silveira *et al*. isolated MV and exosomes of equine ovarian follicular fluid and by proteomics and real-time PCR analysis evidenced proteins and miRNAs presence. The miRNAs were present in surrounding follicular cells (taken), suggesting that

MV and exosomes play a not previously recognized role in mediating cell communication within the mammalian ovarian follicle (Silveira *et al.*, 2012). Also, Sohel *et al*. demonstrated the exosome and non-exosome mediated transport of miRNAs in bovine follicular microenvironment (Sohel *et al.*, 2013). In the same line Ng *et al*. identify and examine the presence and potential role of MV and exosomes in the uterine cavity. MV and exosome 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 of Burns *et al*. support the hypothesis that exosomes and MV are present in uterine luminal fluid of pregnant and cyclic ewes, contain specific proteins, miRNAs, and mRNAs, and are capable of delivering their contents *in vitro* (Burns *et al.*, 2014). Differences in the molecular contents by pregnancy status suggest a differential EV sources (endometrial epithelia-conceptus trophectoderm) serving as evidence of a communication way between the conceptus and endometrium supporting the notion that EV in the uterine fluid have a biological role in conceptus-endometrial interactions important for the establishment and maintenance of pregnancy. Al-Dossary *et al*. reveals the expression and secretion via oviductal exosomes of PMCA4a (Ca^{2+} homeostasis) in the female reproductive tissues and luminal fluids during estrous, and their sperm-uptake, with possible roles in sperm viability during their storage in the oviduct and during capacitation and the acrosome reaction (Al-Dossary *et al.*, 2013).

2. JUSTIFICATION AND OBJECTIVES

2.1. Justification

In vitro embryo production (IVP) in cattle is used for research and for applied purposes. As a research tool, the technology is of great value to address fundamental questions on endocrine control, molecular switches and metabolic pathways that modulate early embryo development. As an applied technology, provides the opportunity to increase the number of offspring from superior phenotypes. However, several aspects need to be addressed at cellular and molecular level to increase its efficiency (Galli *et al.*, 2003).

The IVP is a multifactorial process influenced by extrinsic and intrinsic factors. The embryo development and quality is mainly affected by the origin of the oocyte and by the post-fertilization culture environment (Lonergan and Fair 2008). The culture environment affects the viability of the early embryo, its ability to establish a pregnancy and the characteristics of the newborn calf. This is due to culture media composition which varies from chemically define to semi-define normally supplemented with serum, proteins and macromolecules (Rizos *et al.* 2003).

Embryo development can be assessed by visual observations of blastocysts formation with stereomicroscope on days 6 to 9. The best measure of embryo quality is the ability of the embryo to establish a pregnancy after transfer. However, this is not always feasible due to the big number of embryos produced *in vitro* and the high cost of animal maintains. Thus, invasive and not invasive methods have been used successfully such as the assessment of the ability of the embryo to withstand cryopreservation (Rizos *et al.* 2008); the embryo cell number and proliferation (Trigal *et al.* 2013); and gene expression patterns of gene transcripts related with molecular processes, epigenetics, implantation, etc. (Bermejo-Alvarez *et al.* 2012).

The oviduct is a dynamic organ where fertilization and early embryo development occur. Studying the oviductal environment is crucial to further our understanding of the underlying regulatory mechanisms controlling embryo

development (Aviles *et al.* 2015). The advantages of the oviductal environment have been demonstrated in different models, through the substances isolated and the effect of environment *per se*. Many physiological aspects have been clarified; however, many others are still unknown (Hunter 2012).

The BOEC as intimal structure of the oviduct has been used as *in vitro* model to study the oviduct physiology. In the IVP, the embryo co-culture improved embryo quality by secretion of embryotrophic substances and regulation of culture environment, however, it has been associated with a disadvantage, of lack of repeatability between processes (Myers *et al.* 1994). On the other hand, many secretions of cell culture are present in the CM. Thus, CM is an alternative of embryotrophic substances, which can replace co-culture systems (Mermillod *et al.* 1993).

The study of the oviductal environment under *in vivo* conditions is difficult, so, *in vitro* models are necessary to study mechanisms, pathways, molecules etc. related with embryo-maternal interactions. The OF is considered the main component of oviductal environment. Based on the OF composition, culture media (e.g. SOF) have been developed supporting early embryo development. Recently, it was evidenced the positive effect of OF in porcine fertilization (Coy *et al.* 2008), however, there are no evidence of its effect on embryo culture in vitro in any mammalian species.

The EV, as somatic cell secretions are present in the body fluids. The lipids, proteins, miRNA and mRNA content, convert them as mediators of intercellular transport (Raposo and Stoorvogel 2013). Although their rapid progress in areas such as immunology, currently very little is known about the role of these particles in the reproductive sphere and even less in preimplantation environment.

To summarize, the content of the oviductal environment and its effect on early embryo development is of great importance and may provide information and new insights on embryo-maternal communication, which will improve our current IVP systems. The challenge today is to develop *in vitro* culture conditions that will

allow a physiological growth of the embryo based on the *in vivo* situation to enhance the development of high quality embryos.

2.2. Objectives

The main objective of this thesis was to study the role of the oviductal environment through oviductal cells, oviductal fluid and their extracellular vesicles on *in vitro* embryo development and quality in bovine.

To achieve this general objective, specific objectives have been employed to comprise this thesis:

1. To characterize the extracellular vesicles (EV) secreted by the BOEC
2. To evaluate the effect of different BOEC lines, conditioned media, and BOEC secreted vesicles (EV) in *in vitro* culture,

   On the developmental capacity of bovine zygotes to reach blastocyst stage, and

   On the quality of the produced blastocysts in terms of:

   - Cryotolerance
   - Differential cell number
   - Gene expression analysis
3. To evaluate the effect of oviductal fluid (OF) supplementation during *in vitro* culture,

   On the developmental capacity of bovine zygotes to reach blastocyst stage, and

   On the quality of the produced blastocysts in terms of:

   - Cryotolerance
   - Differential cell number
   - Gene expression analysis
4. To characterize the extracellular vesicles (EV) present in the oviductal fluid

5. To evaluate the effect of extracellular vesicles from the ampulla and isthmus of oviductal fluid isolated at different centrifugal forces in *in vitro* culture,

   On the developmental capacity of bovine zygotes to reach blastocyst stage and

   On the quality of the produced blastocysts in terms of:

   - Cryotolerance
   - Gene expression analysis

3. MATHERIALS AND METHODS

3. Materials and methods

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

3.1. Oocyte Collection and *In vitro* Maturation

Immature cumulus oocyte complexes (COCs) were obtained by aspirating follicles (2-8 mm) from the ovaries of mature heifers and cows collected at slaughter. COCs were matured for 24 h in 500 µl of maturation media (TCM 199 - M4530 supplemented with 10% (v⁄v) foetal calf serum (FCS), and 10 ng ⁄ml epidermal growth factor (E4127) in a four well dish, in groups of 50 COCs per well at 38.5 ºC under an atmosphere of 5% CO_2 in air, with maximum humidity.

3.2. Sperm preparation and *In vitro* Fertilization

Frozen semen straws (0.25 ml) from Asturian Valley bulls, were thawed at 37 ºC in a water bath for 1 min and centrifuged for 5 min at 280 x g through a gradient of 1 ml of 40% and 1 ml of 80 % Bovipure according to the manufacturer´s specification (Nidacon Laboratories AB, Göthenborg, Sweden). The sperm pellet was isolated and washed in 3 ml of Boviwash by centrifugation at 280 x g for 5 minutes. The pellet was resuspended in the remaining 300 µl of Boviwash. Sperm concentration was determined and adjusted at a final concentration of 1 x 10^6 sperm⁄ml for the IVF. Gametes were co-incubated for 18-22 h in 500 µl of fertilization media (Tyrode's medium with 25 mM bicarbonate, 22 mM Na lactate, 1mM Na-pyruvate, and 6 mg/ml fatty acid-free BSA supplemented with 10 mg/ml heparin sodium salt, Calbiochem, San Diego, CA) in a four well dish, in groups of 50 COCs per well under an atmosphere of 5% CO_2 in air, with maximum humidity at 38.5 ºC.

3.3. *In vitro* culture of Presumptive Zygotes

At approximately 20 h post-insemination (p.i.), presumptive zygotes were denuded of cumulus cell by vortex and cultured in Synthetic Oviduct Fluid, (SOF) (Holm *et al.*, 1999)- with 4.2 mM sodium lactate, 0.73 mM sodium pyruvate, 30 µl/ml BME amino acids, 10 µl/ml MEM amino acids, 1 µg/ml phenol-red) with or

without BOEC or in CM (see experimental design for clarification) at 38.5 ºC under an atmosphere of 5% CO_2, 5% O_2 and 90% N , with or without BOEC, BOEC-CM, BOEC-EV, OF and OF-EV (see experimental design for clarification).

Depending the experiment zygotes were cultured in SOF supplemented or not with 3mg/ml BSA or 5% FCS in groups of 25 in 25 µl droplets under mineral oil for the first two experiments. While, in the third experiment were cultured in groups of 50 in 500 µl in a four well dish covered with mineral oil.

3.4. Assessment of embryo development and quality

3.4.1 Embryo development

Cleavage rate was recorded at day 2 (48 h post insemination) and cumulative blastocyst yield were recorded at days 7, 8, and 9 p.i. under a stereomicroscope.

3.4.2. Embryo quality

3.4.2.1.Blastocyst vitrification

The ability of the blastocyst to withstand cryopreservation was used as quality indicator. Day 7 and 8 blastocysts were vitrified in holding medium (HM) (TCM199-M7528 supplemented with 20% (v/v) FCS) and cryoprotectants, following the procedures of Rizos *et al.* (2002b), in two steps protocol using the Cryloop® device (Hampton Research. Aliso Viejo, CA). First step: HM with 7.5% ethylene glycol, 7.5 % dimethyl sulfoxide and second step final solution: HM with 16.5 % ethylene glycol, 16.5 % dimethyl sulfoxide and 0.5M Sucrose. The blastocysts are then warmed in two steps in HM with 0.25 M and 0.15 M sucrose and then cultured in 25 µl droplets of HSOF with 5 % FCS. Survival was defined as re-expansion of the blastocoel and its maintenance for 24, 48, and 72 h.

3.4.2.2. Differential Staining of Blastocysts

Differential staining of inner cell mass (ICM) and trophectoderm (TE) cells was carried following the procedures of Thouas *et al.* (2001). Briefly, blastocyst were permeabilized and TE cell stained by incubation in 500 µl of PBS with 0.2% Triton X-100 and 100 µg/ml propidium iodide (PI) in the dark for 60 sec at 37ºC. For fixation and ICM staining, blastocysts were transferred into 500 µl of ethanol

absolute with 25 µg/ml bisbenzimide (Hoechst 33342) for 3 min. Fixed and stained blastocysts were transferred to glycerol and mounted onto a glass microscope slide, gently flattened with a coverslip and visualized for cell counting under a fluorescent microscope.

3.4.2.3. Gene expression analysis

Gene expression analysis was performed using 5 groups of 10 blastocysts per treatment. Poly(A) RNA was extracted using the Dynabeads mRNA Direct Extraction Kit (Dynal Biotech, Oslo, Norway) with minor modifications (Bermejo-Álvarez *et al.*, 2008). Immediately after extraction, the RT reaction was carried out following the manufacturer´s instructions (Bioline, Ecogen, Madrid, Spain) using poly(T) primer, random primers, and MMLV reverse transcriptase enzyme. Tubes were heated to 70 ºC for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 50 units of reverse transcriptase. They were then incubated 10 min at 25 ºC, 30 min at 45 ºC to allow the RT of RNA, followed by 85 ºC for 5 min to denature the RT enzyme. The quantification of all mRNA transcripts was carried out by qPCR with two repetitions for all genes of interest. qPCR was performed by adding a 2 µl aliquot of each cDNA sample to the PCR mix containing the specific primers. Primer sequences and the approximate sizes of the amplified fragments of all transcripts in the 3 experiments are shown in table 1. For quantification, qPCR was performed as described previously (Bermejo-Alvarez *et al.*, 2010); PCR conditions were tested to achieve efficiencies close to 1. The comparative cycle threshold (CT) method was used to quantify expression levels. Values were 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 of one cycle is equivalent to a doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the *H2AFZ* CT value for each sample from each gene CT value of the sample. The calculation of ΔΔCT involved using the highest treatment ΔCT value, i.e. the treatment with the

lowest target expression, as an arbitrary constant to substract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$.

Table 1. Primers used for RT-qPCR

Gene	Primer sequence (5´-3´)	Fragment Size, bp	Gene Bank Accession No.
H2A.Z	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	212	NM_016750
18S	AGAAACGGCTACCACATCCAA CCTGTATTGTTATTTTTCGTCACTACCT	45	NR_036642.1
PLIN2	ACAACACACCCCTCAACTGG CTGCCTGCCTACTTCAGACC	211	NM_173980.2
ACACA	AAGCAATGGATGAACCTTCTTC GATGCCCAAGTCAGAGAGC	196	FN185963.1
IFNT	GCCCTGGTGCTGGTCAGCTA CATCTTAGTCAGCGAGAGTC	564	AF238612
PLAC8	CGGTGTTCCAGAGGTTTTTCC AAGATGCCAGTCTGCCAGTCA	163	NM_016619
PAG1	CAACGTGCCATTTCTGAGCCTG TGATGTCCCGGTGTCCACAAGG	115	NM_174411.2
DNMT3A	CTGGTGCTGAAGGACTTGGGC CAGAAGAAGGGGCGGTCATC	318	XM_001252215.1
TFAM	GGCAGACTGGCAGGTGTA CGAGGTCTTTTTGGTTTTCCA	164	AF311909
CX43	TGGAATGCAAGAGAGGTTGAAAGAGG AACACTCTCCAGAACACATGATCG	293	NM_174068.2
GPX1	GCAACCAGTTTGGGCATCA CTCGCACTTTTCGAAGAGCATA	116	NM_174076.3
MnSOD	CCCATGAAGCCTTTCTAATCCTG TTCAGAGGCGCTACTATTTCCTTC	307	S67818.1
GLUT1	AGCGTCATCTTCATCCCAGC CCACAATGCTCAGGTAGGAC	540	NM_174602.2
GAPDH	GGGCGTGAACCACGAGAAGTA CCCTCCACGATGCCAAAGT	120	NM_001034034.2
G6PD	CGCTGGGACGGGGTGCCCTTCATC CGCCAGGCCTCCCGCAGTTCATCA	347	NM_001244135.1
SCL2A1	CTGATCCTGGGTCGCTTCAT ACGTACATGGGCACAAAACCA	68	NM_174602.2
GAPDH	ACCCAGAAGACTGTGGATGG AYGCCTGCTTCACCACCTTC	247	BC102589
LDHA	TTCTTAAGGAAGAACATGTC TTCACGTTACGCTGGACCAA	310	NM_174099.2
LDLR	CAAAACCCCGATCATCCCCA TCGACCCTGAACTGGAAACG	194	NM_001166530
CYP51	GGCCCAADDTDATTTCCATTTC CTCCCAAGAAACCCTGCACTGG	167	BC149346.1
FADS1	GCTGCCCAATCTGAGCAAAGC TCCTGTCATGGTGTGGGTCCTG	208	Bt.4959.1.A1_at
DNMT3A	CTGGTGCTGAAGGACTTGGGC CAGAAGAAGGGGCGGTCATC	318	AY271299
IGF2R	GCTGCGGTGTGCCAAGTGAAAAAG AGCCCCTCTGCCGTTGTTACCT	201	NM_174352.2
UBE2A	GGGCTCCGTCTGAGAACAACATC CATACTCCCGCTTGTTCTCCTGG	336	XM_864331
AQP3	CGGTGGTTTCCTCACCATCA CAGAGGGGTAGGTGGCAAAG	299	NM_001079794.1
AQP11	GCTTCGTATCCACCTGCTGT TGCAGTGAAAGTGCCAAAGT	102	NM_001110069.1
ATP1A1	GCGGACACGACAGAGAATCA GAGGCATCACCCGCTACAG	158	NM_001076798.1
LDLR	CAAAACCCCGATCATCCCCA TCGACCCTGAACTGGAAACG	194	NM_001166530
LDHA	TTCTTAAGGAAGAACATGTC TTCACGTTACGCTGGACCAA	310	NM_174099.2
DNMT3A	CTGGTGCTGAAGGACTTGGGC CAGAAGAAGGGGCGGTCATC	318	AY271299
IGF2R	GCTGCGGTGTGCCAAGTGAAAAAG AGCCCCTCTGCCGTTGTTACCT	201	NM_174352.2
GRB10	GAAGACGGGACAAGCAAAGT CTGGCACCACGTAACCATCTG	291	XM_010803961.1
SNRPN	AACAGCACGTACCAGAGGTG ATGCCTGTCTCAGTTCTGCC	144	NM_001079797.1

3.5. BOEC in suspension, monolayers and conditioned media preparation

The establishment of the BOEC cell line was carried out following the procedures of Ramos-Ibeas *et al.* (2014) with minor modifications. Briefly, ipsilateral oviducts at the mid-luteal phase of the estrous cycle were collected from slaughtered heifers. Oviductal mucosa sample was washed 2 times with PBS by centrifugation at 300 x*g* for 10 min. The pellet was resuspended in 2 ml of trypsin / EDTA and incubated for 3 min at 37°C. After, the action of the trypsin was blocked by adding 2 ml of SOF + 5% de FCS and pipetting until obtaining a single cell suspension. Oviductal cells were counted in a hemocytometer and diluted in the appropriate volume of culture medium to give a final concentration of $1x10^6$ cells/ml, and plated for culture at 38.5°C, 5% CO_2 and saturated humidity until confluence. During BOEC cultures half of the media (SOF+5% FCS) were replaced every 48 h.

BOEC were cultured in (i) 4-well dishes with SOF+5% FCS for suspension cells (SC) and conditioned media (CM) production, or in (ii) 100 mm petri dish with Dulbecco's modified Eagle medium (DMEM plus 4.5 mg/l glucose, GlutaMAX, and pyruvate; Invitrogen, Carlsbad, CA) supplemented with 10% FCS, 2mM glutamine, 1mM MEM nonessential amino acids solution, and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) for freezing after confluence (cell line establishment).

SC: At day 2 of cell culture (SOF+5% FCS), BOEC-SCs were isolated from the supernatant media and washed twice before used for embryo culture.

CM–Fresh: At day 5 to 7 of BOEC culture (SOF+5% FCS) cell confluence was 100%. Supernatant was removed and monolayers were washed with PBS before new SOF+5% FCS was added for CM production after and additional 72 h of culture. Then supernatant was filtered through a 0.22 μm nitrocellulose membrane and used for embryo culture and EV isolation.

BOEC Established line: At day 5 to 7 of BOEC culture (DMEM) cell confluence was 100%. Monolayers were frozen in FCS+10% DMSO at -80ºC. After

thawing cells were cultured until 100% confluence and used for embryo co-culture or CM production as explained above. Same BOEC frozen/thawed line was used for all experiments.

3.6. Bovine Oviductal Fluid Collection

Bovine OF (OFA3 - NaturArts, Murcia, Spain) consists of oviductal fluid collected from oviducts ipsilateral to the corpus luteum from ovaries of slaughtered heifers at the early-luteal phase of the estrous cycle, with a further process of purification and freezing (Carrasco *et al.*, 2008).

3.7. Extracellular Vesicles Isolation and Quantification

From BOEC-CM

Extracellular vesicles were isolated from BOEC CM, by ultracentrifugation following the procedures of Théry *et al.*, (2006). Briefly, filtered BOEC-CM was centrifuged at 100000 x*g* (100k) during 60 min at 4ºC (Avanti J30i, Beckman Coulter). Then, the supernatant was removed and the pellet was re-suspended in PBS for EV washing by repeating the previous step. A representative part (100 µl) of resultant pellet (≈400 µl obtained vesicles) was used to determine the size and number of EV by Nanoparticle Tracking Analysis (NTA) with Nanosight® LM10 and NTA 2.3 Software (Nanosight, Wiltshire, UK). Furthermore, a negative staining (Uranyl acetate) transmission electron microscopy (JEM-1010, JEOL, Tokio, Japan) was used to characterize the EV. After quantification, the EV concentration was standardized and either frozen or used fresh for embryo culture.

From OF

Ipsilateral oviducts from slaughtered heifers at the early-luteal phase of the estrous cycle (corpus luteum observation) were transported in the laboratory in ice. Oviducts were washed twice in PBS, and the ampulla and isthmus were separated on an ice-supported surface. Each oviduct region was washed with 5 mL of PBS (4ºC). After a first centrifugation for BOEC elimination (400x*g*), the extracellular vesicles were isolated under 2 centrifugal (*g*) forces sequentially: (i) 10000 xg (10k)

and (ii) 100k following the procedures of Théry *et al.* (2006) with minor modifications as mentioned before. Briefly, after BOEC removal, the OF was centrifuged at 10k during 60 min at 4ºC. Then, the supernatant was removed and centrifuged again at 100k during 60 min at 4ºC and the resultant pellet recovered (Avanti J30i, Beckman Coulter). The EV recovered at 10k and 100k *g* forces was washed in PBS under the previous conditions. A representative part of resultant pellet was used to determine the size and number of EV as mentioned before. After characterization the EV concentration was standardized, and samples were frozen for embryo culture. During the procedure of EV isolation the temperature was maintained at 4ºC.

3.8. Transmission Electron Microscopy

For negative staining of exosomes, ionized carbon and collodion-coated copper Electron microscopy (EM) grids were floated on a sample drop, washed, and stained with 2% uranyl acetate (in double-distilled water) for 1 min and visualized in a JEM-1010 (JEOL, Tokio, Japan) transmission EM.

3.9. Depletion of FCS Extracellular Vesicles

Heat inactivated FCS (F2442) was subjected to overnight (18 h) centrifugation at 100000 x*g* at 4ºC (Avanti J30i, Beckman Coulter). Then the supernatant were aliquoted and stored at -20ºC for embryo culture media supplement.

3.10. Experimental design

3.10.1. Experiment 1: Extracellular vesicles from BOEC in *in vitro* embryo development and quality

For details see Figure 9.

3.10.1.1. Experiment 1.1: Effect on embryo development and quality of *in vitro* culture with different types of BOEC and Conditioned Media

In this experiment the developmental capacity of bovine zygotes and the quality of the produced embryos were assessed on day 7-8 of *in vitro* culture under

the following conditions: SOF+5% FCS, Control group (used as a basic medium for the remaining groups - C^+); BOEC suspension cells (BOEC-SC); frozen/thawed BOEC monolayer (BOEC-FrM); CM from fresh BOEC monolayer (BOEC-CM); and CM from frozen/thawed BOEC monolayer (BOEC-FrCM).

BOEC primary cultures were preliminary prepared either 3-4 or 5-7 days before embryo culture starts. At approximately 20 h p.i., presumptive zygotes were transferred to culture drops for embryo culture according to the experimental groups. Half of the media was replaced every 48 h. For all consecutive experiments, overall cleavage rate was recorded at 48 h pi and blastocyst development was recorded on days 7, 8, and 9 p.i. To assess blastocyst quality, a representative number of day 7-8 blastocysts from each group were either vitrified/warmed for survival rate analysis every 24 h up to 72 h post-warming or fixed for differential cell count. A total of 11 replicates were carried out.

3.10.1.2. Experiment 1.2: Effect of extracellular vesicles from BOEC on the development and quality of in vitro produced bovine embryos

In this experiment the developmental capacity and quality of bovine zygotes cultured *in vitro* with previously purified EV from BOEC conditioned media were assessed. At approximately 20 h post insemination presumptive zygotes were transferred to culture droplets for embryo culture with recently isolated and characterized EV ("fresh" EV) or frozen/thawed EV (Fr-EV) diluted in SOF+5% FCS (C^+) at different concentrations: $3x10^5$ EV/ml; $1.5x10^5$ EV/ml; and $7.5x10^4$ EV/ml. Due to the absence of information in the literature of EV physiological concentrations, we took as starting dilution the initial concentration of secreted vesicles ($3x10^5$ EV/ml=100%) recovered from 10 mL of CM produced from confluent BOEC monolayer in a 100 mm petri dish ($\approx 5.5x10^6$ cells). After isolation and characterization, BOEC EV were diluted to lower concentrations ($1.5x10^5$ = 50% and $7.5x10^4$ EV/ml = 25%) and either frozen/thawed or used fresh for embryo culture. Blastocyst development and quality was assessed as in Experiment 1.1 and blastocysts from C^+, F-EV and Fr-EV were frozen in LN_2 in groups of 10 and stored at -80°C for gene expression analysis. A total of 13 replicates were carried out.

3.10.1.3. Experiment 1.3: Effect of extracellular vesicles secreted from BOEC cultured in different culture media (DMEM or TCM199) on the development and quality of in vitro produced bovine embryos in the absence of FCS

In this experiment the developmental capacity and quality of bovine zygotes cultured *in vitro* in absence of FCS, with previously purified extracellular vesicles from BOEC conditioned media cultured with specific cell media were assessed. At approximately 20 h post insemination presumptive zygotes were cultured with frozen EV (from CM of BOEC cultured in DMEM or TCM199) in the absence of FCS in SOF (C^-) with $3x10^5$ EV/ml. A positive control of SOF+5% FCS (C^+) was included as well. Blastocyst development and quality was assessed as in Experiment 1.1 and blastocysts from C^-, Fr-EV-DMEM and Fr-EV-TCM199 were frozen in LN_2 in groups of 10 and stored at -80°C for gene expression analysis. A total of 8 replicates were carried out.

3.10.1.4. Experiment 1.4: Effect of EV present in FCS on in vitro bovine embryo development and embryo quality

In this experiment the developmental capacity and quality of bovine zygotes cultured *in vitro* with FCS with or without EV were assessed. At approximately 20 h post insemination presumptive zygotes were cultured in SOF+5% FCS, containing EV (+) or EV-depleted (-). Embryo development and survival after vitrification/warming was assessed as in experiment 1.1. A total of 4 replicates were carried out.

3.10.2. Experiment 2: Effect of bovine oviductal fluid on development and quality of bovine embryos *in vitro*

In this experiment the developmental capacity and quality of bovine zygotes cultured *in vitro* with SOF supplemented with different concentrations of OF were assessed. In a preliminary experiment concentrations of 25, 10 and 5% OF were found to be detrimental for embryo development. Based on that, at 20 h post insemination presumptive zygotes were cultured in SOF with (C+) or without 5%

FCS (C-) and SOF supplemented with 2.5, 1.25 and 0.625% of OF. Overall cleavage rate was recorded at 48 h p.i. and blastocyst development was recorded on days 7, 8, and 9 p.i. To assess blastocyst quality a representative number of day 7-8 blastocysts from each group were either vitrified/warmed and survival rate was recorded every 24 h up to 72 h post-warming or fixed for differential cell count. Blastocysts from C- , 1.25% OF and C+ were frozen in LN_2 in groups of 10 and stored at -80°C for gene expression analysis. A total of 11 replicates were carried out.

3.10.3. Experiment 3: Bovine oviductal fluid extracellular vesicles and their effect on *in vitro* embryo development and quality

In this experiment the developmental capacity and quality of bovine zygotes cultured *in vitro* with previously isolated and characterized EV of oviductal fluid from the ampulla (A) and the isthmus (I) at 10.000x*g* (10k) and 100.000x*g* (100k) were assessed. At approximately 20 h post insemination presumptive zygotes were transferred to culture wells for embryo culture with frozen/thawed OF-EV from the ampulla and isthmus isolated at 10k and 100k and diluted in SOF supplemented with 3mg/ml BSA (C-) under adjusted concentration of $3x10^5$ EV/ml. A group of SOF supplemented with 5% fetal calf serum was included (C+). Embryo development was assessed on days 7, 8, and 9. To assess blastocyst quality a representative number of day 7-8 blastocysts from each group were either vitrified/warmed and survival rate was recorded every 24 h up to 72 h post-warming, and blastocysts from C+, C-, Isthmus 10k and Isthmus 100k were frozen in LN_2 in groups of 10 and stored at -80°C for gene expression analysis. A total of 11 replicates were carried out.

3.11. Statistical analysis

Results of cleavage rates, blastocyst yield, survival after vitrification/warming, relative mRNA abundance for candidate genes and OF-EV concentration were analyzed using one-way analysis of variance ANOVA ($p<0.05$). The embryo cell number (ICM, TE and Ratio) were analyzed by multiple pair-wise

comparisons using a t - test. All analysis was made with the SigmaStat (Jandel Scientific, San Rafael, CA) software package.

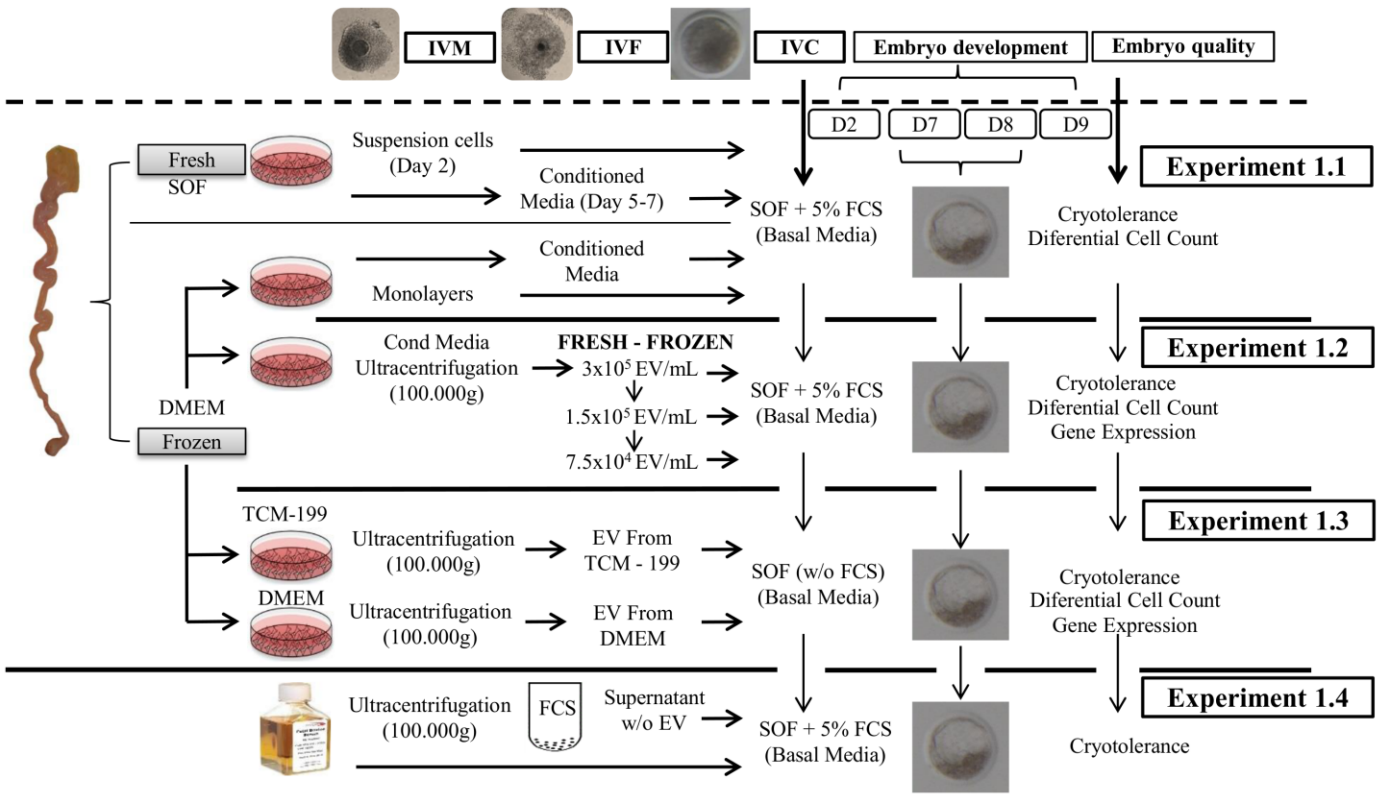

Figure 9. Experimental design of Experiment 1

4. RESULTS

4. Results

4.1. Extracellular vesicles from BOEC in *in vitro* embryo development and quality

4.1.1. The use of conditioned media from an established BOEC cell line has a positive effect on the quality of bovine embryos

In Experiment 1.1 we analyzed embryos cultured either in SOF+5% FCS (Control group); in co-culture with BOEC suspension cells (BOEC-SC) or frozen/thawed BOEC monolayer (BOEC-FrM) or in the presence of CM from fresh BOEC monolayer (BOEC-CM); or CM from frozen/thawed BOEC monolayer (BOEC-FrCM). No differences were found in terms of cleavage rates (range: 87.6-89.4%) and blastocyst yield on day 7 (range: 21.7-27.7%), 8 (range: 31.0-36.0%) or 9 (range: 34.3-39.3%) between groups, as shown in table 2.

Table 2. Effect of co-culture with different types of BOEC and Conditioned Media on embryo development *in vitro*

	n	Cleavage n (% ± S.E)	Blastocyst yield		
			Day 7 n (% ± S.E)	Day 8 n (% ± S.E)	Day 9 n (% ± S.E)
Control (C^+)	682	599 (87.8±1.2)	176 (26.0±2.4)	217 (32.0±2.3)	237 (35.2±2.4)
BOEC-SC	442	387 (87.6±1.2)	105 (24.1±2.0)	145 (33.5±2.8)	147 (34.3±3.8)
BOEC-FrM	424	379 (89.4±1.2)	92 (21.7±3.2)	132 (31.0±4.0)	151 (35.6±3.9)
BOEC-FCM	510	447 (87.9±0.6)	141 (27.7±2.6)	182 (36.0±1.6)	192 (39.3±2.1)
BOEC-FrCM	530	465 (87.6±1.5)	141 (26.8 ±2.2)	174 (33.3±2.5)	186 (35.8±2.7)

n: Total number of presumptive zygotes placed in culture.

The survival rates of vitrified/warmed blastocysts produced in BOEC-FrM and CM was significantly higher when compared to BOEC-SC and C^+ groups at 24h (67.8%; 68.4%; 72.6% vs 49.1%; 54.0% respectively, $p<0.05$). At 72 h only blastocysts cultured in CM from fresh or frozen BOEC survived significantly higher than BOEC-FrM, BOEC-SC and C^+ groups (54.0%; 55.4% vs 14.1%; 17.6%;

16.7% respectively, p<0.05) (Figure 10).

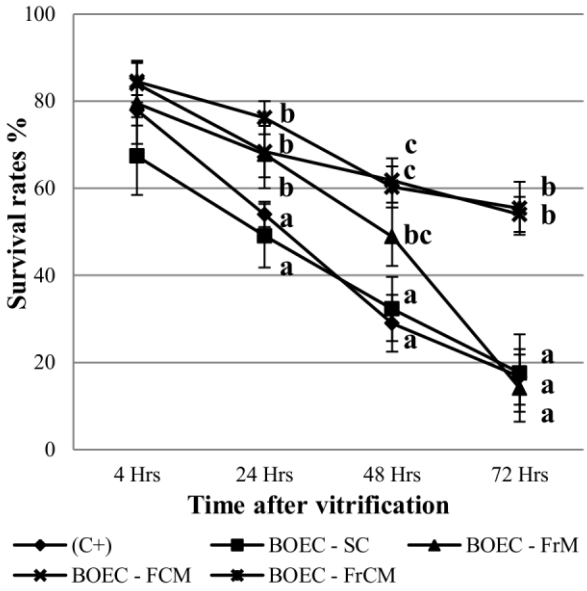

Figure 10. Survival rates after vitrification and warming of D7 blastocysts co-cultured with different types of BOEC (Suspension Cells-SC, Frozen Monolayer -FrM) or Conditioned Media (from fresh-CM, or frozen monolayers-FrCM)
[a,b,c] Different superscripts indicate significant differences at given time points (p<0.05).

These differences in cryotolerance were also reflected in a significant higher number of TE cells on embryos cultured in FrM, CM or FrCM compared to SC and C^+ groups (70.2; 72.1; 71.1 vs 68.7; 67.9 respectively, p<0.05). However, the total embryo cell number was similar for all groups (range: 152.1 to 167.6) (Table 3).

Table 3. Effect of co-culture with different types of BOEC and Conditioned Media on blastocyst nuclei number

	n	Total nuclei Mean ± S.E	ICM nuclei Mean ± S.E	ICM % ± S.E	TE nuclei Mean ± S.E	TE % ± S.E	Ratio ICM/TE
Control (C)	44	152.1±4.7	47.7±1.7	32.1±1.2^{a}	104.4±4.2	67.9±1.2^{a}	0.5±0.02
BOEC-SC	44	158.8±5.3	48.6±1.6	31.3±1.0^{ac}	110.2±4.5	68.7±1.0^{ac}	0.5±0.02
BOEC-FrM	44	161.9±4.1	47.5±1.8	29.8±1.2^{acd}	114.4±3.7	70.2±1.1^{bc}	0.4±0.02
BOEC-FCM	41	163.8±5.4	45.6±1.8	27.8±0.7^{bd}	118.3±4.2	72.1±0.7^{b}	0.4±0.01
BOEC-FrCM	42	167.6±6.9	46.3±1.5	28.9±1.2^{bc}	121.3±6.5	71.1±1.2^{b}	0.4±0.02

n: Number of blastocysts processed.
[a,b,c] Values in the same column with different superscripts differ significantly (p< 0.05).

No significant differences were found in terms of embryo development and embryo differential cell count by using 100% conditioned medium or conditioned medium diluted 50% with DMEM (data not shown).

4.1.2. BOEC conditioned media contain EV

For Experiment 1.2 EV were isolated from BOEC-CM, by ultracentrifugation. Transmission electron microscopy revealed that the isolates contained vesicles approximately 200 nm in diameter (Fig. 11B). For quantification of EV concentration and particle size and distribution, nanoparticle tracking analysis of the isolates was performed (Fig. 11A). This analysis revealed that the vesicles collected from BOEC-CM had an average diameter of 220 nm (Fig. 11A).

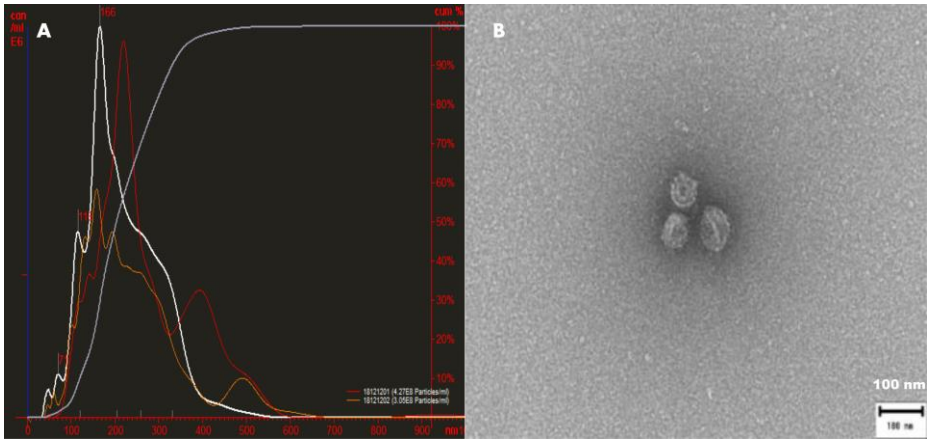

Figure 11. Transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) of vesicles isolated from BOEC-CM. **a**- Relation particle size/concentration of EV secreted by BOEC and measured by Nanosigth®. **b**- Electron microscope image of BOEC-EV like exosomes**.**

4.1.3. Extracellular Vesicles secreted from BOEC *in vitro* cultures have a positive effect on the quality of *in vitro* produced bovine embryos

In Experiment 1.2 we analyzed whether BOEC-derived EV were responsible for the positive effect of CM in the quality of bovine zygotes cultured *in vitro*. No differences were found in terms of cleavage rates (range: 86.2-89.8%) and blastocyst yield on day 7 (range: 26.8-32.1), 8 (37.8-43.4) and 9 (range: 40.9-46.0)

between C^+ and the different concentrations of EV groups, either fresh (F-EV) or frozen (Fr-EV), as shown in table 4.

Table 4. Effect of culture with BOEC-EV at different concentrations on embryo development *in vitro*

	BOEC-EV Dilutions	n	Cleavage n (%±S.E)	Blastocyst yield		
				Day 7 n (%±S.E)	Day 8 n (%±S.E)	Day 9 n (%±S.E)
Control (C^+)		877	778 (88.8±1.0)	241 (27.5±1.2)	329 (37.8±1.7)	357 (40.9±1.8)
F-EV	100%	777	695 (89.6±1.1)	217 (28.3±1.2)	321 (41.2±2.2)	356 (45.5±2.3)
	50%	776	688 (88.6±1.1)	236 (31.2±2.4)	331 (43.4±3.1)	355 (46.0±2.9)
	25%	772	668 (86.2±1.2)	242 (32.1±2.2)	313 (41.2±2.8)	351 (46.0±2.7)
Fr-EV	100%	814	730 (89.9±0.9)	217 (26.8±1.0)	315 (38.7±2.2)	348 (42.7±1.8)
	50%	811	709 (87.2±0.7)	240 (30.2±1.9)	314 (39.7±2.8)	349 (44.1±3.0)
	25%	795	703 (88.6±0.9)	245 (30.9±1.7)	324 (40.5±2.0)	359 (45.1±2.0)

F-EV: Fresh EV. Fr-EV: Frozen/thawed EV.
n: Total number of presumptive zygotes placed in culture.

Embryos cultured with EV, irrespective of concentration and processing, survived significantly higher than C^+ group at all-time points (range at 72h: 48.7-56.5% vs 22.3% respectively, $p<0.05$) (Figure 12).

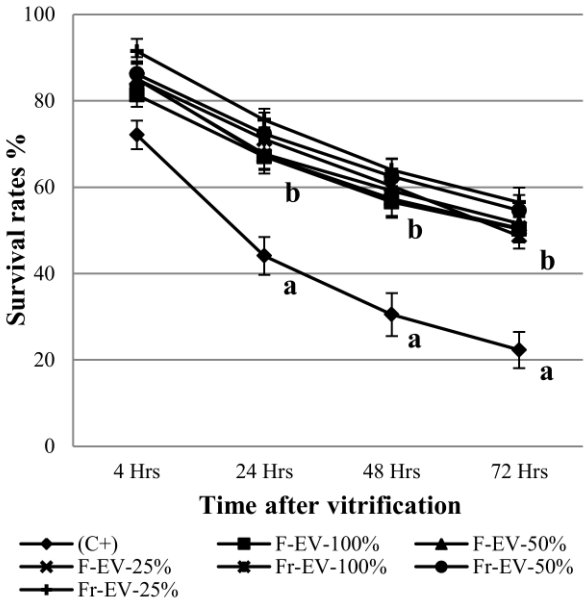

Figure 12. Survival rates after vitrification and warming of D7 blastocysts cultured with different concentrations (100, 50, 25%) of recently purified (F-EV) or frozen/thawed (Fr-EV) BOEC extracellular vesicles.
[a,b]Different superscripts indicate significant differences at given time points ($p<0.05$).

Similarly, blastocysts cultured with EV had significantly more cells than the C^+ group (range: 177.1-191.1 vs 160.4 respectively, $p<0.05$) and also more TE cells (range: 127.5-131.8 vs 111.5 respectively, $p<0.05$), as shown in table 5.

When we assessed, in Experiment 1.3 whether the media employed in the culture of BOEC for the obtention of EV had an effect on embryo development, again no differences were found in cleavage rates between groups (range: 82.6-86.8%). However, blastocyst yield of the C^+ group was significantly higher than C^-, and both EV (FrEV-DMEM, FrEV-TCM199) groups, on Days 7, 8 and 9 (Day 9: 29.9% vs 23.6%; 24.5%; 23.3% respectively, $p<0.05$) as shown in table 6.

Table 5. Effect of culture with BOEC-EV at different concentrations on blastocyst nuclei number

	BOEC-EV Dilutions	n	Total nuclei Mean ± S.E	ICM Nuclei Mean ± S.E	ICM % ± S.E	TE nuclei Mean ± S.E	TE % ± S.E	Ratio ICM/TE
Control (C^+)		40	160.4±7.3^{a}	48.9±3.6	30.3±1.2	111.5±5.4^{a}	69.7±1.2	0.5±0.02
F-EV	100%	40	180.7±8.2^{b}	51.6±2.8	28.8±1.1	129.1±6.3^{b}	71.2±1.1	0.4±0.02
	50%	41	175.3±8.0^{ab}	47.9±2.8	27.1±0.8	127.5±5.8^{b}	72.9±0.8	0.4±0.02
	25%	41	182.5±7.5^{b}	51.6±3.0	28.1±1.0	130.8±5.4^{b}	71.9±1.0	0.4±0.02
Fr-EV	100%	40	177.1±7.1^{b}	49.0±2.9	27.3±0.9	128.0±5.0^{b}	70.7±0.9	0.4±0.02
	50%	39	184.1±9.0^{b}	56.2±3.5	30.6±1.3	127.9±6.5^{b}	69.4±1.3	0.5±0.03
	25%	38	191.1±8.6^{b}	59.4±3.7	30.4±1.1	131.8±5.6^{b}	69.6±1.1	0.5±0.02

n: Number of blastocysts processed.
F-EV: Fresh EV. Fr-EV: Frozen/thawed EV.
^{a,b}Values in the same column with different superscripts differ significantly ($p< 0.05$).

Table 6. Effect of culture with EV secreted by BOEC cultured in DMEM and TCM199 on embryo development *in vitro*

	BOEC-EV from	n	Cleavage n (%±S.E)	Blastocyst yield		
				Day 7 n (%±S.E)	Day 8 n (%±S.E)	Day 9 n (%±S.E)
Control (C^+)		490	405 (82.6±1.0)	117 (24.2±1.2) ^{a}	134 (27.4±1.0) ^{a}	146 (29.9±1.8) ^{a}
Control (C^-)		590	504 (85.5±1.1)	77 (13.7±1.5) ^{b}	122 (20.8±0.6) ^{b}	138 (23.6±0.8) ^{b}
Fr-EV	DMEM	631	541 (85.1±1.3)	94 (16.1±1.9) ^{b}	132 (22.6±2.6) ^{b}	144 (24.5±2.3) ^{b}
	TCM199	630	552 (86.8±1.5)	76 (12.3±1.3) ^{b}	122 (20.3±1.9) ^{b}	142 (23.3±1.5) ^{b}

n: Total number of presumptive zygotes placed in culture.
^{a,b}Values in the same column with different superscripts differ significantly ($p< 0.05$).

The survival rate after vitrification and warming of embryos cultured with EV (FrEV-DMEM or FrEV-TCM199) and without FCS was significantly higher than the C+ group at all-time points (72 h: 37.0%; 36.7% vs 18.4% respectively, $p<0.05$). However, no differences were found between C- and EV groups (Figure 13).

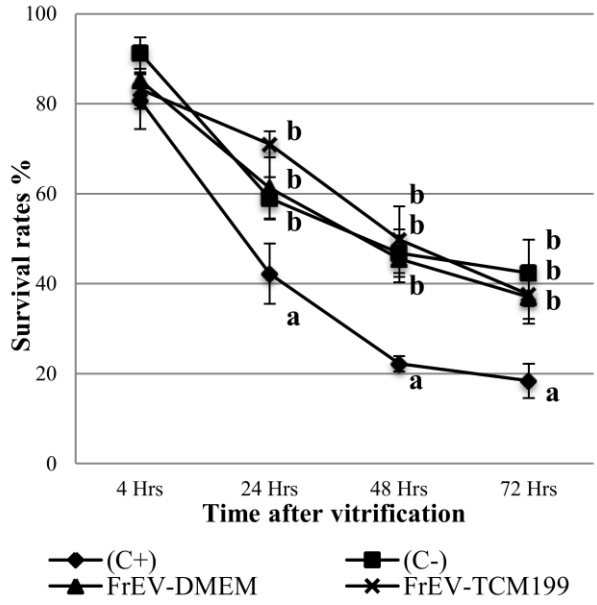

Figure 13. Survival rates after vitrification and warming of D7-8 blastocysts cultured with extracellular vesicles (EV) secreted by BOEC cultured in DMEM and TCM199.
C+: Control+FCS. C-: Control-FCS.
[a,b,]Different superscripts indicate significant differences at given time points ($p<0.05$).

In terms of cell number, blastocysts produced with EV (FrEV-DMEM or FrEV-TCM199) and without FCS and C- had lower number of total (range: 133.6-142.7) and TE cells (range: 101.4-108.6) compared to C+ (155.9 and 117.6 respectively) (Table 7).

Table 7. Effect of culture with EV secreted by BOEC cultured in DMEM and TCM199 on blastocyst nuclei number

	BOEC-EV from	n	Total nuclei Mean ± S.E	ICM Nuclei Mean ± S.E	ICM % ± S.E	TE nuclei Mean ± S.E	TE % ± S.E	Ratio ICM/TE
Control (C^+)		32	155.9±10.8	38.2±2.6	25.4±1.1	117.6±9.1	74.5±1.1	0.3±0.02
Control (C^-)		31	133.6±7.7	31.2±2.2	23.9±1.4	101.4±6.5	76.0±1.4	0.3±0.03
Fr-EV	DMEM	31	142.7±8.7	34.0±1.8	24.6±1.0	108.6±7.4	75.3±1.0	0.3±0.01
	TCM199	31	141.7±7.6	35.2±2.4	24.8±1.2	106.4±5.4	75.0±1.2	0.3±0.02

n: Number of blastocysts processed.

On D7 blastocysts from Experiments 1.2 and 1.3 we analyzed the expression levels of different genes, including housekeeping genes (histone *H2AFZ*, 18 s ribosomal RNA (*18S*), fatty acid related genes such as periplin 2 (*PLIN2*), and acetyl-Coa carboxylase alpha (*ACACA*), implantation-related genes (interferon tau (*IFN-t*), placenta specific 8 (*PLAC8*) and pregnancy associated glycoprotein 1 (*PAG1*), epigenetics-related genes (DNA methyltransferase 3A (*DNMT3A*), transcription factor A, mitochondrial (*TFAM*), gap junctions gene connexin 43 (*Cx43*) and genes involved in the regulation of oxidative stress such as gluthathione peroxidase 1 (G*PX1*), manganese superoxide dismutase (*MnSOD*), solute carrier family 2 (facilitated glucose transporter), member 1 (*SCL2A1*, previously known as *GLUT1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and glucose 6 phospathe dehydrogenase (*G6PD*). The expression level of *PAG1*, an implantation related gene, was upregulated in blastocysts cultured in presence of FCS supplemented with fresh (F-EV) or Frozen (Fr-EV) extracellular vesicles when compared with C^+. No differences were observed for the rest of transcripts studied (Figure 14 - Experiment 1.2). When FCS was removed the expression level of *IFN-τ* was downregulated in both EV groups (FrEV-DMEM and Fr-EV-TCM199) compared to C^-. *PLAC8* was downregulated in Fr-EV-DMEM while *PAG1* and *Cx43* were downregulated in FrEV-TCM199 group. *GAPDH* was upregulated in both EV groups; and *G6PD* was downregulated in FrEV-DMEM group (Figure 15 – Experiment 1.3).

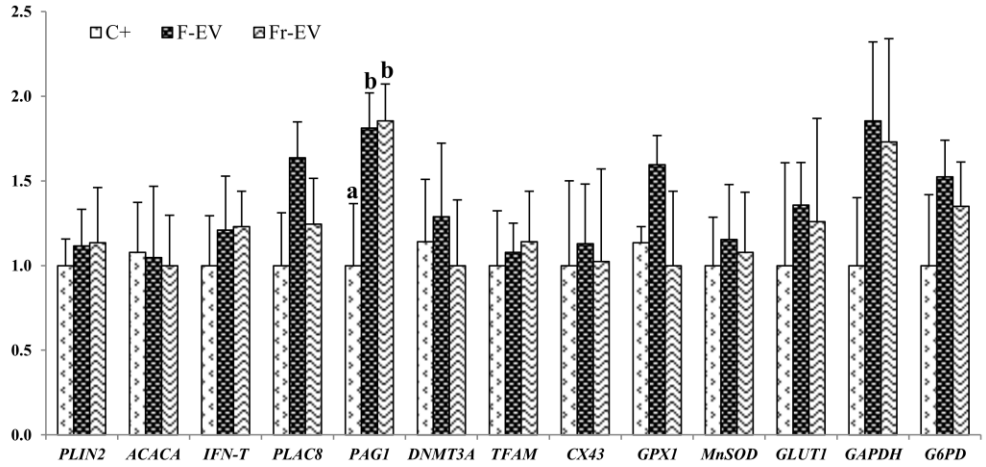

Figure 14. Relative mRNA transcription of developmental related genes in bovine *in vitro* blastocysts (D7 p.i) cultured with or without (C^+) EV secreted by BOEC fresh (F-EV) and frozen (Fr-EV).

[a.b] Different superscripts indicate significant differences for each gene ($p<0.05$).

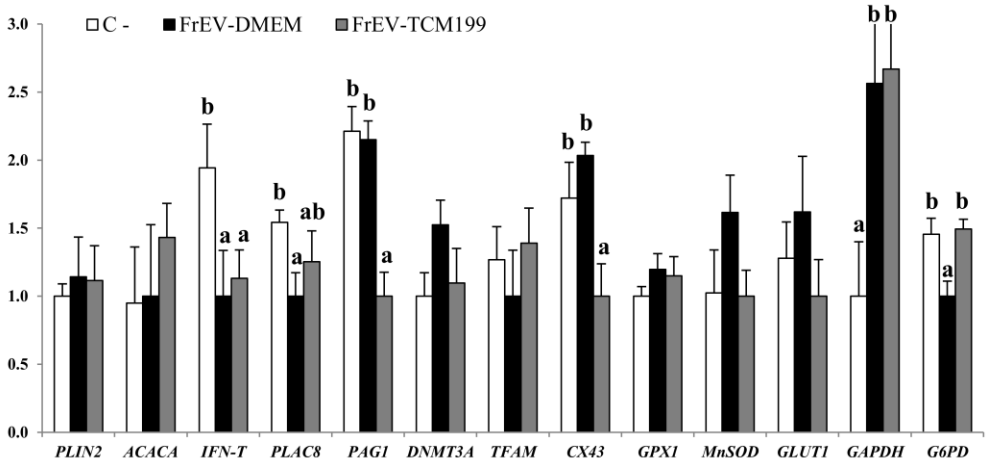

Figure 15. Relative mRNA transcription of developmental related genes in bovine *in vitro* blastocysts (D7-8 p.i) cultured with or without (C-) EV secreted by BOEC cultured in DMEM and TCM199.

[a.b] Different superscripts indicate significant differences for each gene ($p<0.05$).

4.1.4. Depletion of extracellular vesicles from fetal calf serum improves the quality of bovine embryos produced *in vitro*

In Experiment 1.4 we analyzed the effect of EV present in FCS on *in vitro* bovine embryo development and embryo quality. No differences were found between FCS-EV(+) and FCS-EV(-) for cleavage rate (84.0±3.3% vs.82.0±0.7%) or blastocyst yield on day 7 (22.7±2.3%vs.28.8±5.6%) or day 9 (34.0±3.4vs.38.1±5.4%) (Table 8). However, after vitrification/warming, significantly more embryos survived at 48 and 72 h in FCS-EV(-) compared with FCS-EV(+) group (51.2±8.2% and 41.9±2.8% vs. 29.2±6.9% and 19.0±8.6%, respectively) ($p<0.05$) (Figure 16).

Table 8. Effect of culture in presence (+) or absence (-) of FCS-EV on embryo development *in vitro*

	n	Cleavage n (% ± S.E)	Blastocyst yield		
			Day 7 n (% ± S.E)	Day 8 n (% ± S.E)	Day 9 n (% ± S.E)
FCS-EV (+)	333	281 (84.0±3.3)	78 (22.7±2.3)	106 (31.4±4.0)	115 (34.0±3.4)
FCS-EV (-)	321	264 (82.0±0.7)	95 (28.8±5.6)	120 (35.8±4.2)	130 (38.1±5.4)

n: Total number of presumptive zygotes placed in culture.

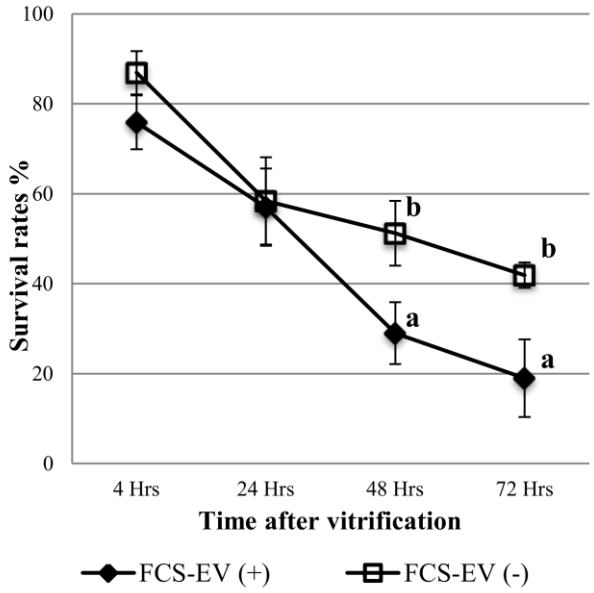

Figure 16. Survival rates after vitrification and warming of D7 blastocysts cultured with normal FCS (containing EV) or EV-depleted FCS.
[a,b] Different superscripts indicate significant differences at given time points ($p<0.05$).

4.2. Bovine oviductal fluid on development and quality of bovine embryos *in vitro*

4.2.1. Low concentrations of OF has a positive effect on the quality of bovine embryos

In Experiment 2, supplementation with 25, 10 and 5% of OF in culture media was detrimental for embryo development with blastocyst yields of 1, 10 and 11% on Day 9 respectively, significantly lower compared to control (data not showed). Therefore, lower concentrations of 2.5, 1.25 and 0.625% were used.

No differences were observed in cleavage rate between both controls and OF groups (range: 86.7-89.1%). Blastocyst yield of the C+ group was significantly higher than C- and 2.5% OF group, on days 7, and 8 (26.6% vs 18.3% and 21.2% respectively, $p<0.05$). However, no differences were found between C+ and 1.25% and 0.625% OF groups between Days 7-9 (Table 9). Blastocysts yield of 1.25% OF group was significantly higher than C+ on days 7, 8 and 9 (Day 9: 27.5% vs 21.5% respectively $p<0.05$). No differences were found between 1.25% and 0.625% OF

groups on Days 7-9. On Day 9 C+, 1.25% and 0.625% OF rates were similar (≈27.5%), and significantly higher to C- (21.5%) as shown in Table 9.

Table 9. Effect of *in vitro* embryo culture with low concentrations of bovine oviductal fluid (OF) on development *in vitro*.

	OF Dilutions	n	Cleavage n % ± S.E	Blastocyst yield		
				Day 7 n % ± S.E	Day 8 n % ± S.E	Day 9 n % ± S.E
Control (C+)		872	747 (86.7±1.5)	198 (22.9±1.2) [a]	226 (26.6±1.2)[a]	236 (27.7±1.9)[a]
Control (C-)		927	823 (88.8±1.2)	96 (12.0±1.7)[c]	164 (18.3±1.2)[c]	195 (21.5±1.4)[b]
OF	2.5%	855	748 (87.3±1.1)	110 (13.9±1.4)[bc]	180 (21.2±1.4)[bc]	192 (22.7±1.5)[b]
	1.25%	964	855 (89.1±1.5)	163 (17.4±1.5)[b]	236 (24.4±1.7)[ab]	266 (27.5±1.7)[a]
	0.625%	1011	898 (89.1±1.3)	160 (16.0±1.2)[b]	230 (22.3±1.0)[b]	279 (27.5±1.2)[a]

n: Total number of presumptive zygotes placed in culture.
[a,b,c,] Values in the same column with different superscripts differ significantly ($p<0.05$).
C-: SOF.
C+: SOF+5% FCS

The survival rates of vitrified/warmed blastocysts produced in C- and 0.625% OF was significantly higher when compared to 2.5%, 1.25% OF and C+ groups at 24h (74.1%; 71.7% vs 58.7%; 63.1%; 41.3% respectively, $p<0.05$). Similarly, at 72 h the survival rates of blastocysts cultured in 1.25%, 0.625% and C- was significantly higher than 2.5% and C+ groups (53.6%; 57.7%; 56.1% vs. 36.1%; 25.9% respectively, $p<0.05$) (Fig. 17).

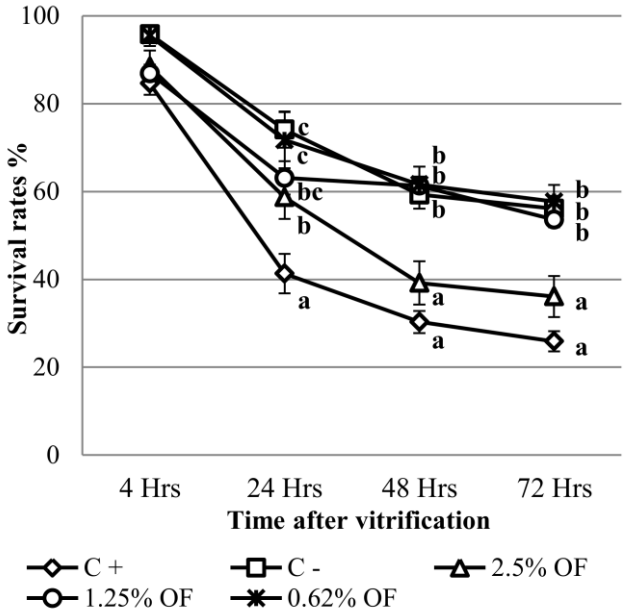

Figure 17. Survival rates after vitrification and warming of D7-8 blastocysts cultured with low concentrations of bovine oviductal fluid (OF)
[a,b,c] Different superscripts indicate significant differences at given time points ($p<0.05$). C-: SOF
C+: SOF+5% FCS

Total cell number was significantly lower in the C+ and C- groups compared with 2.5% and 1.25% (143.1; 127.7 vs.165.1; 156.2 respectively $p<0.05$). Similarly, the number of trophectoderm (TE) cells in embryos in the C+ and C- groups were significantly lower compared to 1.25% and 0.625% OF groups (107.1; 96.6 vs. 127.0; 119.9 respectively, $p<0.05$). Difference between 1.25 and 2.5% groups (127.0 vs. 113.1 $p<0.05$) were also evident, as shown in Table 10.

Table 10. Effect of *in vitro* embryo culture with low concentrations of bovine oviductal fluid (OF) on blastocyst cell number

		n	Total nuclei Mean ± S.E	ICM nuclei Mean ± S.E	ICM % ± S.E	TE nuclei Mean ± S.E	TE % ± S.E	Ratio ICM/TE
Control (C+)		40	143.1±4.9^{a}	36.0±1.4^{a}	25.6±0.9	107.1±4.3^{a}	74.4±0.9	0.3±0.02
Control (C-)		40	127.7±4.9^{b}	31.1±1.4^{b}	24.9±1.1	96.6±4.6^{a}	75.1±1.1	0.3±0.02
OF	2.5%	40	150.2±7.1^{ac}	37.1±1.6^{a}	25.8±1.1	113.1±6.8^{ab}	74.2±1.1	0.3±0.02
	1.25%	40	165.1±4.7^{d}	38.0±1.5^{a}	23.5±0.9	127.1±4.5^{c}	76.5±0.9	0.3±0.02
	0.62%	40	156.2±4.2^{cd}	36.2±1.3^{a}	23.4±0.8	119.9±3.7^{bc}	76.6±0.8	0.3±0.01

n: Number of blastocysts processed.
^{a,b,c,d} Values in the same column with different superscripts differ significantly ($p< 0.05$).
C-: SOF
C+: SOF+5% FCS

The expression levels of 3 genes related with glucose metabolism (*SCL2A1*, *GAPDH* and *LDHA*), three related with lipid metabolism (*LDLR*, *CYP51* and *FADS1*), three related with epigenetics (*DNMT3A*, *IGF2R* and *UBE2A*) and *AQP3* were determined in blastocysts cultured in C+, C- or C- supplemented with 1.25% of OF (Fig. 18). Overall, the groups C- and OF showed no differences in the expression levels of the 10 genes analyzed except for *AQP3* which was significantly upregulated ($p<0.05$) in OF compared with the other two groups. The glucose transporter *SCL2A1* was significantly upregulated ($p<0.05$) in both groups cultured without serum, whereas *GAPDH* expression levels were significantly higher ($p<0.05$) in C- compared with C+ and no differences were observed for *LDHA*. Among the genes related with lipid metabolism, *LDLR* was significantly upregulated ($p<0.05$) in OF compared with C+ and the expression *CYP51* and *FADS1* was significantly higher ($p<0.05$) in both groups cultured without serum (C- and OF). Finally, both *DNMT3A* and *IGF2R* were significantly upregulated ($p<0.05$) in both groups cultured without serum compared with C+, whereas the expression levels of *UBE2A* did not differ between groups.

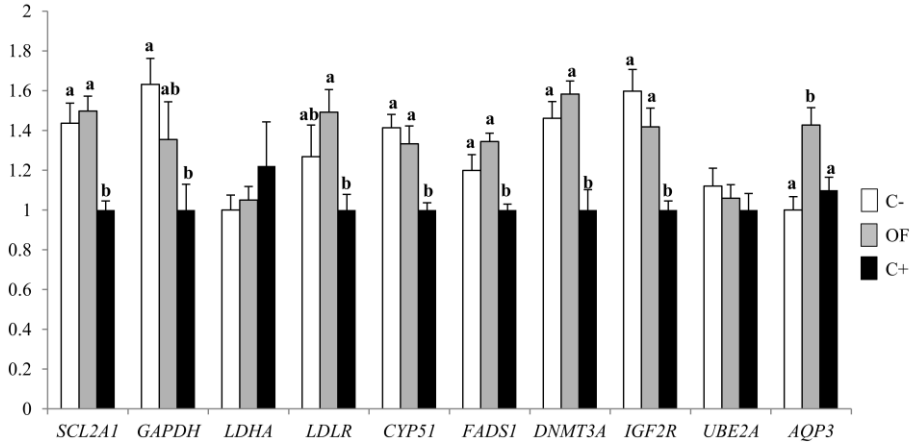

Figure 18. Relative mRNA transcription of developmental related genes in bovine in vitro blastocysts (D7 p.i) cultured with low concentrations of bovine oviductal fluid (OF).
[a.b] Different superscripts indicate significant differences for each gene ($p<0.05$).
C-: SOF
C+: SOF+5% FCS

4.3. Bovine oviductal fluid extracellular vesicles and their effect on *in vitro* embryo development and quality

4.3.1. Characteristics of extracellular vesicles present in the oviductal fluid

For Experiment 3, the size of OF-EV between ampulla and isthmus was similar (≈220 nm) as well as their concentration (≈$7.6x10^8$ EV/ml) irrespective the force used based of nanoparticle analysis system reports (Nanosight®) (Table 11) and transmission electron microscope images analysis (Figure 19). However, the concentration of isthmus OF-EV isolated at 10k was significantly lower compared to ampulla OF-EV at 100k ($3.6x10^8$ vs $10.5x10^8$ EV/ml respectively, $p<0.05$) (Table 11).

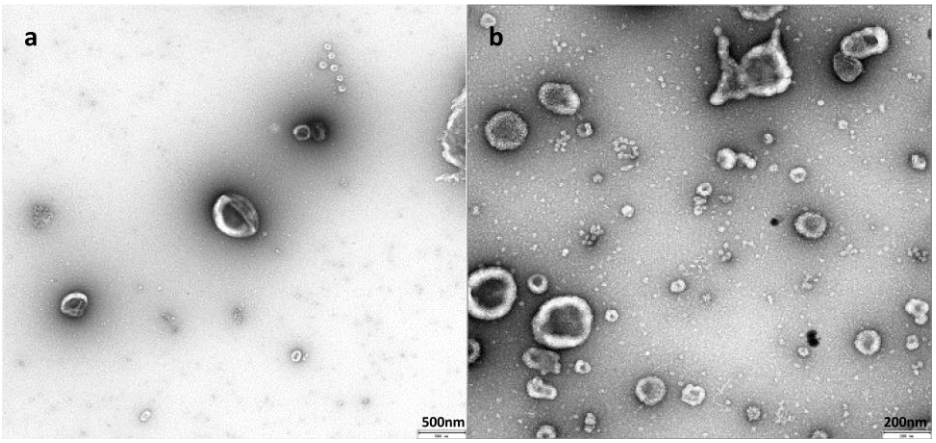

Figure 19. Electron microscope images of OF-EV isolated from the isthmus at 10k (a) and 100k (b) g-forces.

Table 11. Size and concentration OF-EV isolated from the ampulla and the isthmus at different g-forces (10k-100k)

	Centrifugal force (x*g*)	n	Size	Concentration
			nm ± S.D	EV^8 ± S.D
Ampulla	10k	3	195.0±22.5	$9.0±3.4^{ab}$
	100k	3	225.6±31.2	$10.5±3.1^{b}$
Isthmus	10k	3	245.3±63.7	$3.6±2.5^{ac}$
	100k	3	214.6±32.4	$7.5±0.2^{ab}$

n: Number of oviductal samples used in nanoparticle analysis system (Nanosigth®).
k: Represents $1x10^3$
EV^8: $1x10^8$
^{a,b,c} values with different superscripts differ significantly between groups.

4.3.2. Extracellular Vesicles present in oviductal fluid improve the quality of *in vitro* produced bovine embryos

In Experiment 3, no differences were found on cleavage rates (range: 88.0-89.6%) and blastocysts yield at Day 8 (range: 24.6-26.9%) and Day 9 (range: 28.5-30.8%), as shown in Table 12. However, the blastocyst yield at Day 7 in the C+ group was significantly higher (20.6%) compared with the rest of the groups (range: 12.0-13.8) ($p<0.05$).

Table 12. Effect of *in vitro* embryo culture with OF-EV (ampulla - isthmus) isolated at different g-forces (10k-100k xg) on development *in vitro*.

	Centrifugal force (x*g*)	N	Cleavage n % ± S.E	Blastocyst yield Day 7 n % ± S.E	Day 8 n % ± S.E	Day 9 n % ± S.E
Control (C+)		490	432 (88.5±2.2)	99 (20.6±2.1)[a]	129 (26.9±1.9)	143 (29.6±2.3)
Control (C-)		566	498 (88.1±1.3)	75 (13.4±1.8)[b]	139 (24.6±1.7)	168 (29.7±2.5)
Ampulla	10k	514	460 (89.6±1.3)	62 (12.0±1.6)[b]	133 (26.0±2.1)	152 (29.7±2.1)
	100k	462	406 (88.0±1.1)	63 (13.8±1.9)[b]	116 (25.3±2.4)	131 (28.5±2.2)
Isthmus	10k	557	494 (88.6±1.5)	75 (13.7±1.1)[b]	135 (24.6±2.2)	164 (29.6±1.9)
	100k	549	488 (89.1±1.2)	68 (14.3±3.0)[b]	126 (26.1±4.1)	151 (30.8±4.5)

n: Total number of presumptive zygotes placed in culture.
k: Represents $1x10^3$
[a,b] Values in the same column with different superscripts differ significantly ($p< 0.05$).

The survival rate at 24 hours of vitrified/warmed blastocysts produced with OF-EV from I100k group was significantly higher to C+, C- and A10k groups (91.3% vs 48.3%; 71.0%; 62.2% respectively, $p<0.05$). At 48 and 72 hours these differences were increased with a survival rate of I100k group up to 80-89 %. Similarly, the survival rate of the blastocysts from I10k group was significantly higher than C+ in all time points (Table 13).

Table 13. Survival rates after vitrification and warming of D7-8 blastocyst cultured with OF-EV (ampulla - isthmus) isolated at different g-forces (10k-100k xg)

	Centrifugal force (x*g*)	n	Blastocyst survival after vitrification/warming			
			4 h n % ± S.E	24 h n % ± S.E	48 h n % ± S.E	72 h n % ± S.E
Control (C+)		80	65 (80.4±5.2)	43 (48.3±9.1)^{a}	37 (41.3±7.0)^{a}	31 (34.5±8.0)^{a}
Control (C-)		105	92 (87.6±4.5)	77 (71.0±6.6)^{ab}	64 (58.0±7.7)^{ab}	56 (50.5±8.1)^{ab}
Ampulla	10k	88	78 (85.9±4.6)	61 (62.2±10.4)^{ab}	52 (50.8±11.5)^{ab}	48 (46.3±12.2)^{ab}
	100k	64	59 (89.9±3.6)	50 (73.5±7.4)^{abc}	49 (71.9±7.7)^{bc}	45 (66.8±7.6)^{bc}
Isthmus	10k	119	114 (96.6±1.8)	100 (83.6±4.7)^{bc}	93 (78.6±3.3)^{bc}	86 (70.5±4.4)^{bc}
	100k	97	93 (96.7±1.6)	87 (91.3±3.8)^{c}	84 (89.3±4.3)^{c}	79 (80.1±3.9)^{c}

n: Total number of presumptive zygotes placed in culture.

k: Represents $1x10^{3}$

^{a,b,c} Values in the same column with different superscripts differ significantly ($p<0.05$).

The relative transcript abundance of 3 genes related with membrane trafficking (*AQP3*, *AQP11* and *ATP1A1*), two related with metabolism (*LDLR* and *LDHA*) and four with epigenetic phenomena (*DNMT3A*, *IGF2R*, *GRB10* and *SNRPN*) were determined in blastocysts cultured in presence of FCS (C+), BSA (C-), or supplemented with OF-EV isthmus-derived at either 10k (Isthmus 10k) or 100k (Isthmus 100k) (Figure 20 and 21). The water channel *AQP3* was significantly upregulated in both groups supplemented with isthmus vesicles (10k and 100k) compared with the group supplemented with serum (C+). The lipid receptor *LDLR* was significantly downregulated in C+ compared with the rest of the groups. *DNMT3A* and *SNRPN* were significantly downregulated in C+ compared with I100k. The other genes displayed no significant differences.

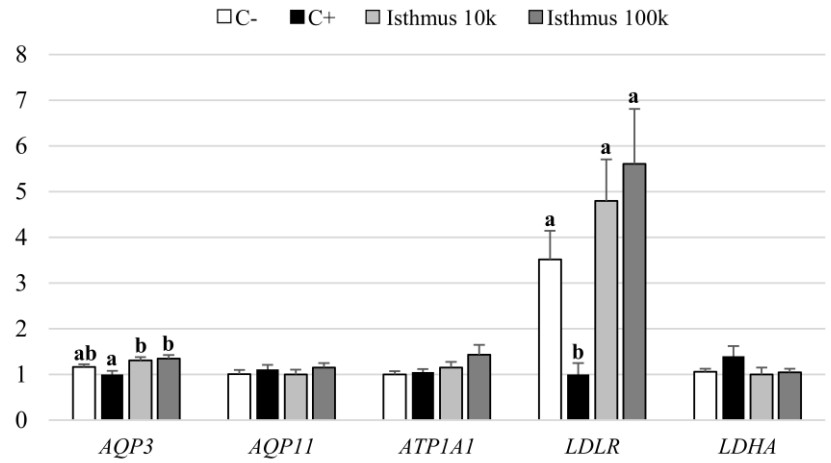

Figure 20. Relative mRNA transcription of developmental related genes in bovine *in vitro* blastocysts (D7-8 p.i) cultured with OF-EV (isthmus) isolated at different g-forces (10k-100k)
[a.b] Different superscripts indicate significant differences for each gene ($p<0.05$)

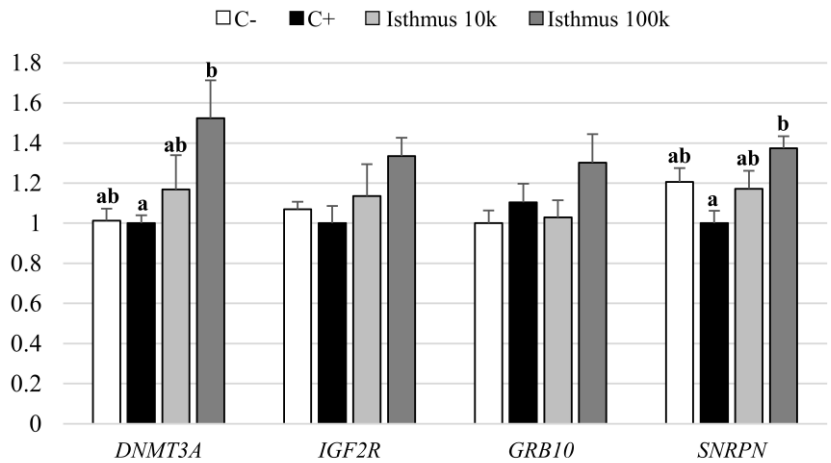

Figure 21. Relative mRNA transcription of developmental related genes in bovine in vitro blastocysts (D7-8 p.i) cultured with OF-EV (Isthmus) isolated at different g-forces (10k-100k)
[a.b] Different superscripts indicate significant differences for each gene ($p<0.05$)

5. DISCUSSION

5. Discussion

The oviduct is the anatomical part of the reproductive tract where fertilization and early embryo development take place, and provides an optimal environment to gametes and early embryo development (Hunter, 2012). Events such as sperm storage, capacitation, sperm release and transport, final oocyte maturation, fertilization, early embryo development and embryo transport to the uterus occur in the oviduct. These sequential events require a dynamic and synchronized system, successfully supported by the oviduct (Aguilar and Reyley, 2005). In the oviductal milieu, molecular mechanisms and pathways represent the first signal exchange between the maternal environment and the embryo (Besenfelder *et al.*, 2012). Many other mechanisms are still unknown that under *in vivo* conditions are difficult to study.

BOEC as *in vitro* model to study the oviductal environment

It is well known that culture environment during embryo development has an impact on the quality of the produced embryos in terms of cryotolerance (Rizos *et al.*, 2002b, 2008), ultrastructure morphology (Fair *et al.*, 2001); embryo cell number (Trigal *et al.*, 2011) and gene expression (Rizos *et al.*, 2002c; Lonergan *et al.*, 2003). Ellington *et al.* reported superior development of bovine zygotes and early embryos in simple medium with BOEC monolayers, compared with a complex medium (Ellington *et al.*, 1993). Recently, a study by Cordova *et al.* confirmed that the presence of BOEC at the early stage of embryo development, up to four days, improve embryo development and embryo quality in terms of specific gene transcripts, concluding that this period reflects the *in vivo* conditions where the embryo is still in the oviduct (Cordova *et al.*, 2014). We clearly showed in Experiment 1 that an established BOEC line can be used successful for co-culture with no differences on embryo development (day 7-9: ≈35%) when compared either with co-culture with fresh recovered cells or normal culture in SOF. This factor gives a great advantage over the classical co-culture systems since it helps to provide homogenous results.

Conditioned Media as vehicle of embryotrophic oviductal secretions

As mentioned earlier, the main aim of embryo co-culture was the capture of oviductal embryotrophic substances such as growth factors (Eyestone and First, 1989; Nancarrow and Hill, 1994) by modulating the media and the surrounding environment. Thus, the use of CM for *in vitro* culture would avoid undesired substances and exclude confounding effects of co-cultured cell/tissue presence (Eyestone *et al.*, 1991). In addition, CM can be prepared in large quantities, freeze and used when needed (Mermillod *et al.*, 1993). Our results in Experiment 1 indeed support that CM from the BOEC line had a similar impact on blastocyst yield than co-culture with fresh BOEC, further reinforcing the advantages of using an established BOEC line. In the same extend, Mermillod *et al.* demonstrate that BOEC CM in absence of FCS induce differential effects of embryonic development in terms of cleavage and blastocyst rates (Mermillod *et al.*, 1993). Furthermore, we clearly showed that embryos cultured with CM from frozen/thawed or fresh BOEC had better cryotolerance with a higher survival rate up to 72h. Similarly, the percentage of trophectoderm cells in embryos cultures with CM was significantly higher than the BOEC co-culture and control groups. TE cells are crucial for blastocoel re-expansion and maintenance after cryopreservation. Thus, higher lipid contents in TE cells (Abe *et al.*, 2002) makes them particularly damaged during cryopreservation. Moreover, in cattle, trophoblastic cells play a crucial role around day 14 when intense trophoblastic proliferation begins together with increased trophoblastic secretion of the pregnancy recognition factor interferon-tau (IFNT) (Ealy and Yang, 2009). IFNT regulates the expression of various uterine-derived factors responsible for placental attachment, modify the uterine immune system, and regulate early conceptus development (Austin *et al.*, 1996). Therefore, trophoblastic cells have an essential role on implantation and placentation.

Clearly then, we provide firm evidence that fresh or frozen BOEC CM improve blastocysts quality to the same extent than classical co-culture with fresh BOEC monolayers.

BOEC Extracellular Vesicles

The positive effect of BOEC CM could be due to soluble factors or to the presence in this medium of extracellular vesicles secreted by these cells. Electron microscopy and nanoparticle tracking analysis support the conclusion that extracellular vesicles are present in these conditioned media from the established BOEC line. By classical ultracentrifugation methods (Théry *et al.*, 2006) we obtained $3x10^5$ EV/ml from initial 10 ml of BOEC CM as assessed by Nanosight, in Experiment 1. Transmission electron microscopy confirms their mean size of 220 nm. To date only Burns *et al.* have demonstrated the presence of extracellular vesicles in the reproductive tract of ruminants (Burns *et al.*, 2014). These authors isolated exosomes (30 to 100 nm in diameter) and microvesicles from uterine luminal fluid of pregnant and cyclic ewes, containing specific proteins, miRNAs, and mRNAs. These studies provide evidence for pre-implantation communication of the conceptus and endometrium via cell secreted or shed vesicles. Recently, (Al-Dossary *et al.*, 2013) showed in mice the effect of oviductal exosomes on sperm motility and fertility. However, to our knowledge this is the first report where EV from BOEC have been isolated, morphologically characterized and used in *in vitro* embryo culture.

The extracellular vesicles (EV, exosomes - microvesicles) are emerging as important mediators of cell–cell communication. These EV produced by most cell types contain both mRNAs and non-coding RNAs such as small regulatory miRNAs, as well as proteins that can be functionally delivered between different cell types and across species. In reproduction, EV have been identified in amniotic fluid (Simpson *et al.*, 2008), uterine fluid (Ng *et al.*, 2013), placenta (Mincheva-Nilsson and Baranov, 2010), follicular fluid (Silveira *et al.*, 2012) and in oviductal environment (Al-Dossary *et al.*, 2013).

When fresh or frozen/thawed BOEC EV at different concentrations, were included in embryo culture in the presence of FCS, the developmental competences between days 7-9 were homogeneous including the control group (range: 40.9±1.8% - 46.0±2.9%). In the absence of FCS, embryo development of EV

groups and C^- was significantly lower (range: 23.3±1.5% - 25.1±1.8%) compared to C^+ (29.9±1.2%) evidencing the embryotrophic effect of FCS (Bavister, 1995). These results are in agreement with previous studies comparing serum with serum free media in *in vitro* culture (George *et al.*, 2008; Gómez *et al.*, 2008). However, we showed clearly the positive effect of BOEC EV in embryo cryotolerance with a significantly higher survival rate after vitrification and warming in serum free media and bypassing the negative effect of serum. It has been showed that embryos developed in serum-containing media have more TE cells than embryos produced in serum-free media (Crosier *et al.*, 2001), which is in line with our results showing that embryos cultured with BOEC EV either in serum or serum free media had higher number of total and TE cells.

Gene expression analysis of blastocysts cultured with BOEC EV in the presence of serum did not show differences compared to control group, apart of one transcript, *PAG1* that was highly expressed in BOEC EV groups. *PAG1* is a member of aspartic proteinase gene family, also related with implantation, which is primarily expressed in trophoblastic binucleate cells (Wooding *et al.*, 2005; Garbayo *et al.*, 2008; Ramos-Ibeas *et al.*, 2014). In cows, pregnancy-associated glycoproteins are released into the maternal circulation soon after implantation (i.e. around Day 25) and thereafter, concentrations rise until parturition. Plasma *PAG1* levels have been used for pregnancy diagnosis and as a marker of placental/foetal connection (Patel *et al.*, 1997; López-Gatius *et al.*, 2007). The fact that no clear differences were observed in gene expression, in clear contrast with the significant effect on cryotolerance and embryo cell number, could be partially explained by the presence of serum that could mask the effect of EV.

In the absence of serum *IFNT* and *PLAC8* expression levels were down regulated in EV groups compared to C^-, indicating a better quality embryo. A bovine embryo begins to express IFNT at the blastocyst stage (Farin *et al.*, 1990) and is primarily dependent on the presence of a functional TE (Hernandez-Ledezma *et al.*, 1993; Wrenzycki *et al.*, 2003). (Kubisch *et al.*, 1998) reported a negative relationship between early *IFNT* production and developmental competence, which

was later confirmed by comparing *in vivo*- and *in vitro*-produced blastocysts, showing that an early and high expression of *IFNT* indicates poor quality embryo (Wrenzycki *et al.*, 2001). *PLAC8 is* a gene related with successful fetal development, and plays an important role in placental development and feto-maternal interaction (Galaviz-Hernandez *et al.*, 2003). It has been linked to cell invasion, and higher expression in *in vitro* produced embryos has been associated with live birth (El-Sayed *et al.*, 2007). Similarly, Hoelker *et al.* found an up-regulation of *PLAC8* in *in vitro* produced embryos in groups of 50 when compared with groups of 16. However, in the same study it was evidenced that *in vivo* produced embryos down-regulate *PLAC8* compared to *in vitro* counterparts (Hoelker *et al.*, 2009). In the same line, Machado *et al.* showed down-regulation of *PLAC8* in *in vivo* produced embryos compared to *in vitro* (Machado *et al.*, 2013).

Additional markers of improved embryo quality would correspond to the upregulated expression of *CX43* and *GAPDH*. *CX43* is a gene related with compaction and cell to cell adhesion (Wrenzycki *et al.*, 2001; Rizos *et al.*, 2003). High expression of *CX43* has been associated with better quality embryos and increased cryotolerance (Gutiérrez-Adán *et al.*, 2004b). With regard to GAPDH, it has multiple functions independent of its role in energy metabolism. Increased GAPDH gene expression and enzymatic function is associated with cell proliferation (Nicholls *et al.*, 2012). *GAPDH* has been used as a housekeeping gene (Luchsinger *et al.*, 2014). However, Garcia-Herreros *et al.* found higher level of GAPDH protein in male embryos compared to female associated with the faster development of male embryos (Garcia-Herreros *et al.*, 2012).

Our results also suggest that the culture media (DMEM or TCM-199) used to cultivate BOEC did not affect the capacity of EV in improving embryo development and quality, although some specific effects were observed on embryo gene expression. The expression levels of *PAG1* from the control and FrEV-DMEM groups corresponded to late gestation, while that from FrEV-TCM199 group, with lower expression, would correspond to mid-gestation considering the classification of (Hashizume *et al.*, 2006).

The G6PD gene expression was either up-or down-regulated in embryos cultured with EV depending on the media used for BOEC culture. The G6PD gene has been coded as an indicator of the pentose phosphate pathway activity (Guérin *et al.*, 2001). A lower expression of *G6PD* has been observed in *in vitro* produced bovine embryos (Lopes *et al.*, 2007) and has been related with lower quality. However, in other studies a significantly higher *G6PD* expression has been observed in *in vitro*-produced embryos compared with *in vivo* cultured in the ewe oviduct (Lonergan *et al.*, 2003) or obtained *in vivo* (Wrenzycki *et al.*, 2002; Balasubramanian *et al.*, 2007). Also, the expression of this gene can be influenced *in vitro* by other factors such as sex of embryo, origin of embryo or respiration rate (Gutiérrez-Adán *et al.*, 2000; Lopes *et al.*, 2007).

Conclusively, we clearly demonstrated that BOEC-CM positive effect on early embryo development and quality can be reproduced by the sole addition of EV from an established BOEC line.

FCS Extracellular Vesicles

The FCS is a commonly used supplement in bovine *in vitro* culture increasing embryo development and the number of embryonic cells (Van Langendonckt *et al.*, 1997). However, embryos cultured with serum present a decreased compaction at the morula stage (Thompson, 1997), greater accumulation of lipid droplets in the cytoplasm (Rizos *et al.*, 2002a); lower cryotolerance (Rizos *et al.*, 2002a) and alterations in gene expression (Rizos *et al.*, 2003) compared to *in vivo* produced embryos. In addition, serum has been linked to the large offspring syndrome (Farin *et al.*, 2001; Lazzari *et al.*, 2002). It has been shown that serum contains an abundance of EV (Caby *et al.*, 2005) with mostly unknown function; however, serum-derived EV may have an influence in cell biology, such as growth of breast cancer cell lines (Ochieng *et al.*, 2009). Eitan *et al.* (2015) showed that EV depleted from bovine and human serum present a reduced capacity to support the cell growth.

Today, it is not clear the role of FCS EV in *in vitro* embryo culture. Thus, in experiment 1, we evidenced that EV from FCS have a deleterious effect on embryo

quality, which was avoided by an addition of BOEC EV in the culture media. So, EVs from FCS may be at least partially responsible for its consequences in short and long-term embryo/fetus development.

The oviductal fluid and *in vitro* embryo culture

Over the last two decades, a considerable amount of research has focused on the quality improvement of the *in vitro* produced blastocyst, which lags behind their *in vivo* counterparts. Studies using the "oviduct" as an intermediate host for embryo culture *in vitro* pointed out that the fundamental part of the process responsible for suboptimal embryo quality is the period after fertilization (Rizos *et al.*, 2010). Moreover, any modification of the *in vitro* culture conditions can have a significant impact for the normality of the embryo. Thus, in an attempt to resemble the *in vivo* conditions we studied the effect of the presence of bovine OF during *in vitro* embryo culture on the developmental competence and the quality of the blastocysts produced.

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

Most media used for *in vitro* embryo culture (e.g Synthetic Oviduct Fluid - (Holm *et al.*, 1999)) have been designed based on the ion, energy substrate and amino acid composition of the OF (Leese *et al.*, 2007). However, the optimization of an *in vitro* culture environment that contains all the substances present in the

oviduct regions may resolve part of the major limitations present in the embryo culture allowing the production and the quality of embryos with comparable rates and quality as those that occur *in vivo*. Thus, by mimicking *in vivo* conditions using animal models we could improve assisted reproductive technologies applied to both domestic species and humans.

Exposure of matured pig and cow oocytes for a short period of time (30 min) before fertilization to pure OF has been used to improve zona hardening, reduce polyspermy and improve embryo quality (Coy *et al.*, 2008; Cebrian-Serrano *et al.*, 2013). To our knowledge, this is the first evidence using OF during the embryo culture period and measuring embryo development and embryo quality. When a high concentration of OF was supplemented, a negative effect on blastocyst development was evident, with a low rate of 7% at Day 7. That may reflect the continuous renewal of OF in the oviduct *in vivo* as the reproductive tract modifies its activity in order to provide the optimal environment for the development of the embryo (Buhi, 2002), while *in vitro* degraded with a negative effect on the embryo. Gradual decreases in OF concentrations (2.5, 1.25 and 0.625%) diminish the detrimental effects, and improve embryo development rates.

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

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

embryo cryotolerance compared to serum group. On the other hand, higher number of total cells and trophectoderm cells were observed in the embryos cultured with 1.25% OF. It is known in cattle that trophoblastic cells play a crucial role around day 14 when intense trophoblast proliferation begins together with increased trophoblast secretion of the pregnancy recognition factor interferon-tau (IFNT) (Ealy and Yang, 2009). Therefore, trophoblastic cells have an essential role on implantation and placentation. The positive effect of OF on embryo development and quality could be attributed to the oviductal embryotrophic secretions which are absent in the defined and serum supplemented media. Ballester *et al.* proposed the OF and/or uterine fluid supplementation as a challenge for improving the *in vitro* culture environment (Ballester *et al.*, 2014). However, it is important to note that many aspects of oviductal environment are still unclear.

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

Recently, Cebrian-Serrano *et al.* evaluated the effect of a short incubation of matured oocytes with OF before fertilization on early embryo development and quality. No effect on embryo development was found; however, embryos derived from OF pre-incubation showed modification in *G6PD* and *SOD32* transcripts (Cebrian-Serrano *et al.*, 2013). Similarly, *in vitro* matured porcine oocytes exposed to bovine OF during 30 min before fertilization increased blastocyst rates, embryo cell number, and the gene expression patterns of apoptotic and developmentally related genes were modified (Lloyd *et al.*, 2009).

Gene expression analysis in our study suggested that glucose and lipid metabolism is affected by the addition of serum to the culture media. In particular, the expression of the glucose transporter *SCL2A1* and the enzyme *GADPH*, whose

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

Our findings evidenced a positive effect of OF supplementation, as a replacement of serum during embryo culture, improving the blastocyst development and quality.

Extracellular vesicles of oviductal fluid

To elucidate the role of bovine oviductal fluid EV on early embryo development and embryo quality the *in vitro* culture was used as a tool. We found that embryo development was not affected from the presence of OF-EV, but the quality of the produced embryos in terms of cryotolerance as well as the expression of metabolism and epigenetics related genes was improved. To our knowledge this is the first attempt isolating and morphologically characterized EV from the ampullary and isthmic OF and evaluating their effect during *in vitro* culture.

The first stages of early embryo development in bovine occur in the oviduct, where the embryo spends around 4 days (Hackett *et al.*, 1993). Embryonic Genome Activation occurs when the embryo starts to synthetize and use its own mRNA (Graf *et al.*, 2014) and a correct process ensures normal preimplantation and early fetal development (Gad *et al.*, 2012). The oviductal environment is represented in the OF, constituted by the secretions of OEC and the transudation into the oviduct lumen of blood plasma (Ellington, 1991). So far, the effect of bovine OF in vitro has been evidenced in porcine and bovine matured oocytes in terms of ZP modulation and embryo quality (Coy *et al.*, 2008; Lloyd *et al.*, 2009; Cebrian-Serrano *et al.*, 2013); and during *in vitro* culture of bovine zygotes with positive effect on embryo development and quality (Lopera *et al.*, 2014). Also, *in vitro* embryo co-culture with BOEC improved the development and quality of the blastocysts (Cordova *et al.*, 2014) and offers the possibility to study molecules, mechanisms and functions that *in vivo* are difficult to study (Ulbrich *et al.*, 2010; Cordova *et al.*, 2014). This knowledge can be used to improve the current *in vitro* production systems and identify models that will provide new information on early embryo maternal interactions.

The EV, are membrane vesicles secreted from the cell surface, and actually are considered mechanisms of intercellular communication (Raposo and Stoorvogel, 2013). A delivery of these components to neighboring cells has effects on recipient cell function (Lee *et al.*, 2012). The role of these intercellular communicators is clearly advanced in immunology and cancer fields (György *et al.*, 2011a). The EV are classified according to their size, biological content, biogenesis and way of release from the cell, as exosomes (30–90nm) (Théry, 2011), and MV and apoptotic bodies (>100nm) (Raposo and Stoorvogel, 2013).

The differential centrifugation is a widely used method to isolate EV from biological fluids (György *et al.*, 2011a). A lower centrifugal forces, sediment the larger EV (10k -20kx*g*), and the higher forces isolate the smaller EV (100k -120k x*g*) (Witwer *et al.*, 2013). Crescitelli *et al.* used differential centrifugation steps to achieve a relative EV separation, distinguishing EV subpopulations (Crescitelli *et al.*, 2013). Different EV populations associate different morphological characteristics and RNA profiles. Jeppensen *et al*. evaluate differential centrifugation forces (from 33k to 200k x*g*) on exosome characteristics in two human cell lines through NTA, protein quantification and immunoblotting, evidencing that different centrifugal forces influences the purity and yield of exosomes, and the sedimented MV profile (Jeppesen *et al.*, 2014). For qualitative and quantitative assessment of EV (characterization), are commonly used the EM to evidence the presence of the vesicular structures (Raposo *et al.*, 1996), and the NTA, for obtain concentration and size distribution of EV populations (Dragovic *et al.*, 2011). The size and concentration of EV from maternal reproductive tract under NTA measurements are heterogeneous. For example, the human follicular fluid EV are polydisperse and included MV and exosome sized EV, with an average of ≈220nm and a mean concentration of ≈$2.7x10^6$ EV/ml (Tannetta *et al.*, 2014). In the ewe uterine fluid the EV show a mean size of ≈148nm in a ≈$200x10^9$ EV/ml (Burns *et al.*, 2014). This is in agreement with our findings with an average size of ≈220nm and average concentration of ≈$7.6x10^8$ EV/ml between ampullary and isthmic OF-EV irrespective the force used for isolation. However, the concentration of isthmic

OF-EV isolated at 10k ($3.6x10^8$ EV/ml) was significantly lower compared to ampulla OF-EV at 100K ($10.5x10^8$ EV/ml) approving the effect of centrifugation force on EV populations.

The role of EV present in the reproductive tract is a very recently field of research. Silveira *et al.* isolated MV and exosomes of equine ovarian follicular fluid and evidenced the presence of proteins and miRNAs (Silveira *et al.*, 2012). These miRNAs were present in the follicular cells, suggesting that MV and exosomes play an important role in cell to cell communication within the mammalian ovarian follicle. In the same extend, Sohel *et al.* demonstrated a miRNA mediated transport by exosome and non-exosome structures in bovine follicular microenvironment, and associated the extracellular miRNA with the growth status of the oocyte (Sohel *et al.*, 2013). Using an *in vitro* model, Ng *et al.* identify and examine the presence and role of EV of the uterine cavity. The miRNA present in the uterine EV, allowed a bioinformatic identification of pathways and the possible influence after the EV taken-up by trophectoderm or endometrial epithelium at the time of implantation, or after sperm transfer during the transit across the uterus (Ng *et al.*, 2013). Burns *et al.* evidenced the presence of EV in the uterine fluid (UF) of pregnant and cyclic ewes, which contain specific proteins, miRNAs, and mRNAs, and their capability of delivering the contents *in vitro*. Moreover, the differences identified in the molecular contents by pregnancy status suggest a different EV sources (endometrium-conceptus), evidencing a conceptus-maternal communication, supporting the idea of UF-EV have a biological role in interactions relevant for the establishment and maintenance of pregnancy (Burns *et al.*, 2014). Recently, Al-Dossary *et al.* reveals the expression and secretion via oviductal exosomes of Plasma Membrane Ca^{2+}-ATPase 4a (PMCA4a - Ca^{2+} homeostasis) in the female reproductive tissues and luminal fluids during estrous, and their sperm-uptake with possible role in sperm viability during storage in the oviduct and during capacitation and acrosome reaction (Al-Dossary *et al.*, 2013).

Today, the role of bovine OF-EV on early embryo development is unknown. Thus, our results showed for first time that EV can be isolated from the ampulla or

isthmus of the bovine oviduct at the early luteal phase by ultracentrifugation and could be used successfully in *in vitro* culture with developmental rates similar to serum containing media. Furthermore, the quality of the blastocysts cultured with OF-EV was higher compared to those produced in semi-defined media with serum or BSA in terms of cryotolerance possibly due to cell-cell communication and other mechanisms related to the dialogue between the mother (oviduct) and the early embryo. In the same extend could be explained the higher survival rates evidenced in blastocysts produced with isthmic OF-EV compared to ampullary OF-EV. Isthmus is the part of the oviduct where *in vivo* the early embryo develop up to 8-16 cell stage before enter the uterus. Thus, the OF complexity is associated with the dynamic of oviductal epithelial cell populations at different stages of estrus cycle (Yániz *et al.*, 2000). In agreement with the above, a recent study from our group Maillo *et al.*, (2015), characterizing the bovine oviductal transcriptome at the beginning of the EGA between cyclic and pregnant heifers, showed 4011 genes differentially expressed between ampulla and isthmus irrespective of the presence of an embryo.

A plausible molecular explanation for the increased survival rates after vitrification in the isthmic OF-EV is the upregulation of the water channel *AQP3*. We have previously reported a similar small, yet significant, upregulation of *AQP3* in blastocysts cultured in the presence of oviductal fluid, which also exhibited higher cryotolerance than those cultured in the presence of serum (Lopera *et al.*, 2014). Aquaporins selectively conduct water molecules allowing the rapid movement of water through the membrane and thereby facilitating survival after freezing and thawing. In particular, the artificial expression of *AQP3* in mouse oocytes (Edashige *et al.*, 2003) improved survival after cryopreservation and *AQP3* has been proposed as a major water and cryoprotectant transporter in bovine morulae (Jin *et al.*, 2011). In contrast, two other genes related with membrane trafficking, another aquaporin (*AQP11*) and the Na/k-ATPase α-1-subunit (*ATP1A1*), essential for blastocysts expansion in mice (Barcroft *et al.*, 2004), did not differ between groups. We also analyzed the expression of a lipid receptor

(*LDLR)* and a glycolytic enzyme (*LDHA*) that has been related with anaerobic glycolysis in bovine cumulus cells (Bermejo-Alvarez *et al.*, 2010). *LDHA* did not display significant differences between groups, but *LDLR* was significantly downregulated in the presence of serum compared with the other groups. This result is in agreement with our previous findings in bovine embryos (Lopera *et al.*, 2014) and may be the consequence of a higher amount of lipids in the serum supplemented media. In this perspective, *LDLR* has been reported to be downregulated in embryos derived from obese mice and it was proposed as a regulator of lipid uptake in blastocysts (Bermejo-Alvarez *et al.*, 2012).

Finally, the *de novo* DNA methyltransferase *DNMT3A* and the imprinting gene *SNRPN* were downregulated in the group supplemented with serum compared with isthmic 100k OF-EV, whereas the imprinting gene *IGF2R* and the putatively imprinting gene *GRB10* did not revealed significant differences, despite showing a similar trend. Altered expression patterns of imprinting genes may be indicative of imprinting disorders. Imprinting disorders caused by artificial reproductive techniques are known to cause phenotypic alterations in the offspring such as the large offspring syndrome (Young *et al.*, 2001). In particular, abnormal *SNRPN* imprinting causes Prader-Willi syndrome in humans (Özçelik *et al.*, 1992). Similar to humans, bovine *SNRPN* is a paternally expressed imprinting gene (Lucifero *et al.*, 2006) and its expression is positively linked with methylation in bovine (Suzuki *et al.*, 2009). Thus, a reduced expression of *DNMT3A* may have caused a reduction in methylation and expression of *SNRPN*, similar to the one observed in bovine placenta derived from IVF embryos compared with AI (Suzuki *et al.*, 2009).

Conclusively, bovine OF-EV (isthmic) modifies the cryotolerance and gene expression patterns, providing evidence of an association between the oviductal environment and the developing embryo confirming an early embryo-maternal dialogue.

Overall, the results from this thesis confirm the need to use *in vitro* models, which mimic the *in vivo* environment, to improve our knowledge in events related to early embryo development and embryo maternal communication and develop new strategies to enhance assisted reproductive technologies.

6. CONCLUSIONS

6. Conclusions

1. An established BOEC line can be used successfully in *in vitro* embryo culture avoiding the reproducibility lack of primary cultures.
2. CM from BOEC primary culture or established line improves embryo quality in terms of cryotolerance and differential cell number.
3. EV from BOEC CM can be isolated, characterized and used successfully in *in vitro* embryo culture as an alternative of FCS, improving the quality of the produced embryos in terms of cryotolerance, differential cell number and developmental gene expression patterns.
4. EV from FCS do not affect embryo development but decrease cryotolerance of the produced embryos.
5. Low concentration of OF from early luteal phase, as a replacement of FCS in culture medium *in vitro*, exert a positive effect on embryo development and the quality of the resulting blastocysts, increasing their cryotolerance and number of trophectoderm cells and modifying the relative abundance of developmentally important gene transcripts, including imprinting genes.
6. EV from ampullary and isthmic OF from early luteal phase can be isolated, characterized and used successfully in *in vitro* embryo culture improving the cryotolerance of the resulting blastocysts.
7. EV from isthmic OF from early luteal phase affect the expression of metabolism and epigenetic related genes in *in vitro* produced embryos.
8. Essential association of the oviductal environment (cells-fluid-EV) and the developing embryo was evidenced, confirming an early embryo-maternal dialogue in bovine.

7. CONCLUSIONES

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1. Una línea establecida de BOEC permite con éxito el cultivo de embriones *in vitro,* evitando la falta de reproducibilidad propia del uso de cultivos primarios.
2. Los CM de cultivos primarios y de líneas establecidas de BOEC mejoran la calidad embrionaria, en términos de criotolerancia y número de células del trofoectodermo.
3. Las EV presentes en los CM de BOEC pueden ser aisladas, caracterizadas y utilizadas con éxito en el cultivo de embriones *in vitro* como una alternativa al FCS, mejorando la calidad de los embriones en términos de criotolerancia, número de células y patrón de expresión de genes relacionados con desarrollo.
4. Las EV presentes en el FCS no afectan al desarrollo del embrión, pero disminuyen su criotolerancia.
5. El empleo de bajas concentraciones de OF presente en la fase lútea temprana, como sustituto del FCS en el medio de cultivo *in vitro*, ejerce un efecto positivo en el desarrollo embrionario y en la calidad de los blastocistos incrementando su criotolerancia y el número de células del trofoectodermo, y modificando la abundancia relativa de genes relacionados con el desarrollo, incluyendo de impronta genómica parental.
6. Las EV presentes en el fluido del istmo y de la ampolla del oviducto en la fase lútea temprana pueden ser aisladas, caracterizadas y utilizadas con éxito en el cultivo de embriones *in vitro* mejorando la criotolerancia de los blastocistos.
7. Las EV presentes en el fluido del istmo oviductal en la fase lútea temprana afecta a la expresión de genes relacionados con el metabolismo y la epigenética en embriones producidos *in vitro*.
8. Se evidencia una asociación esencial entre el ambiente oviductal (células-fluido-EV) y el embrión en desarrollo, confirmando un diálogo materno-embrionario en la especie bovina.

8. BIBLIOGRAPHY

8. Bibliography

Abe H (1996) The mammalian oviductal epithelium: regional variations in cytological and functional aspects of the oviductal secretory cells. *Histology and Histopathology* **11** 743–768.

Abe H and Hoshi H (1997) Bovine oviductal epithelial cells: their cell culture and applications in studies for reproductive biology. *Cytotechnology* **23** 171–183.

Abe H, Onodera M, Sugawara S, Satoh T And Hoshi H (1999) Ultrastructural features of goat oviductal secretory cells at follicular and luteal phases of the oestrous cycle. *Journal of Anatomy* **195** 515–521.

Abe H, Yamashita S, Satoh T and Hoshi H (2002) Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Molecular Reproduction and Development* **61** 57–66.

Adams GP, Jaiswal R, Singh J and Malhi P (2008) Progress in understanding ovarian follicular dynamics in cattle. *Theriogenology* **69** 72–80.

Admyre C, Johansson SM, Qazi KR, Filén J-J, Lahesmaa R, Norman M, Neve EPA, Scheynius A and Gabrielsson S (2007) Exosomes with Immune Modulatory Features Are Present in Human Breast Milk. *The Journal of Immunology* **179** 1969–1978.

Aguilar and Reyley (2005) The uterine tubal fluid: secretion, composition and biological effects. *Anim. Reprod* **2** 91–105.

Almiñana C and Fazeli A (2012) Exploring the application of high-throughput genomics technologies in the field of maternal-embryo communication. *Theriogenology* **77** 717–737.

Almiñana C, Heath PR, Wilkinson S, Sanchez-Osorio J, Cuello C, Parrilla I, Gil MA, Vazquez JL, Vazquez JM, Roca J *et al.* (2012) Early Developing Pig Embryos Mediate Their Own Environment in the Maternal Tract. *PLoS ONE* **7** e33625.

De Antueno RJ, Knickle LC, Smith H, Elliot ML, Allen SJ, Nwaka S and Winther MD (2001) Activity of human Δ5 and Δ6 desaturases on multiple n-3 and n-6 polyunsaturated fatty acids. *FEBS Letters* **509** 77–80.

Asea A, Jean-Pierre C, Kaur P, Rao P, Linhares IM, Skupski D and Witkin SS (2008) Heat shock protein-containing exosomes in mid-trimester amniotic fluids. *Journal of Reproductive Immunology* **79** 12–17.

Austin KJ, Ward SK, Teixeira MG, Dean VC, Moore DW and Hansen TR (1996) Ubiquitin cross-reactive protein is released by the bovine uterus in response to interferon during early pregnancy. *Biology of Reproduction* **54** 600–606.

Avilés M, Gutiérrez-Adán A and Coy P (2010) Oviductal secretions: will they be key factors for the future ARTs? *Molecular Human Reproduction* **16** 896–906.

Aviles M, Coy P and Rizos D (2015) The oviduct: A key organ for the success of early reproductive events. *Animal Frontiers* **5** 25–31.

Babst M (2011) MVB Vesicle Formation: ESCRT-Dependent, ESCRT-Independent and Everything in Between. *Current Opinion in Cell Biology* **23** 452–457.

Baker TG and Franchi LL (1967) The Fine Structure of Oogonia and Oocytes in Human Ovaries. *Journal of Cell Science* **2** 213–224.

Balasubramanian S, Son WJ, Kumar BM, Ock SA, Yoo JG, Im GS, Choe SY and Rho GJ (2007) Expression pattern of oxygen and stress-responsive gene transcripts at various developmental stages of in vitro and in vivo preimplantation bovine embryos. *Theriogenology* **68** 265–275.

Ball PJH and Peters AR (2008) *Reproduction in Cattle*. John Wiley & Sons.

Ballester L, Romero-Aguirregomezcorta J, Soriano-Úbeda C, Matás C, Romar R and Coy P (2014) Timing of oviductal fluid collection, steroid concentrations, and sperm preservation method affect porcine in vitro fertilization efficiency. *Fertility and Sterility* **102** 1762–1768.e1.

Banerjee M and Chowdhury M (1994) Purification and characterization of a sperm-binding glycoprotein from human endometrium. *Human Reproduction (Oxford, England)* **9** 1497–1504.

Barcroft LC, Moseley AE, Lingrel JB and Watson AJ (2004) Deletion of the Na/K-ATPase α1-subunit gene (Atp1a1) does not prevent cavitation of the preimplantation mouse embryo. *Mechanisms of Development* **121** 417–426.

Barnes FL and First NL (1991) Embryonic transcription in in vitro cultured bovine embryos. *Molecular Reproduction and Development* **29** 117–123.

Bauersachs S, Blum H, Mallok S, Wenigerkind H, Rief S, Prelle K and Wolf E (2003) Regulation of Ipsilateral and Contralateral Bovine Oviduct Epithelial Cell Function in the Postovulation Period: A Transcriptomics Approach. *Biology of Reproduction* **68** 1170–1177.

Bauersachs S, Rehfeld S, Ulbrich SE, Mallok S, Prelle K, Wenigerkind H, Einspanier R, Blum H and Wolf E (2004) Monitoring gene expression changes in bovine oviduct epithelial cells during the oestrous cycle. *Journal of Molecular Endocrinology* **32** 449–466.

Bellingham SA, Coleman BM and Hill AF (2012) Small RNA deep sequencing reveals a distinct miRNA signature released in exosomes from prion-infected neuronal cells. *Nucleic Acids Research* **40** 10937–10949.

Bermejo-Álvarez P, Rizos D, Rath D, Lonergan P and Gutierrez-Adan A (2008) Epigenetic differences between male and female bovine blastocysts produced in vitro. *Physiological Genomics* **32** 264–272.

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P and Gutierrez-Adan A (2010) Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. *Proceedings of the National Academy of Sciences* **107** 3394–3399.

Bermejo-Álvarez P, Lonergan P, Rizos D and Gutiérrez-Adan A (2010) Low oxygen tension during IVM improves bovine oocyte competence and enhances anaerobic glycolysis. *Reproductive BioMedicine Online* **20** 341–349.

Bermejo-Alvarez P, Rosenfeld CS and Roberts RM (2012) Effect of maternal obesity on estrous cyclicity, embryo development and blastocyst gene expression in a mouse model. *Human Reproduction* **27** 3513–3522.

Besenfelder U, Havlicek V and Brem G (2012) Role of the Oviduct in Early Embryo Development. *Reproduction in Domestic Animals* **47** 156–163.

Betts DH and King WA (2001) Genetic regulation of embryo death and senescence. *Theriogenology* **55** 171–191.

Bleil JD and Wassarman PM (1980) Mammalian sperm-egg interaction: Identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell* **20** 873–882.

Boilard M, Bailey J, Collin S, Dufour M and Sirard M-A (2002) Effect of Bovine Oviduct Epithelial Cell Apical Plasma Membranes on Sperm Function Assessed by a Novel Flow Cytometric Approach. *Biology of Reproduction* **67** 1125–1132.

Boquest AC and Summers PM (1999) Effects of 17β-oestradiol or oestrous stage-specific cow serum on the ability of bovine oviductal epithelial cell monolayers to prolong the viability of bull spermatozoa. *Animal Reproduction Science* **57** 1–14.

Boquest AC, Smith JF, Briggs RM, Duganzich DM and Summers PM (1999) Effects of bovine oviductal proteins on bull spermatozoal function. *Theriogenology* **51** 583–595.

Bosch P, de Avila JM, Ellington JE and Wright Jr. RW (2001) Heparin and Ca2+-free medium can enhance release of bull sperm attached to oviductal epithelial cell monolayers. *Theriogenology* **56** 247–260.

Braw-Tal R and Yossefi S (1997) Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary. *Journal of Reproduction and Fertility* **109** 165–171.

Breitbart H, Rubinstein S and Lax Y (1997) Regulatory mechanisms in acrosomal exocytosis. *Reviews of Reproduction* **2** 165.

Briton-Jones C, Lok IH, Cheung CK, Chiu TTY, Cheung LP and Haines C (2004) Estradiol regulation of oviductin/oviduct-specific glycoprotein messenger ribonucleic acid expression in human oviduct mucosal cells in vitro. *Fertility and Sterility* **81, Supplement 1** 749–756.

Brouwers JF, Aalberts M, Jansen JWA, van Niel G, Wauben MH, Stout TAE, Helms JB and Stoorvogel W (2013) Distinct lipid compositions of two types of human prostasomes. *PROTEOMICS* **13** 1660–1666.

Buhi WC (2002) Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. *Reproduction* **123** 355–362.

Burns G, Brooks K, Wildung M, Navakanitworakul R, Christenson LK and Spencer TE (2014) Extracellular Vesicles in Luminal Fluid of the Ovine Uterus. *PLoS ONE* **9** e90913.

Caby M-P, Lankar D, Vincendeau-Scherrer C, Raposo G and Bonnerot C (2005) Exosomal-like vesicles are present in human blood plasma. *International Immunology* **17** 879–887.

Cagnone GLM, Dufort I, Vigneault C and Sirard M-A (2012) Differential Gene Expression Profile in Bovine Blastocysts Resulting from Hyperglycemia Exposure During Early Cleavage Stages. *Biology of Reproduction* **86** 50.

Camous S, Heyman Y, Méziou W and Ménézo Y (1984) Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles. *Journal of Reproduction and Fertility* **72** 479–485.

Carrasco LC, Coy P, Avilés M, Gadea J and Romar R (2008) Glycosidase determination in bovine oviducal fluid at the follicular and luteal phases of the oestrous cycle. *Reproduction, Fertility and Development* **20** 808–817.

Cebrian-Serrano A, Salvador I, García-Roselló E, Pericuesta E, Pérez-Cerezales S, Gutierrez-Adán A, Coy P and Silvestre M (2013) Effect of the Bovine Oviductal Fluid on In Vitro Fertilization, Development and Gene Expression of In Vitro-Produced Bovine Blastocysts. *Reproduction in Domestic Animals* **48** 331–338.

Clemente M, Fuente J de L, Fair T, Naib AA, Gutierrez-Adan A, Roche JF, Rizos D and Lonergan P (2009) Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? *Reproduction* **138** 507–517.

Clemente M, Lopez-Vidriero I, O'Gaora P, Mehta JP, Forde N, Gutierrez-Adan A, Lonergan P and Rizos D (2011) Transcriptome Changes at the Initiation of Elongation in the Bovine Conceptus. *Biology of Reproduction* **85** 285–295.

Cocucci E, Racchetti G and Meldolesi J (2009) Shedding microvesicles: artefacts no more. *Trends in Cell Biology* **19** 43–51.

Corcoran D, Fair T, Park S, Rizos D, Patel OV, Smith GW, Coussens PM, Ireland JJ, Boland MP, Evans ACO *et al.* (2006) Suppressed expression of genes involved in transcription and translation in in vitro compared with in vivo cultured bovine embryos. *Reproduction* **131** 651–660.

Cordova A, Perreau C, Uzbekova S, Ponsart C, Locatelli Y and Mermillod P (2014) Development rate and gene expression of IVP bovine embryos cocultured with bovine oviduct epithelial cells at early or late stage of preimplantation development. *Theriogenology* **81** 1163–1173.

Coy P, Cánovas S, Mondéjar I, Saavedra MD, Romar R, Grullón L, Matás C and Avilés M (2008) Oviduct-specific glycoprotein and heparin modulate sperm–zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proceedings of the National Academy of Sciences* **105** 15809–15814.

Coy P, García-Vázquez FA, Visconti PE and Avilés M (2012) Roles of the oviduct in mammalian fertilization. *Reproduction* **144** 649–660.

Crescitelli R, Lässer C, Szabó TG, Kittel A, Eldh M, Dianzani I, Buzás EI and Lötvall J (2013) Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. *Journal of Extracellular Vesicles* **2**.

Crosier AE, Farin PW, Dykstra MJ, Alexander JE and Farin CE (2001) Ultrastructural Morphometry of Bovine Blastocysts Produced In Vivo or In Vitro. *Biology of Reproduction* **64** 1375–1385.

Dinara S, Sengoku K, Tamate K, Horikawa M and Ishikawa M (2001) Effects of supplementation with free radical scavengers on the survival and fertilization rates of mouse cryopreserved oocytes. *Human Reproduction (Oxford, England)* **16** 1976–1981.

Al-Dossary AA, Strehler EE and Martin-DeLeon PA (2013) Expression and Secretion of Plasma Membrane Ca2+-ATPase 4a (PMCA4a) during Murine Estrus: Association with Oviductal Exosomes and Uptake in Sperm. *PLoS ONE* **8** e80181.

Dragovic RA, Gardiner C, Brooks AS, Tannetta DS, Ferguson DJP, Hole P, Carr B, Redman CWG, Harris AL, Dobson PJ *et al.* (2011) Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *Nanomedicine: Nanotechnology, Biology and Medicine* **7** 780–788.

Ealy AD and Yang QE (2009) REVIEW ARTICLE: Control of Interferon-Tau Expression During Early Pregnancy in Ruminants. *American Journal of Reproductive Immunology* **61** 95–106.

Eberhardt DM, Jacobs WG and Godkin JD (1999) Steroid Regulation of Retinol-Binding Protein in the Ovine Oviduct. *Biology of Reproduction* **60** 714–720.

Eckert J and Niemann H (1998) mRNA expression of leukaemia inhibitory factor (LIF) and its receptor subunits glycoprotein 130 and LIF-receptor-beta in bovine embryos derived in vitro or in vivo. *Molecular Human Reproduction* **4** 957–965.

Edashige K, Yamaji Y, Kleinhans FW and Kasai M (2003) Artificial Expression of Aquaporin-3 Improves the Survival of Mouse Oocytes after Cryopreservation. *Biology of Reproduction* **68** 87–94.

Edwards L j., Batt P a., Gandolfi F and Gardner D k. (1997) Modifications made to culture medium by bovine oviduct epithelial cells: Changes to carbohydrates stimulate bovine embryo development. *Molecular Reproduction and Development* **46** 146–154.

Eitan E, Zhang S, Witwer KW and Mattson MP (2015) Extracellular vesicle–depleted fetal bovine and human sera have reduced capacity to support cell growth. *Journal of Extracellular Vesicles* **4**.

Ekstedt E, Holm L and Ridderstråle Y (2004) Carbonic anhydrase in mouse testis and epididymis; transfer of isozyme IV to spermatozoa during passage. *Journal of Molecular Histology* **35** 167–173.

Ellington JE (1991) The bovine oviduct and its role in reproduction: a review of the literature. *The Cornell Veterinarian* **81** 313–328.

Ellington JE, Carney EW, Farrell PB, Simkin ME and Foote RH (1990) Bovine 1-2-cell embryo development using a simple medium in three oviduct epithelial cell coculture systems. *Biology of Reproduction* **43** 97–104.

Ellington JE, Ignotz GG, Ball BA, Meyers-Wallen VN and Currie WB (1993) De novo protein synthesis by bovine uterine tube (oviduct) epithelial cells changes during co-culture with bull spermatozoa. *Biology of Reproduction* **48** 851–856.

Ellington JE, Jones AE, Davitt CM, Schneider CS, Brisbois RS, Hiss GA and Wright RW (1998) Human sperm function in co-culture with human, macaque or bovine oviduct epithelial cell monolayers. *Human Reproduction* **13** 2797–2804.

Ellington JE, Evenson DP, Wright Jr. RW, Jones AE, Schneider CS, Hiss GA and Brisbois RS (1999) Higher-quality human sperm in a sample selectively attach to oviduct (fallopian tube) epithelial cells in vitro. *Fertility and Sterility* **71** 924–929.

El-Sayed A, Hoelker M, Rings F, Salilew D, Jennen D, Tholen E, Sirard M-A, Schellander K and Tesfaye D (2007) Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. *Physiological Genomics* **28** 84–96.

Escola J-M, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O and Geuze HJ (1998) Selective Enrichment of Tetraspan Proteins on the Internal Vesicles of Multivesicular Endosomes and

on Exosomes Secreted by Human B-lymphocytes. *Journal of Biological Chemistry* **273** 20121–20127.

Espey LL (1980) Ovulation as an inflammatory reaction--a hypothesis. *Biology of Reproduction* **22** 73–106.

Eyestone WH and First NL (1989) Co-culture of early cattle embryos to the blastocyst stage with oviducal tissue or in conditioned medium. *Journal of Reproduction and Fertility* **85** 715–720.

Eyestone WH, Leibfried-Rutledge ML, Northey DL, Gilligan BG and First NL (1987) Culture of one- and two-cell bovine embryos to the blastocyst stage in the ovine oviduct. *Theriogenology* **28** 1–7.

Eyestone WH, Jones JM and First NL (1991) Some factors affecting the efficacy of oviduct tissue-conditioned medium for the culture of early bovine embryos. *Journal of Reproduction and Fertility* **92** 59–64.

Fair T, Lonergan P, Dinnyes A, Cottell D c., Hyttel P, Ward F a. and Boland M p. (2001) Ultrastructure of bovine blastocysts following cryopreservation: Effect of method of blastocyst production. *Molecular Reproduction and Development* **58** 186–195.

Farin CE, Imakawa K, Hansen TR, McDonnell JJ, Murphy CN, Farin PW and Roberts RM (1990) Expression of trophoblastic interferon genes in sheep and cattle. *Biology of Reproduction* **43** 210–218.

Farin PW, Crosier AE and Farin CE (2001) Influence of in vitro systems on embryo survival and fetal development in cattle. *Theriogenology* **55** 151–170.

Farin PW, Piedrahita JA and Farin CE (2006) Errors in development of fetuses and placentas from in vitro-produced bovine embryos. *Theriogenology* **65** 178–191.

Fazeli A (2008) Maternal communication with gametes and embryos. *Theriogenology* **70** 1182–1187.

Florman HM and First NL (1988) The regulation of acrosomal exocytosis: I. Sperm capacitation is required for the induction of acrosome reactions by the bovine Zona pellucida in vitro. *Developmental Biology* **128** 453–463.

Fortune JE (1994) Ovarian follicular growth and development in mammals. *Biology of Reproduction* **50** 225–232.

Freudzon L, Norris RP, Hand AR, Tanaka S, Saeki Y, Jones TLZ, Rasenick MM, Berlot CH, Mehlmann LM and Jaffe LA (2005) Regulation of meiotic prophase arrest in mouse oocytes by GPR3, a constitutive activator of the Gs G protein. *The Journal of Cell Biology* **171** 255–265.

Gad A, Hoelker M, Besenfelder U, Havlicek V, Cinar U, Rings F, Held E, Dufort I, Sirard M-A, Schellander K *et al.* (2012) Molecular Mechanisms and Pathways Involved in Bovine Embryonic Genome Activation and Their Regulation by Alternative In Vivo and In Vitro Culture Conditions. *Biology of Reproduction* **87** 100.

Galaviz-Hernandez C, Stagg C, de Ridder G, Tanaka TS, Ko MSH, Schlessinger D and Nagaraja R (2003) Plac8 and Plac9, novel placental-enriched genes identified through microarray analysis. *Gene* **309** 81–89.

Galli C and Lazzari G (1996) Practical aspects of IVM/IVF in cattle. *Animal Reproduction Science* **42** 371–379.

Galli C, Crotti G, Notari C, Turini P, Duchi R and Lazzari G (2001) Embryo production by ovum pick up from live donors. *Theriogenology* **55** 1341–1357.

Galli C, Duchi R, Crotti G, Turini P, Ponderato N, Colleoni S, Lagutina I and Lazzari G (2003) Bovine embryo technologies. *Theriogenology* **59** 599–616.

Gandolfi F and Moor RM (1987) Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. *Journal of Reproduction and Fertility* **81** 23–28.

Gandolfi F, Brevini TA, Richardson L, Brown CR and Moor RM (1989) Characterization of proteins secreted by sheep oviduct epithelial cells and their function in embryonic. *Development* **106** 303–312.

Garbayo JM, Serrano B and Lopez-Gatius F (2008) Identification of novel pregnancy-associated glycoproteins (PAG) expressed by the peri-implantation conceptus of domestic ruminants. *Animal Reproduction Science* **103** 120–134.

Garcia-Herreros M, Aparicio IM, Rath D, Fair T and Lonergan P (2012) Differential glycolytic and glycogenogenic transduction pathways in male and female bovine embryos produced in vitro. *Reproduction, Fertility and Development* **24** 344–352.

Gardner DK, Lane M, Spitzer A and Batt PA (1994) Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. *Biology of Reproduction* **50** 390–400.

Gassart A de, Géminard C, Février B, Raposo G and Vidal M (2003) Lipid raft-associated protein sorting in exosomes. *Blood* **102** 4336–4344.

Gatti J-L, Métayer S, Belghazi M, Dacheux F and Dacheux J-L (2005) Identification, Proteomic Profiling, and Origin of Ram Epididymal Fluid Exosome-Like Vesicles. *Biology of Reproduction* **72** 1452–1465.

George F, Daniaux C, Genicot G, Verhaeghe B, Lambert P and Donnay I (2008) Set up of a serum-free culture system for bovine embryos: Embryo development and quality before and after transient transfer. *Theriogenology* **69** 612–623.

Georgiou AS, Sostaric E, Wong CH, Snijders APL, Wright PC, Moore HD and Fazeli A (2005) Gametes Alter the Oviductal Secretory Proteome. *Molecular & Cellular Proteomics* **4** 1785–1796.

Georgiou AS, Snijders APL, Sostaric E, Aflatoonian R, Vazquez JL, Vazquez JM, Roca J, Martinez EA, Wright PC and Fazeli A (2007) Modulation of the oviductal environment by gametes. *Journal of Proteome Research* **6** 4656–4666.

Ghersevich S, Massa E and Zumoffen C (2015) Oviductal secretion and gamete interaction. *Reproduction* **149** R1–R14.

Gilchrist RB, Ritter LJ and Armstrong DT (2004) Oocyte–somatic cell interactions during follicle development in mammals. *Animal Reproduction Science* **82–83** 431–446.

Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR and Kot K (1996) Selection of the dominant follicle in cattle. *Biology of Reproduction* **55** 1187–1194.

Gómez E, Rodríguez A, Muñoz M, Caamaño JN, Hidalgo CO, Morán E, Facal N and Díez C (2008) Serum free embryo culture medium improves in vitro survival of bovine blastocysts to vitrification. *Theriogenology* **69** 1013–1021.

Gondos B, Westergaard L and Byskov AG (1986) Initiation of oogenesis in the human fetal ovary: Ultrastructural and squash preparation study. *American Journal of Obstetrics and Gynecology* **155** 189–195.

Gordon I (2003) *Laboratory Production of Cattle Embryos*. CABI Publishing.

Graf A, Krebs S, Heininen-Brown M, Zakhartchenko V, Blum H and Wolf E (2014) Genome activation in bovine embryos: Review of the literature and new insights from RNA sequencing experiments. *Animal Reproduction Science* **149** 46–58.

Gualtieri R and Talevi R (2000) In Vitro-Cultured Bovine Oviductal Cells Bind Acrosome-Intact Sperm and Retain This Ability upon Sperm Release. *Biology of Reproduction* **62** 1754–1762.

Gualtieri R and Talevi R (2003) Selection of highly fertilization-competent bovine spermatozoa through adhesion to the Fallopian tube epithelium in vitro. *Reproduction* **125** 251–258.

Guerin B, Nibart M, Guienne BM-L and Humblot P (1997) Sanitary risks related to embryo transfer in domestic species. *Theriogenology* **47** 33–42.

Guérin P, Mouatassim SE and Ménézo Y (2001) Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Human Reproduction Update* **7** 175–189.

Gutiérrez-Adán A, Oter M, Martínez-Madrid B, Pintado B and De La Fuente J (2000) Differential expression of two genes located on the X chromosome between male and female in vitro–produced bovine embryos at the blastocyst stage. *Molecular Reproduction and Development* **55** 146–151.

Gutiérrez-Adán A, Rizos D, Fair T, Moreira P n., Pintado B, Fuente J de la, Boland M p. and Lonergan P (2004a) Effect of speed of development on mRNA expression pattern in early bovine embryos cultured in vivo or in vitro. *Molecular Reproduction and Development* **68** 441–448.

Gutiérrez-Adán A, Rizos D, Fair T, Moreira P n., Pintado B, Fuente J de la, Boland M p. and Lonergan P (2004b) Effect of speed of development on mRNA expression pattern in early bovine embryos cultured in vivo or in vitro. *Molecular Reproduction and Development* **68** 441–448.

György B, Szabó TG, Pásztói M, Pál Z, Misják P, Aradi B, László V, Pállinger É, Pap E, Kittel Á *et al.* (2011a) Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cellular and Molecular Life Sciences* **68** 2667–2688.

György B, Módos K, Pállinger É, Pálóczi K, Pásztói M, Misják P, Deli MA, Sipos Á, Szalai A, Voszka I *et al.* (2011b) Detection and isolation of cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters. *Blood* **117** e39–e48.

Hackett AJ, Durnford R, Mapletoft RJ and Marcus GJ (1993) Location and status of embryos in the genital tract of superovulated cows 4 to 6 days after insemination. *Theriogenology* **40** 1147–1153.

Hashizume K, Ushizawa K, Patel OV, Kizaki K, Imai K, Yamada O, Nakano H and Takahashi T (2006) Gene expression and maintenance of pregnancy in bovine: roles of trophoblastic binucleate cell-specific molecules. *Reproduction, Fertility and Development* **19** 79–90.

Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE and Trimmer SA (1995) Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* **43** 141–152.

Hemler ME (2003) Tetraspanin Proteins Mediate Cellular Penetration, Invasion, and Fusion Events and Define a Novel Type of Membrane Microdomain. *Annual Review of Cell and Developmental Biology* **19** 397–422.

Hergenreider E, Heydt S, Tréguer K, Boettger T, Horrevoets AJG, Zeiher AM, Scheffer MP, Frangakis AS, Yin X, Mayr M *et al.* (2012) Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. *Nature Cell Biology* **14** 249–256.

Hernandez-Ledezma JJ, Villanueva C, Sikes JD and Roberts RM (1993) Effects of CZB versus medium 199 and of conditioning culture media with either bovine oviductal epithelial cells or buffalo rat liver cells on the development of bovine zygotes derived by in vitro maturation-in vitro fertilization procedures. *Theriogenology* **39** 1267–1277.

Hess KA, Chen L and Larsen WJ (1999) Inter-α-Inhibitor Binding to Hyaluronan in the Cumulus Extracellular Matrix Is Required for Optimal Ovulation and Development of Mouse Oocytes. *Biology of Reproduction* **61** 436–443.

Hoelker M, Rings F, Lund Q, Ghanem N, Phatsara C, Griese J, Schellander K and Tesfaye D (2009) Effect of the microenvironment and embryo density on developmental characteristics and gene expression profile of bovine preimplantative embryos cultured in vitro. *Reproduction* **137** 415–425.

Holm P, Booth PJ, Schmidt MH, Greve T and Callesen H (1999) High bovine blastocyst development in a static in vitro production system using sofaa medium supplemented with

sodium citrate and myo-inositol with or without serum-proteins. *Theriogenology* **52** 683–700.

Holt CE and Bullock SL (2009) Subcellular mRNA Localization in Animal Cells and Why It Matters. *Science* **326** 1212–1216.

Hugentobler SA, Diskin MG, Leese HJ, Humpherson PG, Watson T, Sreenan JM and Morris DG (2007) Amino acids in oviduct and uterine fluid and blood plasma during the estrous cycle in the bovine. *Molecular Reproduction and Development* **74** 445–454.

Hugentobler S a., Humpherson P g., Leese H j., Sreenan J m. and Morris D g. (2008) Energy substrates in bovine oviduct and uterine fluid and blood plasma during the oestrous cycle. *Molecular Reproduction and Development* **75** 496–503.

Hulboy DL, Rudolph LA and Matrisian LM (1997) Matrix metalloproteinases as mediators of reproductive function. *Molecular Human Reproduction* **3** 27–45.

Hull KL and Harvey S (2001) Growth hormone: roles in female reproduction. *Journal of Endocrinology* **168** 1–23.

Humblot P (2001) Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. *Theriogenology* **56** 1417–1433.

Hunter RH (1994) Modulation of gamete and embryonic microenvironments by oviduct glycoproteins. *Molecular Reproduction and Development* **39** 176–181.

Hunter R (2003) Reflections upon Sperm–Endosalpingeal and Sperm–Zona Pellucida Interactions In Vivo and In Vitro. *Reproduction in Domestic Animals* **38** 147–154.

Hunter R h. f. (2012) Components of oviduct physiology in eutherian mammals. *Biological Reviews* **87** 244–255.

Hunter RH and Wilmut I (1984) Sperm transport in the cow: peri-ovulatory redistribution of viable cells within the oviduct. *Reproduction, Nutrition, Development* **24** 597–608.

Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, Xiao T, Schafer J, Lee M-LT, Schmittgen TD *et al.* (2008) Detection of microRNA Expression in Human Peripheral Blood Microvesicles. *PLoS ONE* **3** e3694.

Hussein TS, Thompson JG and Gilchrist RB (2006) Oocyte-secreted factors enhance oocyte developmental competence. *Developmental Biology* **296** 514–521.

Ireland JJ, Coulson PB and Murphree RL (1979) Follicular development during four stages of the estrous cycle of beef cattle. *Journal of Animal Science* **49** 1261–1269.

Iwasaki S, Yoshiba N, Ushijima H, Watanabe S and Nakahara T (1990) Morphology and proportion of inner cell mass of bovine blastocysts fertilized in vitro and in vivo. *Journal of Reproduction and Fertility* **90** 279–284.

Jeppesen DK, Hvam ML, Primdahl-Bengtson B, Boysen AT, Whitehead B, Dyrskjøt L, Ørntoft TF, Howard KA and Ostenfeld MS (2014) Comparative analysis of discrete exosome fractions obtained by differential centrifugation. *Journal of Extracellular Vesicles* **3**.

Jin B, Kawai Y, Hara T, Takeda S, Seki S, Nakata Y, Matsukawa K, Koshimoto C, Kasai M and Edashige K (2011) Pathway for the Movement of Water and Cryoprotectants in Bovine Oocytes and Embryos. *Biology of Reproduction* **85** 834–847.

Johnson MH and Ziomek CA (1981) The foundation of two distinct cell lineages within the mouse morula. *Cell* **24** 71–80.

Kamishita H, Takagi M, Choi YH, Wijayagunawardane MPB, Miyazawa K and Sato K (1999) Development of in vitro matured and fertilized bovine embryos cocultured with bovine oviductal epithelial cells obtained from oviducts ipsilateral to cystic follicles. *Animal Reproduction Science* **56** 201–209.

Killian GJ (2004) Evidence for the role of oviduct secretions in sperm function, fertilization and embryo development. *Animal Reproduction Science* **82–83** 141–153.

Killian G (2011) Physiology And Endocrinology Symposium: Evidence that oviduct secretions influence sperm function: A retrospective view for livestock. *Journal of Animal Science* **89** 1315–1322.

Kim JH, Funahashi H, Niwa K and Okuda K (1993) Glucose requirement at different developmental stages of in vitro fertilized bovine embryos cultured in semi-defined medium. *Theriogenology* **39** 875–886.

Kölle S, Stojkovic M, Prelle K, Waters M, Wolf E and Sinowatz F (2001) Growth Hormone (GH)/GH Receptor Expression and GH-Mediated Effects During Early Bovine Embryogenesis. *Biology of Reproduction* **64** 1826–1834.

Kölle S, Stojkovic M, Boie G, Wolf E and Sinowatz F (2002) Growth hormone inhibits apoptosis in in vitro produced bovine embryos. *Molecular Reproduction and Development* **61** 180–186.

Kouba AJ, Abeydeera LR, Alvarez IM, Day BN and Buhi WC (2000) Effects of the Porcine Oviduct-Specific Glycoprotein on Fertilization, Polyspermy, and Embryonic Development In Vitro. *Biology of Reproduction* **63** 242–250.

Kratz E, Poland DCW, van Dijk W and Kątnik-Prastowska I (2003) Alterations of branching and differential expression of sialic acid on alpha-1-acid glycoprotein in human seminal plasma. *Clinica Chimica Acta* **331** 87–95.

Kubisch HM, Larson MA and Roberts RM (1998) Relationship between age of blastocyst formation and interferon-τ secretion by in vitro–derived bovine embryos. *Molecular Reproduction and Development* **49** 254–260.

Lakkaraju A and Rodriguez-Boulan E (2008) Itinerant exosomes: emerging roles in cell and tissue polarity. *Trends in Cell Biology* **18** 199–209.

Van Langendonckt AV, Donnay I, Schuurbiers N, Auquier P, Carolan C, Massip A and Dessy F (1997) Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium. *Journal of Reproduction and Fertility* **109** 87–93.

Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H and Galli C (2002) Cellular and Molecular Deviations in Bovine In Vitro-Produced Embryos Are Related to the Large Offspring Syndrome. *Biology of Reproduction* **67** 767–775.

Lechniak D, Pers-Kamczyc E and Pawlak P (2008) Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos. *Reproductive Biology* **8** 23–42.

Lee CN and Ax RL (1984) Concentrations and Composition of Glycosaminoglycans in the Female Bovine Reproductive Tract1. *Journal of Dairy Science* **67** 2006–2009.

Lee K-F, Yao Y-Q, Kwok K-L, Xu J-S and Yeung WSB (2002) Early Developing Embryos Affect the Gene Expression Patterns in the Mouse Oviduct. *Biochemical and Biophysical Research Communications* **292** 564–570.

Lee Y, Andaloussi SE and Wood MJA (2012) Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. *Human Molecular Genetics* **21** R125–R134.

Leese HJ (1988) The formation and function of oviduct fluid. *Journal of Reproduction and Fertility* **82** 843–856.

Leese HJ, Tay JI, Reischl J and Downing SJ (2001) Formation of Fallopian tubal fluid: role of a neglected epithelium. *Reproduction* **121** 339–346.

Leese HJ, Hugentobler SA, Gray SM, Morris DG, Sturmey RG, Whitear S and Sreenan JM (2007) Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. *Reproduction, Fertility and Development* **20** 1–8.

Leese HJ, Baumann CG, Brison DR, McEvoy TG and Sturmey RG (2008) Metabolism of the viable mammalian embryo: quietness revisited. *Molecular Human Reproduction* **14** 667–672.

Lepesheva GI and Waterman MR (2004) CYP51—the omnipotent P450. *Molecular and Cellular Endocrinology* **215** 165–170.

Lim J m. and Hansel W (1998) Improved development of in vitro–derived bovine embryos by use of a nitric oxide scavenger in a cumulus–granulosa cell coculture system. *Molecular Reproduction and Development* **50** 45–53.

Lim JM, Reggio BC, Godke RA and Hansel W (1997) Perifusion culture system for bovine embryos: improvement of embryo development by use of bovine oviduct epithelial cells, an antioxidant and polyvinyl alcohol. *Reproduction, Fertility, and Development* **9** 411–418.

Lin Y, Mahan K, Lathrop WF, Myles DG and Primakoff P (1994) A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. *The Journal of Cell Biology* **125** 1157–1163.

Lloyd RE, Romar R, Matás C, Gutiérrez-Adán A, Holt WV and Coy P (2009) Effects of oviductal fluid on the development, quality, and gene expression of porcine blastocysts produced in vitro. *Reproduction* **137** 679–687.

Lonergan P and Fair T (2008) In vitro-produced bovine embryos—Dealing with the warts. *Theriogenology* **69** 17–22.

Lonergan P, Khatir H, Piumi F, Rieger D, Humblot P and Boland MP (1999) Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. *Journal of Reproduction and Fertility* **117** 159–167.

Lonergan P, Gutiérrez-Adán A, Pintado B, Fair T, Ward F, Fuente JDL and Boland M (2000) Relationship between time of first cleavage and the expression of IGF-I growth factor, its receptor, and two housekeeping genes in bovine two-cell embryos and blastocysts produced in vitro. *Molecular Reproduction and Development* **57** 146–152.

Lonergan P, Rizos D, Gutiérrez-Adán A, Moreira PM, Pintado B, Fuente J de la and Boland MP (2003) Temporal Divergence in the Pattern of Messenger RNA Expression in Bovine Embryos Cultured from the Zygote to Blastocyst Stage In Vitro or In Vivo. *Biology of Reproduction* **69** 1424–1431.

Lopera R, Hamdi M, Maillo V, Nunez C, Coy P, Gutierrez-Adan A, Bermejo P and Rizos D (2014) 125 effect of bovine oviductal fluid on development and quality of in vitro-produced bovine embryos. *Reproduction, Fertility and Development* **27** 154–154.

Lopes AS, Wrenzycki C, Ramsing NB, Herrmann D, Niemann H, Løvendahl P, Greve T and Callesen H (2007) Respiration rates correlate with mRNA expression of G6PD and GLUT1 genes in individual bovine in vitro-produced blastocysts. *Theriogenology* **68** 223–236.

López-Gatius F, Garbayo JM, Santolaria P, Yániz JL, Almería S, Ayad A, de Sousa NM and Beckers JF (2007) Plasma pregnancy-associated glycoprotein-1 (PAG-1) concentrations during gestation in Neospora-infected dairy cows. *Theriogenology* **67** 502–508.

Luchsinger C, Arias ME, Vargas T, Paredes M, Sánchez R and Felmer R (2014) Stability of reference genes for normalization of reverse transcription quantitative real-time PCR (RT-qPCR) data in bovine blastocysts produced by IVF, ICSI and SCNT. *Zygote* **22** 505–512.

Lucifero D, Suzuki J, Bordignon V, Martel J, Vigneault C, Therrien J, Filion F, Smith LC and Trasler JM (2006) Bovine SNRPN Methylation Imprint in Oocytes and Day 17 In Vitro-Produced and Somatic Cell Nuclear Transfer Embryos. *Biology of Reproduction* **75** 531–538.

Lussier JG, Matton P and Dufour JJ (1987) Growth rates of follicles in the ovary of the cow. *Journal of Reproduction and Fertility* **81** 301–307.

Machado G m., Ferreira A r., Pivato I, Fidelis A, Spricigo J f., Paulini F, Lucci C m., Franco M m. and Dode M a. (2013) Post-hatching development of in vitro bovine embryos from day 7 to 14 in vivo versus in vitro. *Molecular Reproduction and Development* **80** 936–947.

Machaty Z, Peippo J and Peter A (2012) Production and manipulation of bovine embryos: Techniques and terminology. *Theriogenology* **78** 937–950.

Maillo V, Gaora PÓ, Forde N, Besenfelder U, Havlicek V, Burns GW, Spencer TE, Gutierrez-Adan A, Lonergan P and Rizos D (2015) Oviduct-Embryo Interactions in Cattle: Two-Way Traffic or a One-Way Street? *Biology of Reproduction* biolreprod.115.127969.

Mamo S, Mehta JP, Forde N, McGettigan P and Lonergan P (2012) Conceptus-Endometrium Crosstalk During Maternal Recognition of Pregnancy in Cattle. *Biology of Reproduction* **87** 6.

Martus NS, Verhage HG, Mavrogianis PA and Thibodeaux JK (1997) Enhanced in vitro development of bovine embryos in the presence of a bovine oviductal specific glycoprotein. *Theriogenology* **47** 334.

Maxfield FR and McGraw TE (2004) Endocytic recycling. *Nature Reviews Molecular Cell Biology* **5** 121–132.

Mayor S and Riezman H (2004) Sorting GPI-anchored proteins. *Nature Reviews Molecular Cell Biology* **5** 110–120.

McCaffrey C, McEvoy TG, Diskin MG, Gwazdauskas FC, Kane MT and Sreenan JM (1991) Successful co-culture of 1–4-cell cattle ova to the morula or blastocyst stage. *Journal of Reproduction and Fertility* **92** 119–124.

McNutt-Scott TL and Harris C (1998) Modulation of Intracellular Glutathione and Cysteine Metabolism in Bovine Oviduct Epithelial Cells Cultured In Vitro. *Biology of Reproduction* **59** 314–320.

Memili E and First NL (1999) Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. *Zygote* **8** 87–96.

Mendes Jr. JOB, Burns PD, De La Torre-Sanchez JF and Seidel Jr. GE (2003) Effect of heparin on cleavage rates and embryo production with four bovine sperm preparation protocols. *Theriogenology* **60** 331–340.

Menezo Y and Guerin P (1997) The mammalian oviduct: biochemistry and physiology. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **73** 99–104.

Ménézo YJR and Hérubel F (2002) Mouse and bovine models for human IVF. *Reproductive Biomedicine Online* **4** 170–175.

Mermillod P, Vansteenbrugge A, Wils C, Mourmeaux JL, Massip A and Dessy F (1993a) Characterization of the embryotrophic activity of exogenous protein-free oviduct-conditioned medium used in culture of cattle embryos. *Biology of Reproduction* **49** 582–587.

Mermillod P, Vansteenbrugge A, Wils C, Mourmeaux JL, Massip A and Dessy F (1993b) Characterization of the embryotrophic activity of exogenous protein-free oviduct-conditioned medium used in culture of cattle embryos. *Biology of Reproduction* **49** 582–587.

Mermillod, Schmaltz, Perreau, Tsikis, Martinot, Cordova and Locatelli (2010) Early development of ruminant embryos, autonomous process or the result of a positive dialog with surrounding maternal tissues? *Acta Scientiae Veterinariae* **38** 521–533.

Michael A, Bajracharya S, Yuen P, Zhou H, Star R, Illei G and Alevizos I (2010) Exosomes from human saliva as a source of microRNA biomarkers. *Oral Diseases* **16** 34–38.

Mincheva-Nilsson L and Baranov V (2010) REVIEW ARTICLE: The Role of Placental Exosomes in Reproduction. *American Journal of Reproductive Immunology* **63** 520–533.

Mishra S, Lei ZM and Rao CV (2003) A Novel Role of Luteinizing Hormone in the Embryo Development in Cocultures. *Biology of Reproduction* **68** 1455–1462.

Mittelbrunn M, Gutierrez-Vazquez C, Villarroya-Beltri C, Gonzalez S, Sanchez-Cabo F, Gonzalez MA, Bernad A and Sanchez-Madrid F (2011) Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nature Communications* **2** 282.

Mondéjar I, Grullón LA, García-Vázquez FA, Romar R and Coy P (2012) Fertilization outcome could be regulated by binding of oviductal plasminogen to oocytes and by releasing of plasminogen activators during interplay between gametes. *Fertility and Sterility* **97** 453–461.e3.

Mondéjar I, Martínez-Martínez I, Avilés M and Coy P (2013) Identification of Potential Oviductal Factors Responsible for Zona Pellucida Hardening and Monospermy During Fertilization in Mammals. *Biology of Reproduction* **89** 67.

Moore K, Rodríguez-Sallaberry CJ, Kramer JM, Johnson S, Wroclawska E, Goicoa S and Niasari-Naslaji A (2007) In vitro production of bovine embryos in medium supplemented with a serum replacer: Effects on blastocyst development, cryotolerance and survival to term. *Theriogenology* **68** 1316–1325.

Mucci N, Aller J, Kaiser GG, Hozbor F, Cabodevila J and Alberio RH (2006) Effect of estrous cow serum during bovine embryo culture on blastocyst development and cryotolerance after slow freezing or vitrification. *Theriogenology* **65** 1551–1562.

Murray MK (1997) Morphological features of epithelial cells in the sheep isthmus oviduct during early pregnancy. *The Anatomical Record* **247** 368–378.

Nancarrow CD and Hill JL (1994) Co-culture, oviduct secretion and the function of oviduct-specific glycoproteins. *Cell Biology International* **18** 1105–1114.

Natale DR, Sousa PD, Westhusin ME and Watson AJ (2001) Sensitivity of bovine blastocyst gene expression patterns to culture environments assessed by differential display RT-PCR. *Reproduction* **122** 687–693.

Ng YH, Rome S, Jalabert A, Forterre A, Singh H, Hincks CL and Salamonsen LA (2013) Endometrial Exosomes/Microvesicles in the Uterine Microenvironment: A New Paradigm for Embryo-Endometrial Cross Talk at Implantation. *PLoS ONE* **8** e58502.

Nicholls C, Li H and Liu J-P (2012) GAPDH: a common enzyme with uncommon functions. *Clinical and Experimental Pharmacology & Physiology* **39** 674–679.

Van Niel G, Porto-Carreiro I, Simoes S and Raposo G (2006) Exosomes: a common pathway for a specialized function. *Journal of Biochemistry* **140** 13–21.

Niemann H and Wrenzycki C (2000) Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent development. *Theriogenology* **53** 21–34.

Ochieng J, Pratap S, Khatua AK and Sakwe. AM (2009) Anchorage Independent Growth of Breast Carcinoma Cells is Mediated by Serum Exosomes. *Experimental Cell Research* **315** 1875–1888.

Offenberg H, Barcroft LC, Caveney A, Viuff D, Thomsen PD and Watson AJ (2000) mRNAs encoding aquaporins are present during murine preimplantation development. *Molecular Reproduction and Development* **57** 323–330.

Okada H, Hirose Y, Manonmani P, Uda A, Ito M and Sankai T (2005) Characterization of an immortalized oviduct cell line from the cynomolgus monkey (Macaca fascicularis). *Journal of Medical Primatology* **34** 67–72.

O'Toole CMB, Arnoult C, Darszon A, Steinhardt RA and Florman HM (2000) Ca2+ Entry through Store-operated Channels in Mouse Sperm Is Initiated by Egg ZP3 and Drives the Acrosome Reaction. *Molecular Biology of the Cell* **11** 1571–1584.

Özçelik T, Leff S, Robinson W, Donlon T, Lalande M, Sanjines E, Schinzel A and Francke U (1992) Small nuclear ribonucleoprotein polypeptide N (SNRPN), an expressed gene in the Prader–Willi syndrome critical region. *Nature Genetics* **2** 265–269.

Palasz AT, Rodriguez-Martinez H, Beltran-Breña P, Perez-Garnelo S, Martinez MF, Gutierrez-Adan A and De la Fuente J (2006) Effects of hyaluronan, BSA, and serum on bovine embryo in vitro development, ultrastructure, and gene expression patterns. *Molecular Reproduction and Development* **73** 1503–1511.

Parrish JJ (2014) Bovine in vitro fertilization: In vitro oocyte maturation and sperm capacitation with heparin. *Theriogenology* **81** 67–73.

Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH and First NL (1986) Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology* **25** 591–600.

Parrish JJ, Susko-Parrish J, Winer MA and First NL (1988) Capacitation of bovine sperm by heparin. *Biology of Reproduction* **38** 1171–1180.

Patel OV, Sulon J, Beckers JF, Takahashi T, Hirako M, Sasaki N and Domeki I (1997) Plasma bovine pregnancy-associated glycoprotein concentrations throughout gestation in relationship to fetal number in the cow. *European Journal of Endocrinology* **137** 423–428.

Pauw IMCD, Soom AV, Laevens H, Verberckmoes S and Kruif A de (2002) Sperm Binding to Epithelial Oviduct Explants in Bulls with Different Nonreturn Rates Investigated with a New In Vitro Model. *Biology of Reproduction* **67** 1073–1079.

Pegoraro LMC, Thuard JM, Delalleau N, Guérin B, Deschamps JC, Marquant - Le Guienne B and Humblot P (1998) Comparison of sex ratio and cell number of ivm-ivf bovine blastocysts co-cultured with bovine oviduct epithelial cells or with vero cells. *Theriogenology* **49** 1579–1590.

Peinado H, Alečković M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, Hergueta-Redondo M, Williams C, García-Santos G, Ghajar CM *et al.* (2012) Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nature Medicine* **18** 883–891.

Pisitkun T, Shen R-F and Knepper MA (2004) Identification and proteomic profiling of exosomes in human urine. *Proceedings of the National Academy of Sciences of the United States of America* **101** 13368–13373.

Plante L, L. Shepherd D, Allan King W and Plante C (1994) Cleavage and 3H-uridine incorporation in bovine embryos of high in vitro developmental potential. *Molecular Reproduction and Development* **39** 375–383.

Plourde D, Vigneault C, Laflamme I, Blondin P and Robert C (2012) Cellular and molecular characterization of the impact of laboratory setup on bovine in vitro embryo production. *Theriogenology* **77** 1767–1778.e1.

Pontes JHF, Nonato-Junior I, Sanches BV, Ereno-Junior JC, Uvo S, Barreiros TRR, Oliveira JA, Hasler JF and Seneda MM (2009) Comparison of embryo yield and pregnancy rate between in vivo and in vitro methods in the same Nelore (Bos indicus) donor cows. *Theriogenology* **71** 690–697.

Primakoff P and Myles DG (2000) The ADAM gene family: surface proteins with adhesion and protease activity. *Trends in Genetics* **16** 83–87.

Pushpakumara PG, Robinson RS, Demmers KJ, Mann GE, Sinclair KD, Webb R and Wathes DC (2002) Expression of the insulin-like growth factor (IGF) system in the bovine oviduct at oestrus and during early pregnancy. *Reproduction* **123** 859–868.

Rabinowits G, Gerçel-Taylor C, Day JM, Taylor DD and Kloecker GH (2009) Exosomal microRNA: a diagnostic marker for lung cancer. *Clinical Lung Cancer* **10** 42–46.

Ramos-Ibeas P, Calle A, Pericuesta E, Laguna-Barraza R, Moros-Mora R, Lopera-Vásquez R, Maillo V, Yáñez-Mó M, Gutiérrez-Adán A, Rizos D *et al.* (2014) An Efficient System to Establish Biopsy-Derived Trophoblastic Cell Lines from Bovine Embryos. *Biology of Reproduction* **91** 15.

Raposo G and Stoorvogel W (2013) Extracellular vesicles: Exosomes, microvesicles, and friends. *The Journal of Cell Biology* **200** 373–383.

Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ and Geuze HJ (1996) B lymphocytes secrete antigen-presenting vesicles. *The Journal of Experimental Medicine* **183** 1161–1172.

Reed JC (2000) Mechanisms of Apoptosis. *The American Journal of Pathology* **157** 1415–1430.

Reed KL, Blaeser LL, Dantzer V, Green ML and Simmen RC (1998) Control of secretory leukocyte protease inhibitor gene expression in the porcine periimplantation endometrium: a case of maternal-embryo communication. *Biology of Reproduction* **58** 448–457.

Reinhart KC, Dubey RK, Cometti B, Keller PJ and Rosselli M (2003) Differential Effects of Natural and Environmental Estrogens on Endothelin Synthesis in Bovine Oviduct Cells. *Biology of Reproduction* **68** 1430–1436.

Rief S, Sinowatz F, Stojkovic M, Einspanier R, Wolf E and Prelle K (2002) Effects of a novel co-culture system on development, metabolism and gene expression of bovine embryos produced in vitro. *Reproduction* **124** 543–556.

Rieger D, Grisart B, Semple E, Langendonckt AV, Betteridge KJ and Dessy F (1995) Comparison of the effects of oviductal cell co-culture and oviductal cell-conditioned medium on the development and metabolic activity of cattle embryos. *Journal of Reproduction and Fertility* **105** 91–98.

Rinaudo P and Schultz RM (2004) Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos. *Reproduction* **128** 301–311.

Rizos D, Ward F, Boland MP and Lonergan P (2001) Effect of culture system on the yield and quality of bovine blastocysts as assessed by survival after vitrification. *Theriogenology* **56** 1–16.

Rizos D, Fair T, Papadopoulos S, Boland MP and Lonergan P (2002a) Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro. *Molecular Reproduction and Development* **62** 320–327.

Rizos D, Ward F, Duffy P, Boland MP and Lonergan P (2002b) Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development* **61** 234–248.

Rizos D, Lonergan P, Boland MP, Arroyo-García R, Pintado B, Fuente J de la and Gutiérrez-Adán A (2002c) Analysis of Differential Messenger RNA Expression Between Bovine Blastocysts Produced in Different Culture Systems: Implications for Blastocyst Quality. *Biology of Reproduction* **66** 589–595.

Rizos D, Gutiérrez-Adán A, Pérez-Garnelo S, Fuente J de la, Boland MP and Lonergan P (2003) Bovine Embryo Culture in the Presence or Absence of Serum: Implications for Blastocyst Development, Cryotolerance, and Messenger RNA Expression. *Biology of Reproduction* **68** 236–243.

Rizos D, Pintado B, de la Fuente J, Lonergan P and Gutiérrez-Adán A (2007) Development and pattern of mRNA relative abundance of bovine embryos cultured in the isolated mouse oviduct in organ culture. *Molecular Reproduction and Development* **74** 716–723.

Rizos D, Clemente M, Bermejo-Alvarez P, De La Fuente J, Lonergan P and Gutiérrez-Adán A (2008) Consequences of In Vitro Culture Conditions on Embryo Development and Quality. *Reproduction in Domestic Animals* **43** 44–50.

Rizos D, Ramirez MA, Pintado B, Lonergan P and Gutierrez-Adan A (2010) Culture of bovine embryos in intermediate host oviducts with emphasis on the isolated mouse oviduct. *Theriogenology* **73** 777–785.

Robert C, Nieminen J, Dufort I, Gagné D, Grant JR, Cagnone G, Plourde D, Nivet A-L, Fournier É, Paquet É *et al.* (2011) Combining resources to obtain a comprehensive survey of the bovine embryo transcriptome through deep sequencing and microarrays. *Molecular Reproduction and Development* **78** 651–664.

Rodríguez A, Diez C, Ikeda S, Royo LJ, Caamaño JN, Alonso-Montes C, Goyache F, Alvarez I, Facal N and Gomez E (2006) Retinoids during the in vitro transition from bovine morula to blastocyst. *Human Reproduction (Oxford, England)* **21** 2149–2157.

Rodríguez A, Díez C, Caamaño JN, de Frutos C, Royo LJ, Muñoz M, Ikeda S, Facal N, Álvarez-Viejo M and Gómez E (2007) Retinoid receptor-specific agonists regulate bovine in vitro early embryonic development, differentiation and expression of genes related to cell cycle arrest and apoptosis. *Theriogenology* **68** 1118–1127.

Rodriguez-Martinez H (2007) Role of the oviduct in sperm capacitation. *Theriogenology* **68, Supplement 1** S138–S146.

Rosenkrans CF, Zeng GQ, MCNamara GT, Schoff PK and First NL (1993) Development of bovine embryos in vitro as affected by energy substrates. *Biology of Reproduction* **49** 459–462.

Rottmayer R, Ulbrich SE, Kölle S, Prelle K, Neumueller C, Sinowatz F, Meyer HHD, Wolf E and Hiendleder S (2006) A bovine oviduct epithelial cell suspension culture system suitable for studying embryo–maternal interactions: morphological and functional characterization. *Reproduction* **132** 637–648.

Rüsse I (1983) Oogenesis in cattle and sheep. *Bibliotheca Anatomica* **24** 77–92.

Salumets A, Hydén-Granskog C, Mäkinen S, Suikkari A-M, Tiitinen A and Tuuri T (2003) Early cleavage predicts the viability of human embryos in elective single embryo transfer procedures. *Human Reproduction* **18** 821–825.

Samardzija M, Karadjole M, Matkovic M, Cergolj M, Getz I, Dobranic T, Tomaskovic A, Petric J, Surina J, Grizelj J *et al.* (2006) A comparison of BoviPure® and Percoll® on bull sperm separation protocols for IVF. *Animal Reproduction Science* **91** 237–247.

Schmaltz-Panneau B, Cordova A, Dhorne-Pollet S, Hennequet-Antier C, Uzbekova S, Martinot E, Doret S, Martin P, Mermillod P and Locatelli Y (2014) Early bovine embryos regulate oviduct epithelial cell gene expression during in vitro co-culture. *Animal Reproduction Science* **149** 103–116.

Schultz RM and Kopf GS (1995) Molecular basis of mammalian egg activation. *Current Topics in Developmental Biology* **30** 21–62.

Schultz RM, Davis W, Stein P and Svoboda P (1999) Reprogramming of gene expression during preimplantation development. *Journal of Experimental Zoology* **285** 276–282.

Silveira JC da, Veeramachaneni DNR, Winger QA, Carnevale EM and Bouma GJ (2012) Cell-Secreted Vesicles in Equine Ovarian Follicular Fluid Contain miRNAs and Proteins: A Possible New Form of Cell Communication Within the Ovarian Follicle. *Biology of Reproduction* **86** 71.

Simpson RJ, Jensen SS and Lim JWE (2008) Proteomic profiling of exosomes: Current perspectives. *PROTEOMICS* **8** 4083–4099.

Sirard M-A (2012) Factors Affecting Oocyte and Embryo Transcriptomes. *Reproduction in Domestic Animals* **47** 148–155.

Skog J, Würdinger T, van Rijn S, Meijer DH, Gainche L, Curry WT, Carter BS, Krichevsky AM and Breakefield XO (2008) Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nature Cell Biology* **10** 1470–1476.

Sohel MMH, Hoelker M, Noferesti SS, Salilew-Wondim D, Tholen E, Looft C, Rings F, Uddin MJ, Spencer TE, Schellander K *et al.* (2013) Exosomal and Non-Exosomal Transport of Extra-Cellular microRNAs in Follicular Fluid: Implications for Bovine Oocyte Developmental Competence. *PLoS ONE* **8** e78505.

Van Soom A, Boerjan ML, Bols PE, Vanroose G, Lein A, Coryn M and Kruif A de (1997) Timing of compaction and inner cell allocation in bovine embryos produced in vivo after superovulation. *Biology of Reproduction* **57** 1041–1049.

Van Soom A, Vanroose G and De Kruif A (2001) Blastocyst Evaluation by Means of Differential Staining: a Practical Approach. *Reproduction in Domestic Animals* **36** 29–35.

Spencer TE and Bazer FW (2004) Conceptus signals for establishment and maintenance of pregnancy. *Reproductive Biology and Endocrinology* **2** 49.

Stojkovic M, Kolle S, Peinl S, Stojkovic P, Zakhartchenko V, Thompson JG, Wenigerkind H, Reichenbach HD, Sinowatz F and Wolf E (2002) Effects of high concentrations of hyaluronan in culture medium on development and survival rates of fresh and frozen-thawed bovine embryos produced in vitro. *Reproduction* **124** 141–153.

Sturmey R g., Bermejo-Alvarez P, Gutierrez-Adan A, Rizos D, Leese H j. and Lonergan P (2010) Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. *Molecular Reproduction and Development* **77** 285–296.

Sun T, Lei ZM and Rao CV (1997) A novel regulation of the oviductal glycoprotein gene expression by luteinizing hormone in bovine tubal epithelial cells. *Molecular and Cellular Endocrinology* **131** 97–108.

Sutton ML, Gilchrist RB and Thompson JG (2003) Effects of in-vivo and in-vitro environments on the metabolism of the cumulus–oocyte complex and its influence on oocyte developmental capacity. *Human Reproduction Update* **9** 35–48.

Suuroia T, Aunapuu M, Arend A and Sépp E (2002) [‘Light’ epithelial cells of swine and bovine oviducts]. *Tsitologiia* **44** 656–660.

Suzuki J, Therrien J, Filion F, Lefebvre R, Goff AK and Smith LC (2009) In vitro culture and somatic cell nuclear transfer affect imprinting of SNRPN gene in pre- and post-implantation stages of development in cattle. *BMC Developmental Biology* **9** 9.

Tadros W and Lipshitz HD (2009) The maternal-to-zygotic transition: a play in two acts. *Development* **136** 3033–3042.

Talbot P, Shur BD and Myles DG (2003) Cell Adhesion and Fertilization: Steps in Oocyte Transport, Sperm-Zona Pellucida Interactions, and Sperm-Egg Fusion. *Biology of Reproduction* **68** 1–9.

Talevi R and Gualtieri R (2004) In vivo versus in vitro fertilization. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **115, Supplement** S68–S71.

Talevi R and Gualtieri R (2010) Molecules involved in sperm-oviduct adhesion and release. *Theriogenology* **73** 796–801.

Tannetta D, Dragovic R, Alyahyaei Z and Southcombe J (2014) Extracellular vesicles and reproduction–promotion of successful pregnancy. *Cellular & Molecular Immunology* **11** 548–563.

Taylor DD and Gercel-Taylor C (2008) MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecologic Oncology* **110** 13–21.

Taylor DD, Akyol S and Gercel-Taylor C (2006) Pregnancy-Associated Exosomes and Their Modulation of T Cell Signaling. *The Journal of Immunology* **176** 1534–1542.

Tervit HR, Whittingham DG and Rowson LEA (1972) Successful Culture in Vitro of Sheep and Cattle Ova. *Journal of Reproduction and Fertility* **30** 493–497.

Thatcher WW, Guzeloglu A, Mattos R, Binelli M, Hansen TR and Pru JK (2001) Uterine-conceptus interactions and reproductive failure in cattle. *Theriogenology* **56** 1435–1450.

Théry C (2011) Exosomes: secreted vesicles and intercellular communications. *F1000 Biology Reports* **3**.

Théry C, Zitvogel L and Amigorena S (2002) Exosomes: composition, biogenesis and function. *Nature Reviews Immunology* **2** 569–579.

Théry C, Amigorena S, Raposo G and Clayton A (2006) Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids. In *Current Protocols in Cell Biology*. Eds JS Bonifacino, M Dasso, JB Harford, J Lippincott-Schwartz and KM Yamada. Hoboken, NJ, USA: John Wiley & Sons, Inc.

Théry C, Ostrowski M and Segura E (2009) Membrane vesicles as conveyors of immune responses. *Nature Reviews Immunology* **9** 581–593.

Thompson JG (1997) Comparison between in vivo-derived and in vitro-produced pre-elongation embryos from domestic ruminants. *Reproduction, Fertility, and Development* **9** 341–354.

Thompson JG (2000) In vitro culture and embryo metabolism of cattle and sheep embryos — a decade of achievement. *Animal Reproduction Science* **60–61** 263–275.

Thouas GA, Korfiatis NA, French AJ, Jones GM and Trounson AO (2001) Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. *Reproductive Biomedicine Online* **3** 25–29.

Trigal B, Gómez E, Díez C, Caamaño JN, Martín D, Carrocera S and Muñoz M (2011) In vitro development of bovine embryos cultured with activin A. *Theriogenology* **75** 584–588.

Tse P-K, Lee Y-L, Chow W-N, Luk JMC, Lee K-F and Yeung WSB (2008) Preimplantation Embryos Cooperate with Oviductal Cells to Produce Embryotrophic Inactivated Complement-3b. *Endocrinology* **149** 1268–1276.

Ulbrich SE, Kettler A and Einspanier R (2003) Expression and localization of estrogen receptor α, estrogen receptor β and progesterone receptor in the bovine oviduct in vivo and in vitro. *The Journal of Steroid Biochemistry and Molecular Biology* **84** 279–289.

Ulbrich SE, Zitta K, Hiendleder S and Wolf E (2010) In vitro systems for intercepting early embryo-maternal cross-talk in the bovine oviduct. *Theriogenology* **73** 802–816.

Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ and Lötvall JO (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature Cell Biology* **9** 654–659.

Vallée M, Dufort I, Desrosiers S, Labbe A, Gravel C, Gilbert I, Robert C and Sirard M-A (2009) Revealing the bovine embryo transcript profiles during early in vivo embryonic development. *Reproduction* **138** 95–105.

Vanroose G, Van Soom A and De Kruif A (2001) From Co-culture to Defined Medium: State of the Art and Practical Considerations. *Reproduction in Domestic Animals* **36** 25–28.

Vansteenbrugge A, Van Langendonckt A, Scutenaire C, Massip A and Dessy F (1994) In vitro development of bovine embryos in Buffalo rat liver- or bovine oviduct-conditioned medium. *Theriogenology* **42** 931–940.

Voronina E and Wessel GM (2003) The regulation of oocyte maturation. *Current Topics in Developmental Biology* **58** 53–110.

Van Wagtendonk-de Leeuw AM (2006) Ovum Pick Up and In Vitro Production in the bovine after use in several generations: A 2005 status. *Theriogenology* **65** 914–925.

Wang WL, Jiang HS, Lu KH and Gordon I (1990) Effect of condition medium and glucose concentration on the in vitro development of early bovine embryos. *Theriogenology* **33** 343.

Wetscher F, Havlicek V, Huber T, Gilles M, Tesfaye D, Griese J, Wimmers K, Schellander K, Müller M, Brem G *et al.* (2005) Intrafallopian transfer of gametes and early stage embryos for in vivo culture in cattle. *Theriogenology* **64** 30–40.

Winger QA, Rios P de los, Han VK, Armstrong DT, Hill DJ and Watson AJ (1997) Bovine oviductal and embryonic insulin-like growth factor binding proteins: possible regulators of 'embryotrophic' insulin-like growth factor circuits. *Biology of Reproduction* **56** 1415–1423.

Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J, Nolte-'t Hoen EN, Piper MG, Sivaraman S, Skog J *et al.* (2013) Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *Journal of Extracellular Vesicles* **2**.

Woldesenbet S and Newton GR (2003) Comparison of proteins synthesized by polarized caprine oviductal epithelial cells and oviductal explants in vitro. *Theriogenology* **60** 533–543.

Wolf E, Arnold G, Bauersachs S, Beier H, Blum H, Einspanier R, Fröhlich T, Herrler A, Hiendleder S, Kölle S *et al.* (2003) Embryo-Maternal Communication in Bovine – Strategies for Deciphering a Complex Cross-Talk. *Reproduction in Domestic Animals* **38** 276–289.

Wooding FBP, Roberts RM and Green JA (2005) Light and electron microscope immunocytochemical studies of the distribution of pregnancy associated glycoproteins (PAGs) throughout pregnancy in the cow: possible functional implications. *Placenta* **26** 807–827.

Wrenzycki C and Stinshoff H (2013) Maturation Environment and Impact on Subsequent Developmental Competence of Bovine Oocytes. *Reproduction in Domestic Animals* **48** 38–43.

Wrenzycki C, Herrmann D, Keskintepe L, Martins A, Sirisathien S, Brackett B and Niemann H (2001) Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. *Human Reproduction* **16** 893–901.

Wrenzycki C, Lucas-Hahn A, Herrmann D, Lemme E, Korsawe K and Niemann H (2002) In Vitro Production and Nuclear Transfer Affect Dosage Compensation of the X-Linked Gene Transcripts G6PD, PGK, and Xist in Preimplantation Bovine Embryos. *Biology of Reproduction* **66** 127–134.

Wrenzycki C, Herrmann D and Niemann H (2003) Timing of Blastocyst Expansion Affects Spatial Messenger RNA Expression Patterns of Genes in Bovine Blastocysts Produced In Vitro. *Biology of Reproduction* **68** 2073–2080.

Wrenzycki C, Herrmann D, Lucas-Hahn A, Gebert C, Korsawe K, Lemme E, Carnwath JW and Niemann H (2005) Epigenetic reprogramming throughout preimplantation development and consequences for assisted reproductive technologies. *Birth Defects Research Part C: Embryo Today: Reviews* **75** 1–9.

Wrenzycki C, Herrmann D and Niemann H (2007) Messenger RNA in oocytes and embryos in relation to embryo viability. *Theriogenology* **68, Supplement 1** S77–S83.

Wu B and Zan L (2012) Enhance Beef Cattle Improvement by Embryo Biotechnologies. *Reproduction in Domestic Animals* **47** 865–871.

Wubbolts R, Leckie RS, Veenhuizen PTM, Schwarzmann G, Möbius W, Hoernschemeyer J, Slot J-W, Geuze HJ and Stoorvogel W (2003) Proteomic and Biochemical Analyses of Human B Cell-derived Exosomes Potential Implications For Their Function And Multivesicular Body Formation. *Journal of Biological Chemistry* **278** 10963–10972.

Xia P, Han VK, Viuff D, Armstrong DT and Watson AJ (1996) Expression of insulin-like growth factors in two bovine oviductal cultures employed for embryo co-culture. *The Journal of Endocrinology* **149** 41–53.

Yadav PS, Saini A, Kumar A and Jain GC (1998) Effect of oviductal cell co-culture on cleavage and development of goat IVF embryos. *Animal Reproduction Science* **51** 301–306.

Yániz JL, Lopez-Gatius F, Santolaria P and Mullins KJ (2000) Study of the functional anatomy of bovine oviductal mucosa. *The Anatomical Record* **260** 268–278.

Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I and Sinclair KD (2001) Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nature Genetics* **27** 153–154.

9. CURRICULUM VITAE

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Personal Details

Date of birth: 9 august 1981
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Research and Working Experience

PhD student. *Preimplantation Embryology Laboratory. Department of Animal Reproduction (INIA).* Madrid, Spain. April 2011 - to date.

PhD thesis: Embryo Development in vitro in Cattle: Role of Oviduct cells, Oviductal fluid and Extracellular vesicles.

- IVF Laboratory
- Media Preparation
- Ovary selection and recovery at slaughterhouse
- Maturation, fertilization and embryo culture
- Embryo grading
- Embryo vitrification
- Embryo differential staining
- Embryo freezing
- RNA extraction for embryo samples to study gene expression by quantitative real time PCR
- Extracellular vesicles Isolation
- Bovine oviduct epithelial cells culture.

Lecturer. *Veterinary Faculty. Universidad Cooperativa de Colombia.* Villavicencio, Colombia. 2010 - 2011.

- Teaching and research.

Master student. *IVF Laboratory. Animal Technology Center (IVIA).* Segorbe, Spain. October 2008 – 2009.

Master thesis: "Factors affecting goat in vitro fertility after heterologous in vitro fertilization using zona free bovine oocytes".

- Maturation, fertilization and embryo culture
- Embryo grading
- Oocyte staining
- Goat sperm Recovery

Veterinary student. *Genetics and reproduction Laboratory. Universidad de los llanos,* Villavicencio, Colombia. 2005 – 2006.

Final project: Bovine immature oocyte vitrification by open pulled straw method.

- Oocyte maturation
- Oocyte staining
- Oocyte vitrification
- Ovary recovery at slaughterhouse

Education and courses

PhD: Department of Animal Reproduction. Instituto Nacional de Investigaciones Agropecuarias y tecnología agroalimentaria (INIA). Veterinary Faculty. Universidad Complutense de Madrid (UCM). Madrid, Spain. April 2011- July 2015.

Master in "Animal Breeding and Reproduction Biotechnology" (Universidad Politecnica de Valencia - Universidad Autónoma de Barcelona), Valencia, Spain. October 2009.

Specialization in "Animal Breeding and Reproduction Biotechnology". International Centre for Advanced Mediterranean Agronomic Studies (IAMZ). Valencia. Spain. June 2008.

Veterinary degree. Universidad de los Llanos. Villavicencio. Colombia. (2008/H16094) Spain. December 2006.

Languages: Spanish: Mother language**. English:** Intermediate.

Extracellular Vesicles: Implications in Biomedicine. Universidad Internacional Meléndez Pelayo. Valencia, Spain. September 2013.

XXXIV International course of Animal Reproduction. (INIA-AECID). Madrid, Spain. November 2011.

Bovine embryo transfer. Central de transferencia y núcleo de mejoramiento genético las camelias (CTELCA). Puerto Araujo, Colombia. September 2006.

Teaching experience

Reproduction seminars for veterinary degree students (INIA-UCM). Madrid, Spain. 2013 and 2014.

International course of Bovine Reproduction. INIA - CONIAF - CIMPA - Universidad ISA. S. de los Caballeros, Dominican Republic. May 2013.

Practical-Commercial assessment of IVF in cattle as reproductive technique (INIA - Universidad León - In vitro Brasil). León, Spain. April 2013.

Veterinary Faculty. Universidad Cooperativa de Colombia. Villavicencio, Colombia. 2010-2011.

Oral Communications at international conferences

Lopera R, Hamdi M, Fuertes B, Maillo V, Beltran P, Redruello A, Gutierrez-Adan A, Yañez-Mo M, Ramirez MA, Rizos D (2013). Extracellular vesicles secreted by bovine oviductal epithelial cells increase the quality of in vitro produced bovine embryos. *29th Scientific Meeting of the European Embryo Transfer Society (AETE). Istambul, Turkey.*

Lopera R, Beltran P, Ramos-Ibeas P, Gutierrez-Adan A, Ramirez MA, Rizos D (2012). The effect of embryo co-culture with different types of bovine oviductal epithelial cells and conditioned media in vitro on embryo development and quality. *28th Scientific Meeting of the European Embryo Transfer Society (AETE). Saint-Maló, France.*

Lopera R, Mendez I, Parra MR, Peña M, Gongora A (2007). Bovine immature oocyte vitrification by open pulled straw method. *II International Meeting of animal science researchers (ENICIP). Medellín, Colombia.*

Posters presented at international conferences

Lopera R, Hamdi M, Maillo V, Lloreda V, Nuñez C, Coy P, Gutierrez-Adan A, Bermejo P, Rizos D. Effect of bovine oviductal fluid on development and quality of in vitro-produced bovine embryos. *41th Annual Conference of the International Embryo Transfer Society (IETS). Versailles, France*. January 2015. Reproduction, Fertility and Development; 2015. p. 154. doi:http://dx.doi.org/10.1071/RDv27n1Ab125.

Lopera R, Hamdi M, Fuertes B, Maillo V, Beltran P, Redruello A, Yañez Mo M, Ramirez MA, Rizos D. Depletion of extracellular vesicles from fetal calf serum improves the quality of bovine embryos produced in vitro. *12° Congreso de la Asociación Española de Reproducción Animal (AERA). Alicante, Spain.* October 2014. Reproduction and Domestic Animals; 2014. p. 112. doi: 10.1111/rda.12402

Lopera R, Hamdi M, Maíllo V, Lloreda V, Núñez C, Gutiérrez-Adán A, et al. Effect of extracellular vesicles of bovine oviductal fluid on in vitro embryo development and quality *Conference: Epigenetics and Periconception Enviroment EPICONCEPT - COST. Vilamoura, Portugal.* October 2014. p 45.

Lopera R, Hamdi M, Maíllo V, Lloreda V, Núñez C, Coy P, Rizos D. Effect of bovine oviductal fluid on in vitro bovine embryo production. *30th Scientific Meeting of the European Embryo Transfer Society (AETE). Dresden, Germany*. September 2014. p. 128. Available: http://www.aete.eu/publications.php

Lopera R, Hamdi M, Fuertes B, Maillo V, Beltran P, Redruello A, Gutierrez-Adan A, Yañez-Mo M, Ramirez MA, Rizos D. Effect of extracellular vesicles secreted by bovine oviductal epithelial cells in in vitro bovine embryo production. *International Society for Extracellular Vesicles (ISEV). Rotterdam, the Netherlands.* May 2014. Journal of Extracellular Vesicles; 2014. p. 100. doi:http://dx.doi.org/10.3402/jev.v3.24214.

Lopera R, Hamdi M, Fuertes B, Maillo V, Beltran P, Redruello A, Gutierrez-Adan A, Yañez-Mo M, Ramirez MA, Rizos D. Extracellular vesicles secreted by bovine oviductal epithelial cells increase the quality of in vitro produced bovine embryos. *29th Scientific Meeting of the European Embryo Transfer Society (AETE). Istambul, Turkey.* September 2013. p. 174. Available: http://www.aete.eu/publications.php

Lopera R, Beltran P, Ramos-Ibeas P, Gutierrez-Adan A, Ramirez MA, Rizos D. The effect of embryo co-culture with different types of bovine oviductal epithelial cells and conditioned media in vitro on embryo development and quality. *28th Scientific Meeting of the European Embryo Transfer Society (AETE). Saint-Maló, France*. September 2012. p. 182. Available: http://www.aete.eu/publications.php

Publications at peer reviewed journals - SCI

Lopera-Vásquez R, Hamdi M, Maillo V, Lloreda V, Coy P, Gutierrez-Adan A, Bermejo-Álvarez P, Rizos D. Effect of bovine oviductal fluid on development and quality of bovine embryos in vitro. PLoS ONE. 2015 (Under review).

Lopera-Vásquez R, Hamdi M, Fernandez-Fuertes B, Maillo V, Beltrán-Breña P, Redruello A, Gutierrez-Adán A, Yañez-Mó M, Ramirez MA, Rizos D. Extracellular vesicles from BOEC in in vitro embryo development and quality. Reproduction fertility and development. 2015 (Under review).

Ramos-Ibeas P, Calle A, Pericuesta E, Laguna-Barraza R, Moros-Mora R, **Lopera-Vásquez R**, et al. An Efficient System to Establish Biopsy-Derived Trophoblastic Cell Lines from Bovine Embryos. Biol Reprod. 2014;91: 15. doi:10.1095/biolreprod.114.118430

Ahumada CJ, Salvador I, Cebrian-Serrano A, **Lopera R**, Silvestre MA (2013) Effect of supplementation of different growth factors in embryo culture medium with a small number of bovine embryos on in vitro embryo development and quality. *animal* **7**, 455–462. doi:10.1017/S1751731112001991.

In preparation

Lopera-Vásquez R, Hamdi M, Maillo V, Núñez C, Gutiérrez-Adán A, Bermejo-Álvarez P, Ramírez Miguel A, Yáñez-Mó M, Rizos D. Bovine oviductal fluid extracellular vesicles and their effect on *in vitro* embryo development and quality.