Master Thesis

"Animal breeding and reproduction biotechnology"

Competence of oviductal and uterine Extracellular Vesicles in sequential culture of in vitro bovine embryos

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Tutors at INIA:

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Valencia, June 2020

INFORME DE LOS TUTORES

El doctor Dimitrios Rizos Profesor de Investigación del Departamento de Reproducción Animal del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) y la doctora María Encina González Martínez Profesora Titular del Departamento de Anatomía y Embriología de la Facultad de Veterinaria de la Universidad Complutense de Madrid declaran que la memoria de Trabajo de Fin de Máster presentada por Analía Aracelli Yaryes Estaque con el título: **Competence of oviductal and uterine Extracellular Vesicles in sequential culture of in vitro bovine embryos**, ha sido realizada bajo nuestra dirección y que tras su revisión consideramos que tiene la debida calidad para su presentación y defensa.

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INDEX

LIST OF ABREVIATION

ANOVA: One-way analysis of variance
ART: Assisted reproductive technology
BOEC: Bovine oviduct epithelial cells
BSA: Bovine serum albumin
CL: Corpus luteum
CM: Conditioned media
COCs: Cumulus–oocyte complexes
dFCS: Depleted fetal calf serum
DNA: Deoxyribonucleic acid
EGA: Embryonic genome activation
EGF: Epidermal growth factor
ET: Embryo transfer
EVs: Extracellular vesicles
FCS: Fetal calf serum
ICM: Inner cell mass
IFNT: Interferon tau
IVC: In vitro culture
IVF: In vitro fertilization
IVM: In vitro maturation
IVP: In vitro embryo production
kW: Kilowatt
LOS: Large Offspring Syndrome
LH: Luteinizing hormone
MII: Metaphase II
mRNA: Messenger Ribonucleic acid
OF: Oviductal fluid
PBS: Phosphate-buffered saline
RNA: Ribonucleic acid
ROS: Reactive oxygen species
S1: Early-luteal phase
S2: Early-mid-luteal phase
SOF: Synthetic oviductal fluid

TALP: Tyrode's albumin lactate pyruvate

TCM-199: Tissue culture medium 199

TE: Trophectoderm cells

UF: Uterine fluid

ZP: Zona pellucida

LIST OF FIGURES

Page

LIST OF TABLES

Page

SUMMARY

In vitro embryo production (IVP) is a biotechnology applied to improve animal reproductive performance, efficiency and genetic gain. The primary focus of IVP is to maximize the number of offspring from genetically superior animals and disseminate germplasm worldwide. Despite the fact that IVP is used commercially successfully, it still has some limiting factors for cost reduction and greater dissemination related to the lower quality of the embryos generated in vitro and the consequent greater sensitivity to cryopreservation. It is known that embryo-maternal communication that occurs before implantation plays a vital role in the establishment and maintenance of pregnancy in mammals, so it is necessary to imitate what happens during this communication in in vitro cultures to improve the quality of the obtained embryos. This maternal-embryonic communication is mediated by various growth factors and molecular signaling pathways between the oviduct, the uterus and the embryo, which affect its development in the short and long term. Small particles called extracellular vesicles (EVs), released naturally by cells and containing various products such as proteins and RNAs among other molecules, play an important role in this interaction. Currently, it is still unknown how they work and how they affect the embryo in vivo or in vitro, but it has already been observed that embryos are capable of capturing these EVs. Therefore, the objective of this study was to isolate and characterize the EVs of the oviductal and uterine fluids and to evaluate their effects in an in vitro sequential culture system on the development and quality of bovine embryos. For this, bovine zygotes were cultured in synthetic oviductal fluid (SOF) supplemented with 3 mg/ ml BSA or 5% serum depleted of EVs in the presence or absence of $3x10^5$ EVs of the oviductal (Day 1-4) and uterine fluids (Day 4-9), mimicking the physiological conditions of early embryonic development in vivo. Pooled EVs from five oviducts (early-luteal phase) and five uterine horns (early-mid-luteal phase) from slaughtered heifers were isolated using the size exclusion chromatography kit (Hansa BioMed). The size and concentration of the EVs were evaluated by means of a nanotracking analysis system (NTA) and their morphology by transmission electron microscopy (TEM). Cleavage rate and blastocyst yield was recorded on day 2 and days 7 to 9 respectively. On day 7/8 day blastocyst quality was evaluated by staining with Hoechst 33342 for total number of cells, Bodipy 493/503 for lipid content, Mitotracker Deep Red for mitochondrial activity and survival rate after vitrification/warming. The

The concentration of EVs was $2.97x10^{10}$ and $7.98x10^{10}$ particles/ml, and the mean size of 177.5 and 216.5 nm for the oviductal (OF) and uterine fluids (UF), respectively. Through TEM we confirmed the presence of exosomes among EVs, showing the typical cup-shaped morphology. Western blotting confirmed the expression of classic EVs markers described for exosomes such as CD9 and CD63 tetraspanins and HSP70. Our results showed that supplementation of culture media with EVs from OF and UF has no effect on embryo development. However, their use had a positive effect on the quality of the produced embryos giving rise to blastocysts with a greater number of cells, lower lipid content and greater mitochondrial activity. In contrary the survival rate after vitrification / warming was not affected by the use of EVs in culture. In conclusion, mimicking physiological conditions using EVs from OF and UF in a sequential in vitro culture does not affect embryo development, but improves embryo quality by increasing total cell number, decreasing lipid content, and increasing mitochondrial activity. These results evidenced the association of the reproductive tract environment with the development of the early embryo, which confirms the maternal-embryonic communication.

Key words: Bovine, Embryo-maternal interaction, Extracellular Vesicles, In vitro

RESUMEN

La producción de embriones in vitro (PIV) es una biotecnología aplicada para mejorar el rendimiento reproductivo, la eficiencia productiva y la ganancia genética animal. El objetivo principal de la PIV es maximizar el número de descendientes de animales genéticamente superiores y diseminar germoplasma en todo el mundo. A pesar de que la PIV se usa comercialmente con éxito, todavía tiene algunos factores limitantes para la reducción de costos y una mayor difusión, relacionados con la menor calidad de los embriones generados in vitro y la consecuente mayor sensibilidad a la criopreservación. Se sabe que la comunicación materno-embrionaria que ocurre antes de la implantación juega un papel vital en el establecimiento y mantenimiento del embarazo en mamíferos, por lo que es necesario imitar lo que sucede durante esta comunicación en cultivos in vitro para mejorar la calidad de los embriones obtenidos. Esta comunicación materno-embrionaria está mediada por varios factores de crecimiento y vías de señalización molecular entre el oviducto, el útero y el embrión, que afectan su desarrollo a corto y largo plazo. Pequeñas partículas llamadas vesículas extracelulares (EVs), liberadas naturalmente por las células contienen diversos productos como proteínas y ARN entre otras moléculas, juegan un papel importante en esta interacción. Actualmente, aún se desconoce cómo funcionan y cómo afectan al embrión in vivo o in vitro, pero ya se ha observado que los embriones son capaces de capturar estas EVs. Es por eso que el objetivo de este estudio fue aislar y caracterizar las EVs de los fluidos oviductal y uterino y evaluar su efecto en un sistema de cultivo in vitro secuencial sobre el desarrollo y la calidad de los embriones bovinos. Para esto, los cigotos bovinos se cultivaron en fluido oviductal sintético (SOF) suplementado con 3 mg / ml de BSA o 5% de suero depletado de 5% de EVs en presencia o ausencia de $3x10^5$ EVs de los fluidos oviductales (Día 1-4) y uterino. (Día 4-9), imitando las condiciones fisiológicas del desarrollo embrionario temprano in vivo. Las EVs agrupadas de cinco oviductos (fase lútea temprana) y cinco cuernos uterinos (fase lútea temprana media) de vaquillas sacrificadas se aislaron utilizando el kit de cromatografía de exclusión por tamaño (Hansa BioMed). El tamaño y la concentración de las EVs se evaluaron por medio de un sistema de análisis de nanotracking (NTA) y la morfología por microscopía electrónica de transmisión (TEM). La tasa de división y el rendimiento de blastocito se registraron el día 2 y los días 7 a 9, respectivamente. El día 7/8 días se evaluó la calidad del blastocito mediante tinción con

Hoechst 33342 para el número total de células, Bodipy 493/503 para el contenido de lípidos, Mitotracker Deep Red para la actividad mitocondrial y la tasa de supervivencia después de la vitrificación / calentamiento. La concentración de EVs fue de $2.97x10^{10}$ y $7.98x10^{10}$ partículas / ml, y el tamaño medio fue de 177.5 y 216.5 nm para el fluido oviductal (OF) y el fluido uterino (UF), respectivamente. A través de TEM confirmamos la presencia de EVs, mostrando la típica morfología en forma de copa. El Western Blott confirmó la expresión de marcadores de EVs clásicos descritos para exosomas tales como tetraspaninas CD9 y CD63 y HSP70. Nuestros resultados mostraron que la suplementación de medios de cultivo con EVs de OF y UF no tiene ningún efecto en el desarrollo embrionario. Sin embargo, su uso tuvo un efecto positivo en la calidad de los embriones producidos, dando lugar a blastocitos con un mayor número de células, un menor contenido de lípidos y una mayor actividad mitocondrial. Por el contrario, la tasa de supervivencia después de la vitrificación / calentamiento no se vio afectada por el uso de EVs en el cultivo. En conclusión, imitar las condiciones fisiológicas usando EVs de OF y UF en un cultivo in vitro secuencial no afecta el desarrollo del embrión, pero mejora la calidad del embrión al aumentar el número total de células, disminuir el contenido de lípidos y aumentar la actividad mitocondrial. Estos resultados evidenciaron la asociación del ambiente del tracto reproductivo con el desarrollo del embrión temprano, lo que confirma la comunicación materno-embrionaria.

Palabras clave: bovino, interacción materno-embrionaria, vesículas extracelulares, in vitro

RESUM

La producció d'embrions in vitro (PIV) és una biotecnologia aplicada per a millorar el rendiment reproductiu, l'eficiència productiva i el guany genètic animal. L'objectiu principal de la PIV és maximitzar el numere de descendents d'animals genèticament superiors i disseminar germoplasma a tot el món. A pesar que la PIV s'usa comercialment amb èxit, encara té alguns factors limitants per a la reducció de costos i una major difusió, relacionats amb la menor qualitat dels embrions generats in vitro i la conseqüent major sensibilitat a la criopreservació. Se sap que la comunicació matern-embrionària que ocorre abans de la implantació juga un paper vital en l'establiment i manteniment de l'embaràs en mamífers, per la qual cosa és necessari imitar el que succeeix durant aquesta comunicació en cultius in vitro per a millorar la qualitat dels embrions obtinguts. Aquesta comunicació matern-embrionària està mediada per diversos factors de creixement i vies de senyalització molecular entre l'oviducte, l'úter i l'embrió, que afecten el seu desenvolupament a curt i llarg termini. Xicotetes partícules anomenades vesícules extracelul·lars (EVs), alliberades naturalment per les cèl·lules contenen diversos productes com a proteïnes i ARN entre altres molècules, juguen un paper important en aquesta interacció. Actualment, encara es desconeix com funcionen i com afecten l'embrió in vivo o in vitro, però ja s'ha observat que els embrions són capaços de capturar aquestes EVs. És per això que l'objectiu d'aquest estudi va ser aïllar i caracteritzar les EVs dels fluids oviductal i uterí i avaluar el seu efecte en un sistema de cultiu in vitro seqüencial sobre el desenvolupament i la qualitat dels embrions bovins. Per a això, els zigots bovins es van cultivar en fluid oviductal sintètic (SOF) suplementado amb 3 mg / ml de BSA o 5% de sèrum depletado de 5% d'EVs en presència o absència de $3x10^5$ EVs dels fluids oviductales (Dia 1-4) i uterí. (Dia 4-9), imitant les condicions fisiològiques del desenvolupament embrionari primerenc in vivo. Les EVs agrupades de cinc oviductes (fase lútia primerenca) i cinc banyes uterines (fase lútia primerenca mitjana) de vaquillas sacrificades es van aïllar utilitzant el kit de cromatografia d'exclusió per grandària (Hansa BioMed). La grandària i la concentració de les EVs es van avaluar per mitjà d'un sistema d'anàlisi de nanotracking (NTA) i la morfologia per microscòpia electrònica de transmissió (TEM). La taxa de divisió i el rendiment de blastocito es van registrar el dia 2 i els dies 7 a 9, respectivament. El dia 7/8 dies es va avaluar la qualitat del blastocito mitjançant tinció amb Hoechst 33342 per al nombre total de cèl·lules, Bodipy 493/503 per al contingut de lípids, Mitotracker Deep Red per a l'activitat mitocondrial i la taxa de supervivència després de la vitrificació / calfament. La concentració d'EVs va ser de $2.97x10^{10}$ i $7.98x10^{10}$ partícules / ml, i la grandària mitjana va ser de 177.5 i 216.5 nm per al fluid oviductal (OF) i el fluid uterí (UF), respectivament. A través de TEM confirmem la presència d'EVs, mostrant la típica morfologia en forma de copa. El Western Blott va confirmar l'expressió de marcadors d'EVs clàssics descrits per a exosomas com ara tetraspaninas CD9 i CD63 i HSP70. Els nostres resultats van mostrar que la suplementació de mitjans de cultiu amb EVs de OF i UF no té cap efecte en el desenvolupament embrionari. No obstant això, el seu ús va tindre un efecte positiu en la qualitat dels embrions produïts, donant lloc a blastocitos amb un major nombre de cèl·lules, un menor contingut de lípids i una major activitat mitocondrial. Per contra, la taxa de supervivència després de la vitrificació / calfament no es va veure afectada per l'ús d'EVs en el cultiu. En conclusió, imitar les condicions fisiològiques usant EVs de OF

i UF en un cultiu in vitro seqüencial no afecta el desenvolupament de l'embrió, però millora la qualitat de l'embrió en augmentar el nombre total de cèl·lules, disminuir el contingut de lípids i augmentar l'activitat mitocondrial. Aquests resultats van evidenciar l'associació de l'ambient del tracte reproductiu amb el desenvolupament de l'embrió primerenc, la qual cosa confirma la comunicació matern-embrionària.

Paraules clau: boví, interacció matern-embrionària, vesícules extracelul·lars, in vitro

INTRODUCTION

In vitro embryo production is a useful tool to study early embryonic development in mammals, to solve reproductive issues in humans and to conserve gametes from animals with high genetic merit or endangered species (Lopera *et al*., 2016).

While the number of in vivo produced embryos that are collected and transferred worldwide seems to have stabilized in recent years, the transfer of embryos derived from IVP continues to grow (Figure 1). In 2016, and for the first time in recorded history more IVP embryos were transfered than in vivo produced embryos, based on data recorded by the International Embryo Transfer Society (IETS). According to the annual embryo production statistics presented by the IETS in recent years, more than 400000 bovine IVP embryos were transferred worldwide (Figure 1). Of the total embryos transferred, around 80% of them were transferred fresh (Figure 2). This reflects the fact that embryos produced in vitro have lower cryotolerance than their in vivo counterparts (For review see Ferré *et al*., 2019).

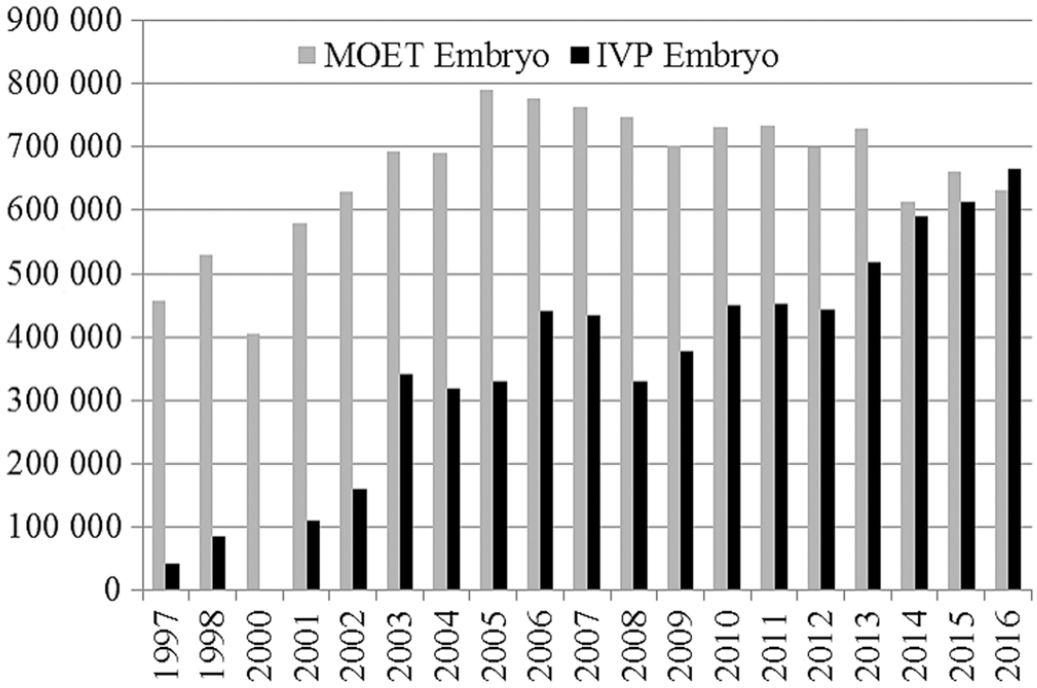


Figure 1. Evolution of Bos indicus and Bos taurus in vivo–produced embryos (MOET) and in vitro–produced (IVP) embryos worldwide as registered by the International Embryo Transfer Society (IETS, http://www.iets.org/comm_data.asp; For review see Ferré *et al*., 2019).

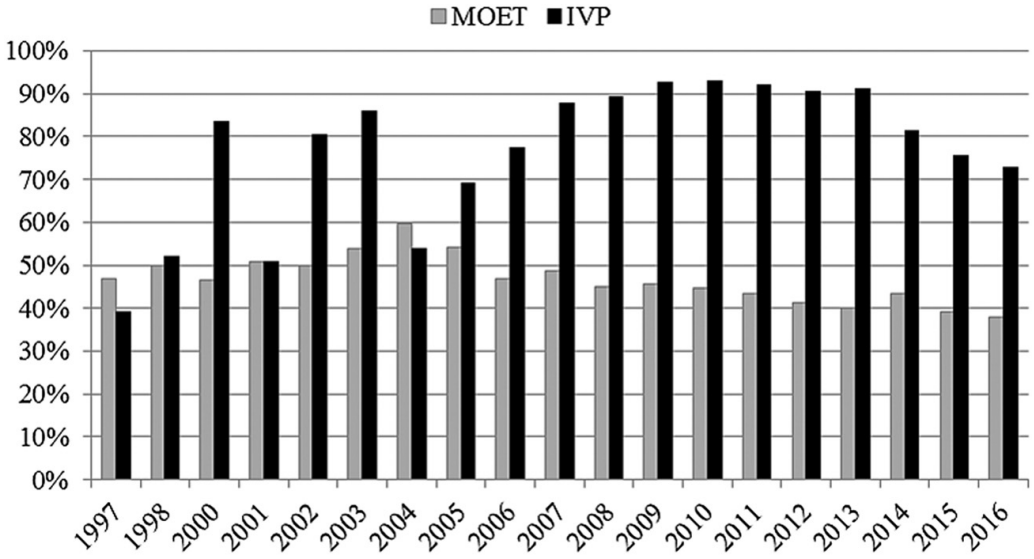


Figure 2. Percentage of fresh Bos indicus and Bos taurus in vivo–produced embryos (MOET) and in vitro–produced (IVP) embryos transferred worldwide registered by the IETS (http://www.iets.org/comm_data.asp; For review see Ferré *et al*., 2019).

The physiological environment where early embryo development occurs has a critical influence on its subsequent growth in short and long terms (Rizos *et al*., 2017). In vivo studies are difficult to perform, not only as they are expensive, but also because it is not possible to differentiate between the outcome of fertilisation and early embryonic death (Maillo *et al*., 2016). Thus, in vitro studies which mimic these physiological mechanisms and interactions in the maternal reproductive tract are necessary for this understanding.

Despite considerable improvements in assisted reproductive technologies in the last several decades, conditions of in vitro embryo production are far from the physiological (Lonergan & Fair 2008). The in vitro deficiencies are reflected in lower embryo developmental rates and quality of the blastocysts produced when compared to their in vivo counterparts (Lonergan 2007), evidenced by lower cryotolerance (Rizos *et al*., 2008), altered inner cell mass/trophectoderm cell ratio (Plourde *et al*., 2012), altered gene expression patterns (Niemann & Wrenzycki 2000) and lower pregnancy rates of transferred embryos (Pontes *et al*., 2009).

1. In vivo embryo development

An embryo is defined as an organism in the early stages of development, with no anatomical forms recognizable, whereas a foetus is defined as a potential offspring that is still within the uterus but is generally recognizable as a member of a given specie (Senger, 2003).

Mammalian embryos develop in a complex and dynamic environment. Once the oocyte grows and matures in the ovarian follicle, achieving full developmental competence, it undergoes fertilisation and early embryonic developmental in the oviduct while then enters the uterus and implants (Figure 3). The first stages of bovine embryo development occur in the oviduct, where the embryo spends around 4 days (Hackett *et al*., 1993). The oviduct is an active organ that maintains and modulates the milieu for sperm capacitation, transport and fertilization of the mature oocyte and early embryonic development (Rodriguez-Martinez, 2007. Lloyd *et al.*, 2009). In the cow, as in other mammals, the fertilisation occurs in the middle segment of the oviduct, the ampulla. Then, approximately 1 to 2 days later, the first cleavage takes place in the isthmus. Between days 3,5 and 4, when the embryo is at the 8- to 16- cell stage, it moves from the oviduct to the uterus. In cattle, when the embryo reached the 8-16 cells stage, a switching from maternal to embryonic control is occurred (Barnes and First, 1991), during which, there is an activation of transcription of mRNA molecules of maternal origin (Memili and First, 2000), an activation of transcription of embryonic genome, a developmental arrest and a marked change in the pattern of protein synthesis; is the process known as the major phase of embryonic genome activation (Telford *et al*., 1990). Beyond the day 5 and 6 after fertilization is the time of compaction, it occurs at 32-64 cell stage (morula) (Bondioli *et al*., 1990; Van Soom *et al*., 1992), when the embryo has already entered the uterus (Hackett *et al*., 1993). At this stage the first tight junctions between adjacent blastomeres are formed (Boni *et al*., 1999). Thus, during the morula stage, cells begin to separate into two distinct populations, the inner cells (unit for gap junctions) and the outer cells, which develop cell-to-cell adhesions know as tight junctions (altering the permeability of the outer cells). After these tight junctions are formed, fluid begins to accumulate inside the embryo and a cavity called blastocoele forms (Senger, 2003). At day 7 to 8 while the embryo is still within the zona pellucida (ZP), a blastocoelic cavity develops and the cells of the early blastocyst differentiate into inner cell mass cells (ICM), destined to form the

foetus, and trophectoderm cells (TE), destined to form the placental tissues (Senger, 2003). For the events of early embryogenesis to continue into an established pregnancy, luteolysis must be prevented. Progesterone must be maintained at sufficiently high levels so that embryogenesis and attachment of the developing embryo to the endometrium can take place. For this to happen, maternal recognition of pregnancy must occur prior luteolysis (Senger, 2003). On days 8-9 post-fertilisation, ZP begins to fragment and the blastocyst `hatches´ (Wolf *et al*., 2003). After that, the blastocyst develops into an ovoid then tubular form and on days 14-15 it elongates to form filamentous conceptus that occupies the entire length of the uterine horn (Spencer *et al*., 2008).

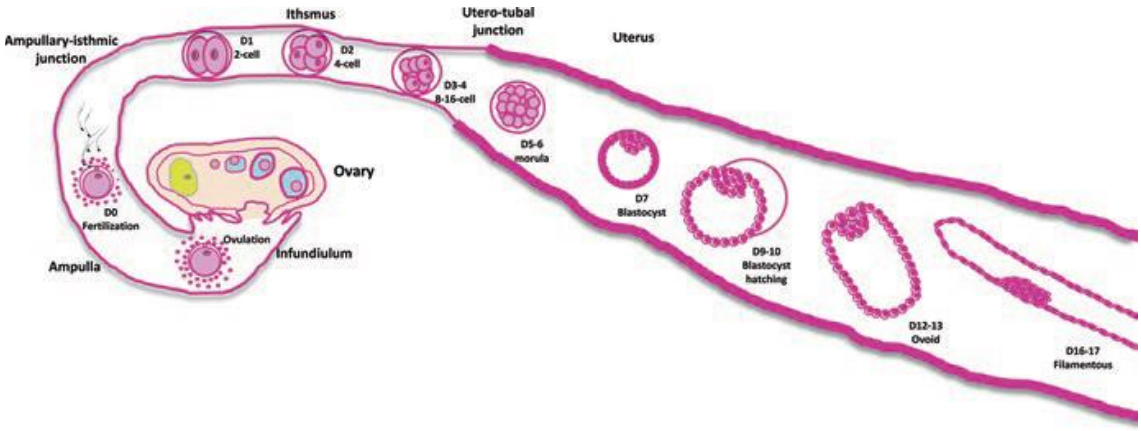


Figure 3. Schematic representation of the process of early embryo development *in vivo* in cattle. After the ovulation, the matured oocyte is fertilized (*D0*) at the ampullary-isthmic junction, while the first cleavage division takes place around 24-30h later in the isthmus (*D1*) followed by subsequent mitotic divisions up to 16-cell stage (*D4*). At this stage, the embryo passes into the uterus through the uterotubal junction and forms a morula (*D5-D6*) and then a blastocyst (*D7*). After hatching from the ZP (*D8*), the morphology of the embryos changes to ovoid (*D12-D13*), then to tubular and filamentous (*D16-D17*) before implantation begins D19 (Rizos *et al.*, 2017).

In cattle, the majority of pregnancy loss is attributed to early embryonic loss prior to maternal recognition of pregnancy (Diskin and Morris 2008) which occurs by approximately day 16 following conception (Northey and French 1980). Therefore, the knowledge of mechanisms controlling embryo-maternal communication should help to

increase the pregnancy rate following embryo transfer (ET) and to avoid early embryonic losses (Wolf *et al*., 2003).

Thus, as the knowledge concerning embryo requirements during early embryonic development increases, in vitro culture systems have evolved to mimic more precisely the physiological conditions in vivo (Rizos *et al*., 2017).

2. In vitro embryo production

Nowadays, in cattle, approximately 90% of oocytes cultured in vitro undergo nuclear and cytoplasmic maturation from which 80% are fertilized and cleave at least once, while only between 30% and 40% reach the blastocyst stage (Rizos *et al*., 2008). In vitro, embryos are typically cultured until day 7 or 8 after fertilization, which corresponds to the blastocyst stage, when they are usually transferred into recipients. In heifers, the pregnancy rate following transfer of in vitro produced blastocysts is approximately 40–50% compared to about 70% when it comes to in vivo derived embryos (Hasler *et al*., 1995; Hoshi 2003). Thus, the challenge today is to improve current in vitro procedures providing high-quality embryos capable of continuing development and implantation after transfer to recipient and resulting in viable births (Rizos *et al*., 2017).

In vitro fertilisation and embryo culture try to mimic as closely as possible the conditions that occur in vivo to provide high quality embryos capable of continued development and implantation and resulting in viable births (Menezo *et al*., 1998). 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 genetic value animals when they die, as well as to continue the existence of valuable endangered species (Wu and Zan 2012).

The in vitro embryo production (IVP) is divided in three steps: oocyte in vitro maturation (IVM), in vitro fertilisation (IVF) and in vitro culture (IVC) (Figure 4).

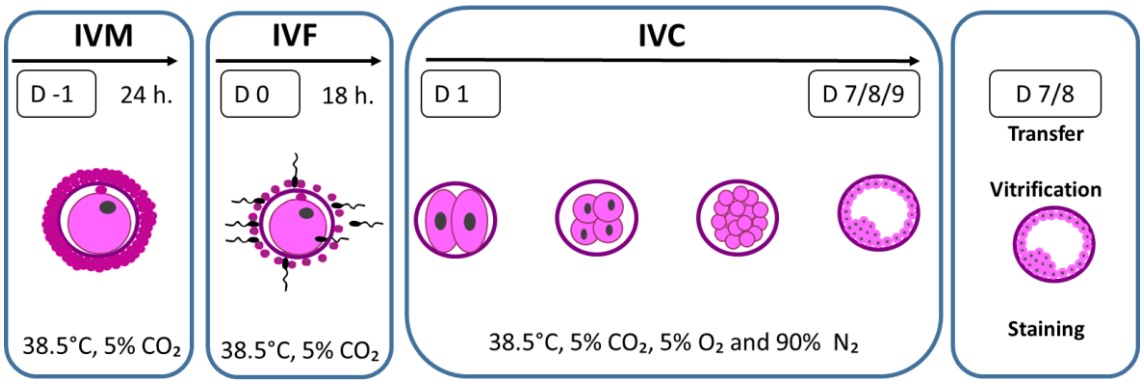


Figure 4. Schematic representation of the in vitro embryo production process.

3. Oocyte in vitro maturation

The oocyte in vitro maturation is designed to support the development of the immature oocyte from the meiotic arrest to the metaphase II stage. In the meantime, the oocyte becomes mature cytoplasmatically and nuclearly (Gilchrist *et al*., 2004).

Cumulus-oocyte complex (COCs) for research use are usually aspirated from ovaries recovered in the slaughterhouse, normally from small- to medium- size follicles (2-8 mm) and COCs are selected for in vitro maturation bases on morphological criteria, as the compactness and thickness of cumulus cells and oocyte ooplasm homogeneity (Rizos *et al*., 2017). The cumulus cells play a critical role in the development of the oocyte by providing metabolites and nutrients, like pyruvate, oxaloacetic acid, and amino acids, thus stimulating them to resume meiosis and progress to metaphase II. Furthermore, cumulus cell expansion is an important marker for oocyte maturation, which is induced by gonadotrophin stimulation in vivo and in vitro leading to massive production of mucoid extracellular matrix protein (Chen *et al*., 1990).

Oocyte developmental competence, often defined as the ability of the oocyte to mature, be fertilized, and develop to the blastocyst stage, has been associated with the size of the antral follicle from which is recovered, the follicular stage and the site of maturation (Rizos *et al*.,2017).

Oocytes matured in vivo are of better quality than those matured in vitro, and this is reflected in the number of embryos obtained subsequently. Indeed, it has been shown that irrespective of whether in vitro culture (IVC) occurred in vivo or in vitro, when oocytes

were matured in vivo, the resultant blastocyst rate was almost 80%, while when oocytes were matured in vitro, it was limited to about 35% (Rizos *et al*., 2002). In relation to in vitro maturation, oocytes originating from follicles bigger than 6 mm resulted in significantly more blastocysts than those from 2 to 6 mm follicles and those recovered prior to the LH surge (Rizos *et al*., 2002).

IVM is performed by placing the oocytes in a suitable culture medium for 24 h in 38.5 ºC, 5% of CO_2 and saturated humidity (Gordon 2003). They can be culture in different media: simple or complex as tissue culture medium 199 (TCM-199). Media used to be supplemented with macromolecules like those contained in fetal calf serum (FCS) or bovine serum albumin (BSA) (Gordon 2003). Also, the supplementation of gonadotropins, steroids or growth factors improved the oocyte developmental competence (Lonergan and Fair 2008).

After 24 hours of incubation, the oocytes are mature with the extrusion of the first polar body, so it is ready to fertilize in vitro (Galli *et al*., 2003). Recent evidence has shown that suboptimal conditions during oocyte IVM have an effect at the epigenetic level and on genomic imprinting (Anckaert and Fair 2015).

4. In vitro fertilization

In vitro fertilisation has the purpose of enabling the union of gametes. For this, culture media such as Tyrode's Albumin Lactate Pyruvate (TALP) or Synthetic oviductal fluid (SOF) are used, both designed with a specific ionic balance for oocyte and sperm requirements (Parrish *et al*., 1988). And it usually contains heparin that capacitates the sperm and prepares it for the acrosome reaction to have a successful fertilization (Parrish 2014).

As the propose is to imitate in vivo fertilisation, sperm selection must take place in a similar manner to that which occurs in the female reproductive tract. Moreover, the extensive use of cryopreserved sperm increases the need for selection of motile sperm as the proportion of fully functional sperm in a frozen- thawed sample is quite low (Samardzija *et al*., 2006; Holt 1997). There are several methods to select motile sperm in

bovine such as swim-up, Percoll® and Bovipure®. The last one is less toxic than Percoll® with the same efficiency (Samardzija *et al*., 2006).

The fate of an embryo is determined at fertilization. Delays in fertilization or fertilization by a damaged spermatozoon could conceivably lead to oocyte aging or the formation of a defective embryo, respectively (Tarín *et al*., 2000). Any damage to the sperm after ejaculation can lead not only to a reduced fertilization rate but also to the formation of embryos with reduced ability to develop at the blastocyst stage. This phenomenon has been demonstrated for embryos formed from sperm exposed to gossypol (Brocas *et al*. 1997), oxidative stress (Silva *et al*., 2007), and sorting for gender by flow cytometry (Wheeler *et al*., 2006; Wilson *et al*., 2006; Bermejo-Alvarez *et al*., 2008; Bermejo-Alvarez *et al*., 2010).

Once oocytes and spermatozoa put in contact, the time of co-incubation is between 17 and 22 hours at 38.5ºC, 5% CO_2 and saturated humidity.

5. In vitro culture

Embryo culture is the longest period during the process of IVP and the step during which the greatest reduction in development occurs, achieving only 30%-40% of blastocyst rate (Rizos *et al*., 2017). It has been clearly showed that the intrinsic quality of the oocyte is the main factor affecting blastocyst yield, while the post-fertilisation culture environment affects the quality of the produced blastocyst (Rizos *et al*., 2002).

The presumptive zygotes obtained after IVF are selected based on their morphological characteristics like homogeneity of the ZP, perivitelline space and cytoplasm and put into culture (Tervit *et al*., 1972).

The embryos can be culture in defined or semi-defined media; co-culture with oviductal, granulosa or Vero cells; or with their conditioned media (CM). Today, the most used media is synthetic oviductal fluid (SOF) which is constituted by chemically defined elements based on the biochemical composition of the oviductal fluid (OF) and it is usually supplemented with 5% of foetal calf serum (FCS) or 3% of bovine serum albumin (BSA) (Holm *et al*., 1999). Embryos secrete factors that sustain their development, so

they grow better in groups than alone (Gardner *et al*., 1994) and their incubation is performed at 38.5ºC in 5% CO_2, 5%O_2 and 90% N_2-at maximum humidity.

Usually, zygotes cultured in vitro until Day 8 or 9. Day 7 is the day which ET in vivo take place and is normally used in vitro for embryo cryopreservation and to measure the quality of the produced blastocysts by the use of invasive or non-invasive techniques. (Hamdi, 2019).

1. Culture conditions

Embryo culture environment is a crucial factor that affects embryo development in vitro, embryo gene expression, and post-implantation development in mammalian embryos (Saadeldin *et al*., 2014).

The embryo is capable of developing in different culture media, from the simplest, made up solely of salts and carbohydrates such as CR1 medium (Rosenkrans and First, 1994), to more complex media such as TCM-199 (Summers and Biggers, 2003).

The in vitro deficiencies are noted in lower embryo development and quality of the blastocysts produced when compared to their in vivo counterparts (Lonergan 2007). In vitro conditions are suboptimal, as evidenced by lower blastocyst yields (30–40%), lower cryotolerance (Rizos *et al*., 2008), altered inner cell mass/trophectoderm cell ratio (Plourde *et al*., 2012), altered gene expression patterns (Niemann & Wrenzycki 2000) and lower pregnancy rates of transferred embryos (Pontes *et al*., 2009).

It is well known that embryos cultured in a group can create a microenvironment through secretion of autocrine and paracrine factors that can support and improve the embryos' development when compared to the embryos cultured individually (Saadeldin *et al*., 2014).

In vitro co-culture with bovine oviductal epithelial cells (BOECs) has been considered to help with the production of good quality embryos (Ulbrich *et al*., 2010). Cordova *et al*. (2014) showed that the use of monolayer BOEC in in vitro embryo culture at the early stages of embryo development, improves embryo development and embryo quality in terms of expression of specific gene transcripts. This period of culture coincides with the in vivo period when the embryo is still in the oviduct. However, the problem is that

monolayer can differentiate, losing important morphological characteristics including reduction of cell height, loss of cilia, and loss of secretory granules and bulbous protrusions (Rottmayer *et al*., 2006).

As mentioned before, nowadays, one of the most commonly used media for the culture of bovine embryos is SOF which is frequently supplemented with fetal calf serum (FCS) and/or bovine serum albumin (BSA). The presence of serum in the IVC media has a stimulatory effect on the development, with more blastocysts on day 6 of culture than either in its absence or with BSA (Rizos *et al*., 2003). However, the addition of the serum can have a negative effect on embryo quality as manifested by reduced cryotolerance and altered gene expression and pregnancy rate after transfer (Lazzarri *et al*., 2002). Moreover, Lazzari *et al*. (2002) evidenced that IVC of bovine embryos in the presence of FCS were associated with a significantly elevated incidence of deviations in embryonic development and a higher proportion of calves with increased birth weight. In a recent study, it was shown that removing BSA over a 24 h period (from D6 to D7) in an individual embryo culture system has a negative effect in embryo development and cell counts in the inner cell mass, although the embryos improve their survival after vitrification (Murillo-Rios *et al*., 2017).

Recently, embryos have been cultured with oviductal and uterine fluid or their extracellular vesicles (Maillo *et al*. 2016). Oviductal fluid (OF) and uterine fluid (UF) contain carbohydrates, ions, lipids, phospholipids and proteins (Aguilar and Reyley 2005; Avilés *et al*., 2010), as well as other unknown components. Some molecules identified have been used in vitro to improve embryo production. The possible role of extracellular vesicles as a mean for nanomaterial-mediated delivery into gametes and embryos under in vitro conditions recently gained more interest (Pavani, *et al*., 2019).

Porcine oocytes treated with OF before fertilisation showed significantly increased cleavage rate and blastocyst yield, suggesting that OF protects the embryo against adverse effects on mitochondrial DNA transcription or replication and apoptosis (Lloyd *et al*., 2009). Recently, it was evidenced that low concentrations of OF and UF (<5%) in bovine embryo culture media as a substitute for serum had a positive effect on development and quality in terms of cryotolerance, cell number, and expression of qualitatively related genes (Lopera-Vasquez *et al*., 2015; Hamdi *et al*., 2018).

The best method of embryo evaluation 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. Thereby a number of invasive and non-invasive methods are used in the laboratory to measure the quality of the in vitro produced embryos. An example of widely used non-invasive method is embryo cryotolerance by measuring the survival rate of the embryo after freezing/thawing. As invasive methods, it is used the embryo staining, which provides the total number of cells or the relation between the number of cells from the ICM and the TE; and also the expression of the relative abundance of genes related with embryo quality (i.e. apoptosis, cell connections, antioxidant stress, metabolism, implantation etc) (Hamdi, 2019).

6. Embryo quality

1. Morphology

The blastocyst morphology is the first parameter considered for the immediate transfer 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 5). Rizos *et al*. (2002a) 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. 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).

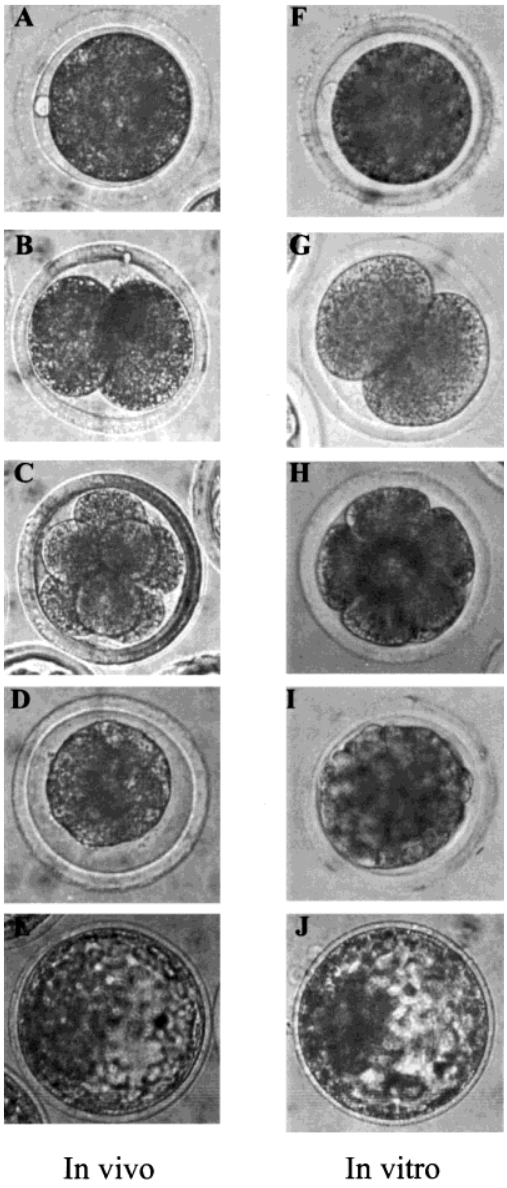


Figure 5. Morphology of bovine embryos produced in vivo (A-E) and in vitro (F-J). Images are representatives of matured oocytes (A and F), 2-cell embryos (B and G), 8 cell embryos (C and H) morulas (D and I) and blastocysts (E and J) (Rizos *et al.* 2002a).

2. Gene expression

Analyzing the expression pattern of certain relevant genes during early embryonic development is a very useful tool to assess the normality of the embryos produced and to optimize assisted reproduction technologies. Currently, transcriptomic studies (mRNA analysis) in bovine embryos they are a rapidly emerging field in which there is a large number of publications [some of them (Laskowski *et al*., 2016; Nagatomo *et al*., 2015)].

Mainly studied genes involved in various biological processes including metabolism, growth factors, stress adaptation, epigenetic regulation of transcription, apoptosis, compaction and blastocyst formation (Wrenzycki *et al*., 2005).

Animal and humans' studies have revealed a link between different ARTs and imprinting disorders, via altered DNA-methylation patterns and histone codes (Urrego *et al*., 2014). Imprinting disorders are more prevalent in gametes and embryos after ARTs than in their counterparts derived from in vivo production (Urrego *et al.,* 2014). These alterations to the epigenetic profile may have a direct effect on the subsequent embryo and fetal development.

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

In the same extend, Rizos *et al*. (2007) after culturing bovine zygotes ex vivo in isolated mouse oviducts showed that most of the 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.

In 2006, Corcoran *et al*. (2006) in a microarray study found that 85% of the differentially expressed transcripts were downregulated in in vitro produced blastocyst compared with their in vivo counterparts. Moreover, in vitro embryo culture has also been associated with epigenetic alterations in the embryo. During this period, the embryo is especially vulnerable to in vitro induced epigenetic defects (El Hajj and Haaf 2013).

3. 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*., 2002a).

It has been demonstrated that culture conditions have a major influence on survival and development of in vitro -produced embryos following cryopreservation (Rizos *et al*., 2008). The in vitro embryos are more sensitive to cryoinjury than in vivo embryos (Hasler *et al*., 1995). Several ultrastructural and biochemical characteristics of IVP embryos, such as their greater lipid content, reduced network of intercellular junctions, fewer mitochondria and microvilli, larger perivitelline space and more cell debris, suggest their reduced tolerance to cryopreservation compared with that of in vivo-produced embryos (Fair *et al*., 2001; Abe *et al*., 2002; Rizos *et al*., 2002b). This lesser cryotolerance of IVP embryos, especially of embryos cultured in serum-supplemented medium, has been correlated with excessive lipid droplet accumulation during in vitro embryo development and gives rise to a lower pregnancy rate (Rizos *et al*., 2008). This lipid accumulation plays an important role reducing embryo cryotolerance (Abe *et al*., 2002).

Embryo co-culture with granulosa cells monolayers reduced blastocyst yield 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*. (2010) 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%). Furthermore, 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). This was confirmed by Rizos *et al.* (2002b) 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%). Furthermore, culture with VE derived from conditioned BOEC media induces cryoprotection to the same extent as classical coculture with fresh BOEC monolayers (Lopera-Vásquez *et al*.,2016).

4. Lipids

Lipids are among the most abundant and essential chemical components of cells, playing important roles in their structure, metabolism, and regulation (Walther and Farese, 2009). Lipid droplets (LDs), such as cytoplasmic membranes, are sensitive to non-physiological temperatures; thus, they can be affected by cryopreservation (Polge, 1977). As

mentioned, Abe *et al*. (2002) "Cytoplasmic LDs have different functions and structures. They contain lipids for energy storage with a hydrophobic core surrounded by a phospholipid monolayer, mostly consisting of triacylglycerols (TGs) and sterol esters, such as cholesterol. LDs are regarded as active organelles playing crucial roles in cellular energy homeostasis". In mammalian species studied, in oocytes and early embryos there are LD with a diameter vary from 0.1 to 1.0 μm, and they can reach up to 5–6 μm or even larger at later embryonic stages (Abe *et al*., 2002).

In addition to energy homeostasis, recent findings demonstrate the involvement of LDs in cell protection, regulation of protein metabolism, and nuclear functions. As proposed "Importantly, LD concentration is associated with the capability of oocytes and embryos to survive after cryopreservation" (Amstislavsky. *et al*., 2019).

The oocytes different listed species are poor in such fatty acids as myristic acid (sheep), stearic acid (rabbit), linolenic acid (cow, pig, and human), and lignoceric acid (rat and mouse) (Reviewed by Amstislavsky S. *et al*., 2019).

When ooplasm is dark-looking in bovine oocytes it might due to an accumulation of lipids and it is also a mark of a good developmental potential (Jeon *et al*., 2009). Kim *et al*. (2001) proposed "The ratios of different fatty acids differed in bovine oocytes of different grades: oleic acid along with palmitic acid were the most abundant fatty acids in high-quality, fresh and frozen–thawed immature and in vitro matured oocytes, whereas stearic and palmitic acids dominated in low-quality oocytes" (Kim *et al*., 2001).

Difficulties in freezing oocytes and preimplantation embryos with high lipid content have already been known for more than 40 years (Polge, 1977), and they are still a major limitation in many mammalian species. Also, various approaches, such as delipidation (partial removal of LDs), polarization of LDs in cells by centrifugation, lipolysis, and chemical modification of lipid profiles, have been attempted to increase the effectiveness of cryopreservation in lipid-rich oocytes/embryos (Reviewed by Amstislavsky S. *et al*., 2019). Abe *et al*. (2002) have proven that the use of TCM199 supplemented with calf serum increases the LDs cytoplasmatic in in vitro embryos since the cleavage till expanded blastocyst in comparison with serum free culture medium, which it has a better embryo production as well as greater survival up to 96 hours of culture after vitrification (Abe *et al*., 2002).

5. Embryo cell number

As mentioned earlier, assessing embryo viability by morphological observations is the most widely method used; however, the cell number of the embryos is a valid indicator of the viability of pre-implantation embryos (Papaioannou and Ebert 1988) Jiang *et al*. (1992) reported that the cell number of bovine IVF blastocysts varied depending on the morphological grade and that the later developing blastocysts were of poor quality as judged by the cell number.

Recently, in the analysis of the EVs transcriptome of bovine oviducts, Almiñana *et al* (2018) detected several transcripts for cell cycle regulation genes, which could potentially be transferred to embryos, stimulating cell division, in order to increase the number of cells in embryos.

7. Embryo-maternal interaction

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).

Disturbance in this unique communication system is associated with high rates of early pregnancy loss, and it is becoming increasingly evident that it also influences the developmental potential of the offspring into adulthood (Almiñana *et al.,* 2020).

In fact, transcriptomic and proteomics studies have demonstrated that these interactions start in the oviduct, the anatomical tube connecting with the ovary and the uterus (reviewed by Almiñana and Bauersachs, 2019). 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).

Traditionally, there are known three different mechanisms of intercellular communication. These are gap junctions, short-range paracrine signaling via secreted

soluble molecules and long-range endocrine signaling via secreted hormones. Recent studies have uncovered the existence of extracellular vesicles (EVs), which are released by cells into the extracellular environment, as novel mediators of intercellular communication that can transfer proteins, lipids and RNAs between cells (Reviewed by Machtinger *et al*., 2015).

8. Extracellular vesicles

Extracellular vesicles (EVs) are a diverse population of endogenous nano- and micro-sized cell-derived membrane vesicles released by prokaryotic and eukaryotic cells (Willms C, *et al*., 2016).

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 (da Silveira *et al*., 2012), and milk (Admyre *et al*., 2007).

These cell-secreted vesicles contain bioactive components (i.e. proteins, lipids, mRNAs and miRNAs), which can be transferred among cells. Extracellular vesicles secreted by donor cells can travel through body fluids without being degraded and deliver their contents to target cells leading to physiological responses in recipient cells. (Da Silveira, *et al.,* 2017). Thus, EVs are an important tool in intercellular communication playing a key role in the regulation of several physiological and pathological processes (Andreu, *et al*., 2014) The EVs are involved in cell adhesion and signal transfer and provide an important method for cell communication (van der Pol *et al*., 2012).

EVs can be categorized into three main classes (Figure 6), based on their biogenesis pathways: exosomes, microvesicles and apoptotic bodies (Théry *et al.*, 2018) Microvesicles (MVs) originate from the cell surface, where they are released by direct outward budding of the plasma membrane. Their heterogeneous size ranges from 50 nm to 1,000 nm in diameter. Apoptotic bodies are released through outward blebbing and fragmentation of the cell membrane of apoptotic cells, and have a broad size range of 50–2,000 nm in diameter. Exosomes are derived from the endolysosomal pathway and are formed within multivesicular bodies (MVBs). They are released by cells upon fusion of

MVBs with the plasma membrane. In contrast to MVs, exosomes are presumed to represent a more homogenous population of EVs, ranging in size from 30 nm to 120 nm in diameter. Sorting of cargo into exosomes involves specific proteins associated with the endosomal sorting complex required for transport (ESCRT), such as ALG-2-interacting protein X (ALIX) and tumour susceptibility gene 101 protein (TSG101)10. As a result, ALIX and TSG101 are commonly used as marker proteins for exosomes (for review see Willms, *et al.*, 2016).

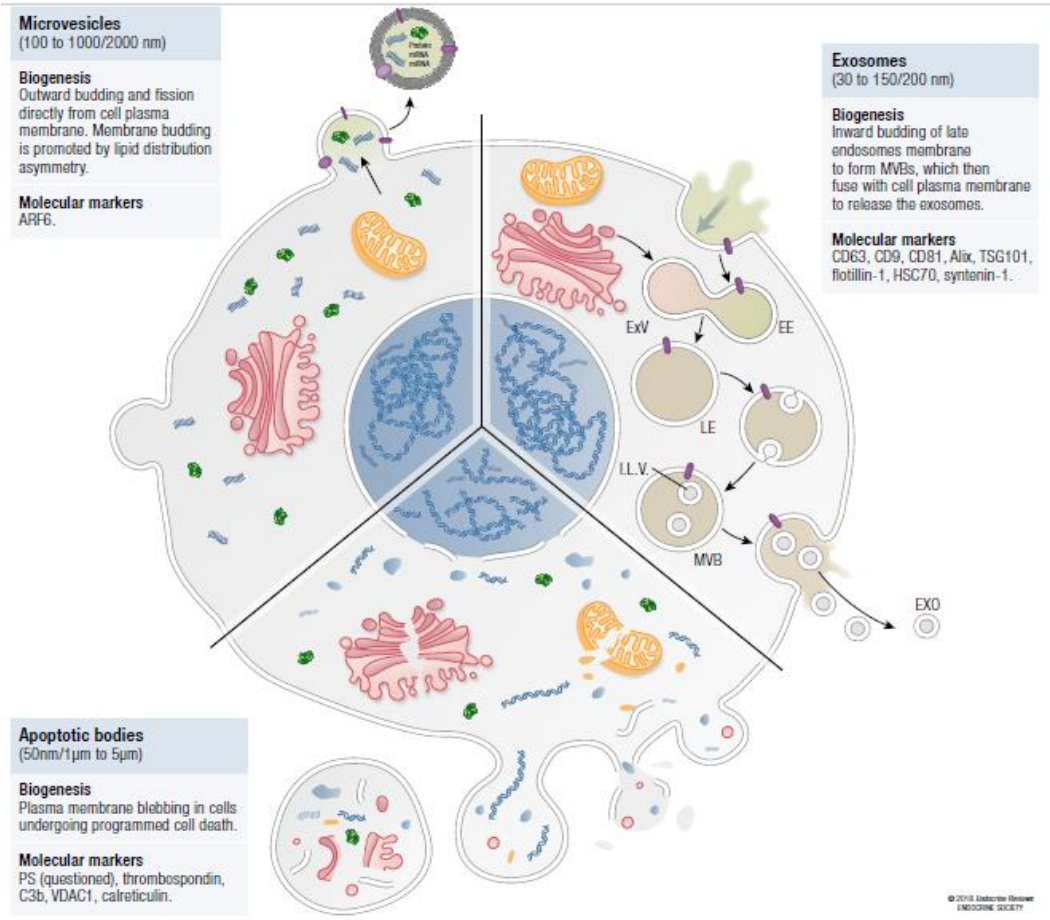


Figure 6. Main types of extracellular vesicles (EVs) present in body fluids and culture media. EVs are classified in three groups according to their biogenetic pathways. Exosomes (EXOs) are produced in the endosomal pathway by invagination of the membrane of late endosomes to form intraluminal vesicles (ILVs) enclosed in multivesicular bodies (MVBs). MVBs can, then, fuse with lysosomes and degrade their content, or fuse with cell plasma membrane to release ILVs, now regarded as EXOs. MVs are produced directly from the cell plasma membrane by outward budding. Apoptotic bodies (Abs) are generated as blebs in cells undergoing programmed cell death. EE, early endosome; ExV, exocytic vesicle; LE, late endosome (Simon *et al.*, 2018).

Also, EVs contain endosome- associated proteins coming from the different cell types, some of which are involve in the biogenesis of Multi vesicles body's (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 (Kleijmeer *et al*., 1998). EVs are also enriched with proteins associated with lipid rafts, including lycosylphosphatidylinositol- anchored proteins and flotillin (Wubbolts *et al*., 2003).

Typically, the isolation and identification of EVs are based on specific EVs characteristics such as size, morphology, flotation density, and the presence of marker proteins, i.e., Alix, HSP70, and CD9 (Pavani, *et al*., 2019). Differential ultracentrifugation (UC) is the most common EVs isolation method.

However, this isolation method is time-consuming and may yield EVs aggregates after pelleting. Recently, it was demonstrated that OptiPrepTM density gradient ultracentrifugation is a reliable EVs isolation technique, which assure EVs isolation without contamination of other nanoparticles (high density lipoproteins, ribonucleoproteins) (Pavani, *et al*., 2019).

Nowadays, commercial exosome isolation kits are available (e.g., PURE-EVs Size Exclusion Chromatography) able to simplify the isolation of exosomes from small starting volumes from a wide range of material/fluids.

Characterization of EVs is fundamental to enable differentiation among the different subpopulations of the sample. Quantitative and qualitative assessment of EVs electron microscopy (EM) techniques are well established and have proven very useful in EVs research (Raposo *et al*., 1996), providing direct evidence for the presence of vesicular structures.

Nanoparticle tracking analysis (NTA) is an optical particle tracking method for obtaining concentration and size distribution of EVs populations. It uses the properties of both light scattering and Brownian motion to obtain particle size distribution in samples in a liquid suspension. (Dragovic *et al*., 2011). 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 EVs subsets in heterogeneous mixtures.

Furthermore, as mentioned earlier Western Blot (WB) is commonly used to confirm the EVs recovery with validated antibodies for a selection of positive exosome markers like CD63, CD9, CD81 and HSP70 (Raposo and Stoorvogel 2013).

In reproduction, secreted vesicles are present in the follicular fluid (da Silveira, *et al*., 2012), endometrial environment (Ng, *et al*.,2013) and seminal plasma (Piehl, *et al.*,2013). Recently, exosomes and microvesicles have been identified as essential components of reproductive tract (Amiñana, 2017; Lopera-Vasquez *et al*., 2017).

Although its presence in the field of reproduction has been described previously, the effect of EVs on embryo production in vitro has not been analyzed until recently. Saadeldin, Oh, & Lee, (2015) showed that in vitro derived embryos secrete EVs in their conditioned media, while Burns, *et al*. (2016) showed in sheep that the isolated EVs in the uterus come from the embryonic trophoectoderm and the uterine epithelium and that are involved in intercellular communication between these tissues during the establishment of pregnancy.

However, limited data on uterine tract EVs content is available today, a part of the identification of the PMCA4a protein, which is essential for sperm hyperactivated motility and fertility in oviductal EVs (Al-Dossary *et al*., 2013) and a recent study demonstrating the possible role of the EVS in the embryo–oviduct dialog (Almiñana and Bauersachs, 2019).

Da Silveira *et al*. (2012) isolated MV and exosomes of equine ovarian follicular fluid and evidenced proteins and miRNAs presence suggesting that MV and exosomes play a role in mediating cells communication within the mammalian ovarian follicle as miRNAs were present in surrounding follicular cells. Furthermore, the presence of an exosome-mediated transport of miRNAs in the bovine follicle has been also evidenced (Sohel *et al*., 2013). The expression and secretion via oviductal exosomes of PMCA4a (Ca2+ homeostasis) in female reproductive tissues and luminal fluids during oestrus, and uptake by sperm, suggest possible roles in sperm viability during storage in the oviduct and during capacitation and the acrosome reaction (Al-Dossary *et al*., 2013).

The study of EVs and exosome-specific miRNA in the uterus has enabled the identification of pathways that could be influenced if the exosomes are taken up by trophectoderm or epithelial cells during implantation or transferred to sperm during transit through the uterus cavity (Ng *et al*., 2013). Burns *et al*. (2014) demonstrated that EVs present in uterine fluid of pregnant and cyclic ewes contain specific proteins, miRNAs and mRNAs, capable of delivering their content in vitro. Furthermore, the differences found in molecular content by pregnancy status suggested a differential EVs source (endometrial epithelia-conceptus trophectoderm). This was demonstrated recently by the same group who described extracellular vesicles emanating from both the conceptus trophectoderm and uterine epithelia supporting the notion that MV in uterine fluid have a biological role in conceptus–endometrial interactions, which may be important for the establishment and maintenance of pregnancy (Burns *et al*., 2016).

On the other hand, Saadeldin *et al*. (2014) showed that the addition of exosomes isolated from the conditioned medium of parthenogenetic embryos increased the developmental competence of cloned embryos. While, in a recent study was evidence that EVs from conditioned media of an extended culture BOEC monolayer can be isolated, morphologically characterized and successfully used for in vitro embryo culture, improving the quality of the produced blastocysts with the objective of trying to mimic the intercellular communications between oviductal tissue and embryo in vitro (Lopera-Vasquez *et al*., 2016). Furthermore, the same group demonstrated also that EVs from bovine isthmic OF can be isolated and their supplementation in in vitro embryo culture has a positive effect on the expression patterns of developmental related genes (Lopera-Vasquez *et al*., 2017).

OBJECTIVE

The objective of the present study was to isolate and characterize the EVs from OF and UF and further evaluate their effect on a sequential in vitro culture system on the development and quality of bovine embryos.

MATERIALS AND METHODS

1. Oviductal and uterine flushing collection

Five oviducts and five uteri from slaughtered heifers were selected according to the stage of the corpus luteum and transported to the laboratory on ice. Only oviducts corresponding to Stage I (from Day 1 to Day 4) and uteri to Stage II (from Day 4 to Day 9) of the oestrous cycle (Ireland *et al*., 1980) ipsilateral to the corpus luteum were used. Each oviduct and uterine horn were trimmed free of associate tissue and washed in PBS free of BSA, Ca2+, Mg2+ (PBS*) keeping both ends closed. Then, each oviduct was flushed with 1ml of cold PBS* towards ampulla to isthmus while each uterine horn was flushed with 2 ml of cold PBS* towards UTJ using 2 ml syringe and a 25G blunt needle. During all process the temperature was maintained at 4 ºC. Each oviduct and uterine flush was centrifuged at 300 g for 7 min and the supernatant from each sample was centrifuged again at 10.000 g for 30 min at 4ºC to remove cellular debris. Supernatant from each sample was stored at 4ºC for 24 h before EVs isolation.

2. EVs Isolation by Size Exclusion Chromatography

A commercial kit called PURE-EVs Size Exclusion Chromatography columns for Exosome and EVs isolation (Hansa Biomed) was used following the manufacturers instruction. PURE-EVs columns were first rinsed with 30 ml of PBS*. Then 1 ml from each OF or 2 ml from each UF flushes were loaded onto the column and when it is completely within, before it is dried, 11 ml of PBS* was introduced on top. Immediately then, the first 6 fractions (each fraction: 500 μL) were eliminated (1-6), while the following 5 fractions (from 7 to 11) where EVs are present were collected (total volume of 2.5 mL). Finally, columns were washed with 20 mL of PBS* and stored at 4ºC to be reused (max of 4 times). To concentrate EVs, each OF or UF sample (2.5 mL) was ultra-centrifuged at 100.000 g and 4ºC for 1 hour using an Optima L-90K ultracentrifugation "Beckman Coulter". Then, under the laminal hood the supernatant was gently eliminated and 100 μL of PBS* was introduced to resuspend the pelleted EVs. The five individual samples from OF or UF were pooled having a final volume of 500 μL from each group.

Then, 50 μL from each group were used for EVs characterization. When EVs concentration was determined the final sample concentration for OF and UF EVs groups was standardized to $3x10^5$ (Lopera-Vasquez *et al*, 2016, 2017), and samples were frozen at -20ºC for embryo culture.

3. EVs characterization

1. Nanoparticletracking analysis (NTA)

Nanoparticle Tracking Analysis (NTA) uses the properties of both light scattering and Brownian motion to obtain particle size distribution in samples in a liquid suspension. A representative part (10 μL) of the obtained vesicles was used to determine the size and number (concentration) of EVs by NTA with Nanosight LM10 and NTA 2.3 Software (Nanosight, Wiltshire, UK). A laser beam is passed through the sample chamber, and the suspended particles in the path of the beam scatter the light so that they can be easily seen through a 20 magnification microscope as points of light scattering. A video camera captures the Brownian movement of the light scattering points. NTA software analyses the movement of particles individually and uses the Stokes-Einstein equation to calculate their hydrodynamic diameters.

2. Transmission electron microscopy (TEM)

For negative staining of EVs (5 μL), ionized carbon and collodion-coated copper 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, Tokyo, Japan) transmission EM.

3. Western blot

EVs preparations were lysed in 2% SDS+1mM Tris and resolved in a 10% gradient SDS-PAGE gel (Biorad). Proteins were transferred to a PVDF membrane (Biorad), blocked

with 3% BSA and incubated with the following primary antibodies: CD9 (D3H4P) Rabbit mAb monoclonal antibody, CD63 (MCA2042GA) Mouse antibovine monoclonal antibody and HSP70 (C92F3A-5) mouse monoclonal antibody. Next membranes were treated either with goat anti-rabbit IgG-HRP (sc-2004) or goat anti-mouse IgG-HRP (sc-2005) secondary antibodies and revealed by Enhanced Chemiluminescence kit and detected by an ImageQuant LAS500 biomolecular imager (GE LifeSciences).

4. EVs depletion of FCS by ultracentrifugation

Heat inactivated FCS (F2442 – Sigma) was ultra-centrifuged at 100.000g and 4ºC for 18 hours using an Optima L-90K ultracentrifugation "Beckman Coulter". Under the laminal hood the supernatant (FCS depleted from the EVs) was collected, aliquoted and store at -20ºC for later use on embryo culture.

4. In vitro embryo production

1. Oocyte recovery

Ovaries from cyclic heifers and cows were collected after slaughter and transported to the laboratory in saline solution (0.9% NaCl (Sigma), supplemented with 0.1% of gentamicin (Sigma G1272)) at 35-37ºC.

Once in the laboratory, the collected ovaries were washed with tempered water at 35-37ºC to clean them from blood and then in saline solution at 35-37ºC. Then, ovaries were maintained in a water bath at 37ºC during the oocyte recovery procedure. Follicular fluid was aspirated from 2-8mm follicles using a 5 ml syringe and 18g needle. The recovered follicular fluid was placed in a sterile falcon tube maintained also in the water bath at 37ºC.

The aspirated follicular fluid was let to settle, and after the supernatant was discarded while the pellet, where the cumulus-oocyte complex (COC´s) are, was resuspended in phosphate buffer solution (PBS, appendix 1). This suspension was placed in a 100mm

cell and tissue culture dish (Biofil) to locate the COC´s under a stereomicroscope using a heated plate at 37ºC. Only grade 1 and 2 COC´s were selected.

2. In vitro maturation of cumulus oocyte complexes (COC´s)

Grade 1 and 2 COC´s were washed in IVM medium (TCM-199 supplemented with 10% FCS and 10ng/ml EGF, appendix 2). After that, selected COC´s were placed in groups of 50 in a four-well dish (Thermo Scientific) containing 500 µL of IVM medium/per well for 22-24 hours at 38.5ºC under atmosphere of 5% CO_2 in air, with maximum humidity.

3. Sperm preparation and in vitro fertilization

Frozen semen from a previously tested Asturian Valley bull (ASEAVA, Asturias, Spain) was thawed at 37ºC in a water bath for 1 min and centrifuged for 10 min at 280 g through a gradient of 1 mL of 40% and 1 mL of 80% Bovipure according to the manufacturer's specification (Nidacon Laboratories AB, Gothenburg, Sweden). The sperm pellet was isolated and washed in 3 mL of Boviwash (Nidacon) by centrifugation at 280 g for 5 min. The pellet was re-suspended in the remaining 300 µL of Boviwash. Sperm concentration was determined and adjusted to a final concentration of 1 x 10^6 sperm/mL for IVF. Gametes were co-incubated for 18–22 h in 500 µL of fertilisation media (Tyrode's medium with 25 mM bicarbonate, 22 mM Na lactate, 1 mM Na-pyruvate, and 6 mg/mL fatty acid-free BSA supplemented with 10 mg/mL heparin sodium salt, Calbiochem, appendix 3) 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 38.5ºC.

4. In vitro culture of presumptive zygotes

At approximately 20 h post insemination (p.i.), presumptive zygotes were denuded of cumulus cells by vortex. Groups of 20-25 zygotes cultured in 25 µL droplets of 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, appendix 4) supplemented with 3 mg/mL BSA or 5% EVs-depleted fetal calf

serum (dFCS) in the presence (BSA^{EV}, and $dFCS^{EV}$) or absence of 3×105 EVs/mL from OF (D1- D4) and UF (D4-D9), under mineral oil, at 38.5ºC under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

5. Assessment of embryo development

Cleavage rate was recorded at Day 2 (48 hours p.i.), and cumulative blastocyst yield was recorded at Days 7, 8 and 9 p.i. under a stereomicroscope.

6. Assessment of embryo quality

1. Blastocyst vitrification

The ability of the blastocyst to withstand cryopreservation was used as a quality indicator. Day 7 blastocysts were vitrified in holding medium (HM) (TCM199 supplemented with 20% FCS) and cryoprotectants, following the procedures of Rizos *et al.* (2002) (Figure 7), in a two-step protocol using the Cryloop device (Hampton Research, Aliso Viejo, CA, USA). 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 0.5 M sucrose. The blastocysts were warmed in two steps in HM with 0.25 M and 0.15 M sucrose and then cultured in 25 μL droplets of SOF with 5% FCS. Survival was defined as re- expansion of the blastocoele and its maintenance for 24, 48 and 72 h.

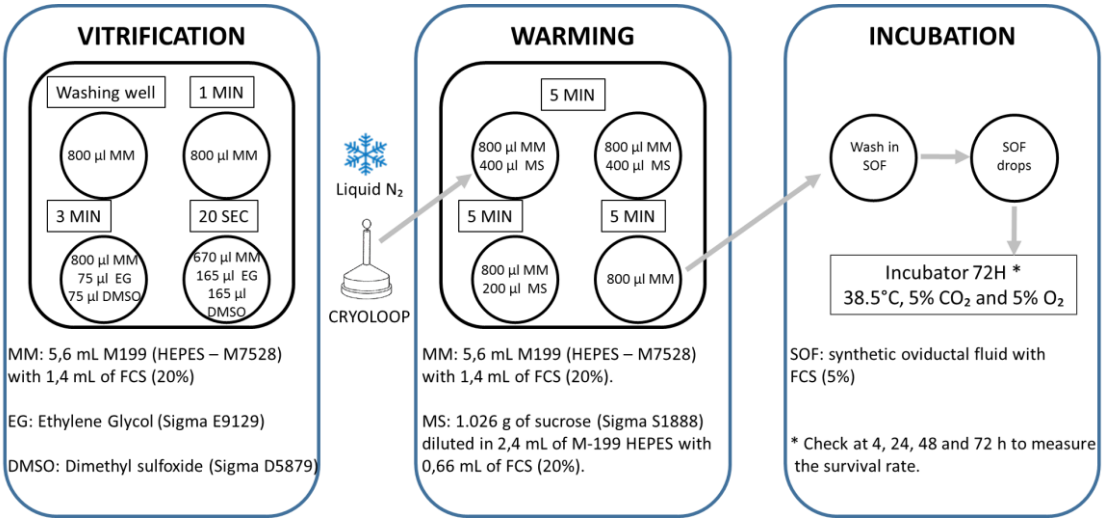


Figure 7. Schematic illustration of the vitrification technique with Cryoloop.

2. Fluorescence staining for embryo mitochondrial activity, lipid contents and total cell number

For staining, embryos D7-D8 (blastocysts and expanded blastocysts) were stained with Mitotracker Deep Red for 30 min (400ng / ml) in a CO_2 oven (5% at r at 38.5oC) to mark the active mitochondria. Subsequently, the embryos were washed and fixed at 4% paraformaldehyde in PBS with 0.1% PVP (PBSpp) for 30 min at room temperature. Then washed 3 times (5 min each) in PBSpp and permeabilized with 0.1% saponin in PBSpp for 1 h at room temperature. They were then washed in PBS and incubated for 1 h with Bodipy 493/503 (10 µg / ml) to stain lipids. After being washed again with PBS, the embryos were incubated for 30 min with Hoechst 33342 (10 µg / ml) to label the nuclei. Finally, washed again PBSpp and mounted on slide in 3.8 µl ProLong® mounting medium (Thermo Fisher Scientific) covered with coverslip and the slides were sealed with nail polish and examined by confocal laser scanning microscope (Leica TCS SP2).

For the assessment of mitochondrial activity, the fluorescence signal intensity (pixels) was quantified. Serial sections of 5 µm were made for each blastocyst and a maximum projection was accomplished for each one. Images obtained were evaluated using the ImageJ program (NIH, USA; http://rsb.info.nih.gov/ ij/). After selection using the freehand selection tool, each blastocyst was measured to determine its area and its integrated density (IntDen), which corresponds to pixel intensity. In addition, the background fluorescence of an area outside the blastocyst was measured. Fluorescence

intensity in each blastocyst was determined using the following formula: Relative fluorescence = IntDen - (area of selected blastocyst x mean fluorescence of background readings). Fluorescence intensities are expressed in arbitrary units (a.u.).

The lipid quantity in blastocysts was obtained by analysis of the total area of lipids in each blastocyst. We captured three images of each blastocyst: one in the middle of the blastocyst (the image with largest diameter) and the other two in the middle of the resulting halves. We used a 63X objective at a resolution of 1024 x 1024 and images were analyzed used the 'nucleus counter' tool, set to detect, distinguish and quantify droplet areas with the ImageJ program (NIH, USA; http://rsb.info.nih.gov/ ij/). For blastocysts, lipid quantity was corrected by area, to account for varying blastocyst sizes. After verification of a significant correlation ($r2 = 0.84$ and $P < 0.0001$ by Pearson's correlation test) between lipid quantity of three sections in 30 blastocysts (10 per group) we chose the section with the largest area per embryo to be analyzed (del Collado *et al*., 2016).

The number of cells per blastocyst was determined by counting the Hoëchst stained cells under an epifluorescence microscope (Nikon 141731) equipped with a fluorescent lamp (Nikon HB-10104AF) and UV-1 filter.

7. Statistical Analysis

Results for cleavage rate, blastocyst yield, survival after vitrification/warming, lipid content, mitochondrial activity and total cell numbers were analysed using one-way ANOVA followed by Tukey test ($P < 0.05$). All analyses were performed with the SigmaStat software package (Jandel Scientific, San Rafael, CA, USA).

8. Experimental design

The developmental capacity of bovine zygotes and the quality of the produced blastocysts cultured in vitro with EVs from OF and UF, mimicking the physiological preimplantation environment in vivo was assessed. At approximately 20 h after insemination, presumptive zygotes were cultured in 4 groups: (i) BSA: SOF with 3 mg/mL BSA; (ii) dFCS: SOF with 5% EVs-depleted fetal calf serum; (iii) BSA^{EV}: SOF with 3 mg/mL BSA

supplemented with 3×10^5 EVs/mL from OF (D1 to D4) and 3×10^5 EVs/mL from UF (D4 to D9); and (iv) dFCS^{EV}: SOF with 5 % dFCS supplemented with 3×10^5 EVs/mL from OF (D1-D4) and 3×10^5 EVs/mL from UF (D4 to D9). BSA and dFCS groups underwent media renewal on Day 4 (Figure 8). Embryo development was assessed on Days 7, 8 and 9. To assess blastocyst quality a representative number of Days 7–8 blastocysts from each group were either vitrified/warmed, and survival rate was recorded every 24 h up to 72 h after warming or fixed and stained for total cell number, lipid content and mitochondrial activity analysis.

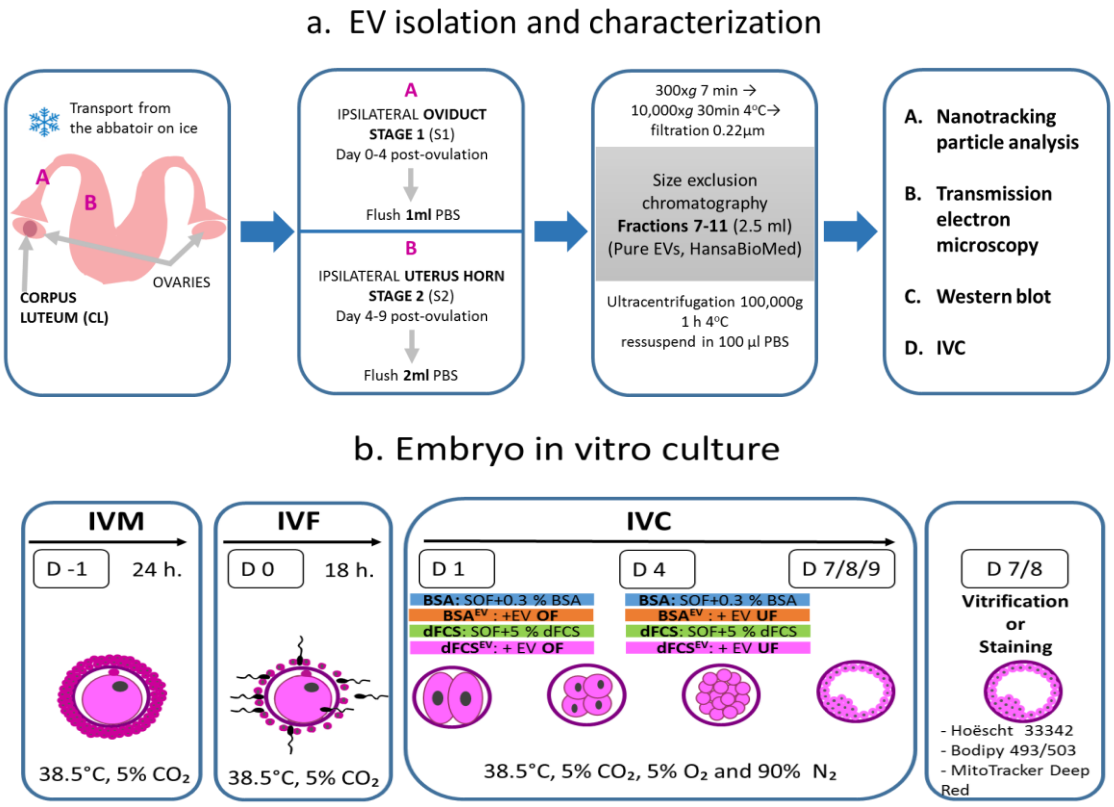


Figure 8. Experimental design. Part a. EVs isolation and characterization, Part b. Embryo in vitro culture.

RESULTS

1. EV´s characterization

1. Nanoparticle tracking analysis (NTA)

Extracellular vesicles were enriched from OF and UF by Size Exclusion Chromatography. Nanosight analyses showed EVs concentrations were 2.97 $x10^{10}$ and 7.98 $x10^{10}$ particles/ml, and mean size 177.5 and 216.5 nm for OF and UF, respectively (Table 1 and Figure 9 A-B respectively).

Table 1 – Concentration (particles/ml), mean (nm) and mode size (nm) of EVs in the pool of 5 samples of OFS1 and UFS2, determined by NTA.

Pool of 5 animals	particles/ml X 10^{10}	Mean size EVs nm	Mode size EVs nm
OFS1	2.97	177.5	137.2
UFS2	7.98	216.5	151.2

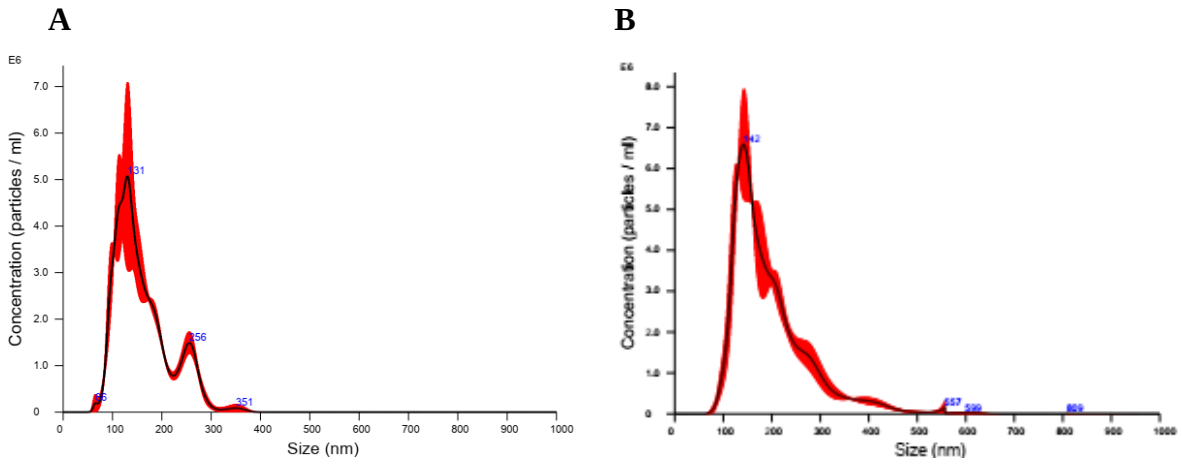


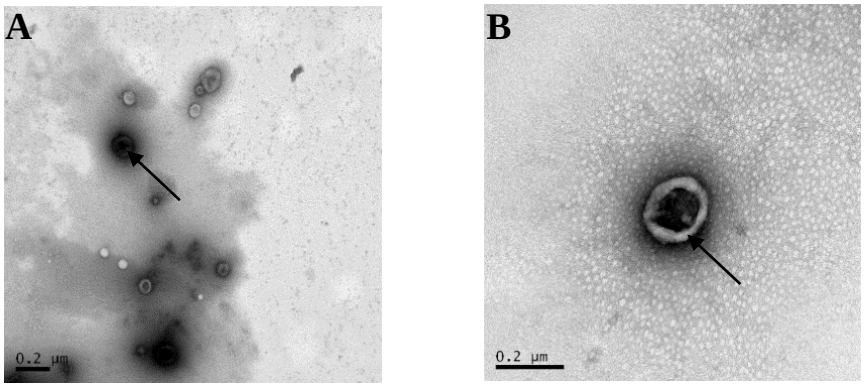
Figure 9. Nanoparticle Tracking Analysis (NTA) of oviductal fluid (OFS1) (A) and uterine fluid (UFS2) (B). Particles size, size distribution and concentration.

2. Transmission electronic microscopy

In Figure 10, representative images of the analyzes by transmission electron microscopy are observed to assess the presence and morphology of the EVs in the oviductal and uterine fluids in both stages.

OFS1

High mag 50 kW **High mag 100 kW**



UFS2

High mag 25 kW **High mag 120 kW**

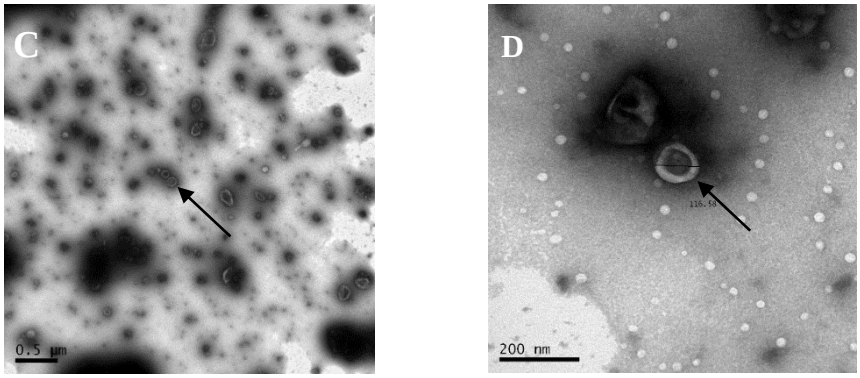


Figure 10. Representative transmission electron microscopy (TEM) images of negatively stained EVs from OFS1 and UFS2 samples. Arrows indicate the presence of EVs of various sizes.

3. Western blotting

Positive bands were detected in samples of isolated oviduct EVs and in the positive controls (blood for CD9, kidney for CD63 and pancreas for HSP70) some of the classical markers described for exosomes such as tetraspanins CD9 and CD63 and HSP70 (Figure 11).

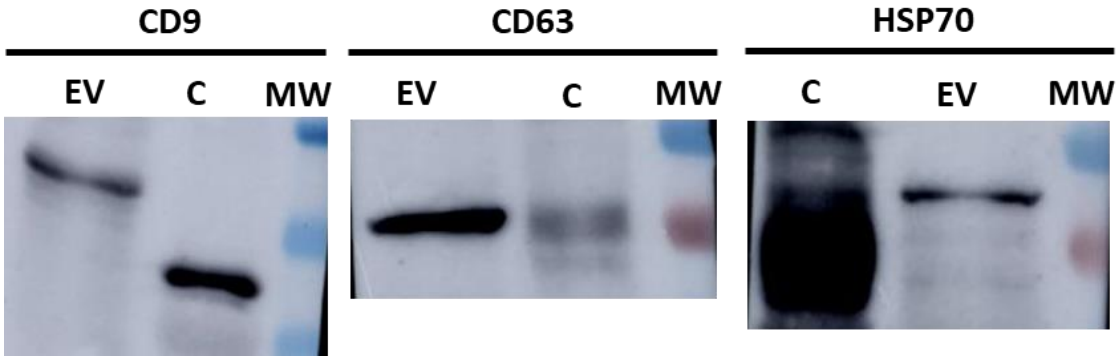


Figure 11. Western blot analysis of OF-EVs with EVs markers. EVs: extracellular vesicles, C: positive control (CD9 – blood, CD63 – kidney, HSP70 – pancreas) – Similarly, all EVs markers were confirmed for UF-EVs sample.

2. Embryo development

No differences were found in cleavage rates (range: 86.7- 88.6%) between the four experimental groups.

Blastocyst yield was significantly lower ($p<0.05$) on Day 7 in the BSA groups (BSA:15.7± 1.8 and BSA^{EV}: 15.7±2.0%) compared with serum groups (dFCS: 28.1±2.2 and $dFCS^{EV}$: 30.1±2.4%) irrespective of EVs supplementation; however, these differences were compensated at Days 8 (range: 28.8±2.4 - 39.0±3.2%) and 9 (range: 31.7±2.3 –40.8±3.2%) as shown in table 2 and figure 12.

Table 2. Effects of in vitro embryo culture with EVs from bovine oviductal fluid (Days 1–4) and from uterine fluid (Days 4–9) on development.

Experimental Group		Cleavage	Blastocyst Yield		
	N	Day 2 N (Mean ± sem)	Day 7 N (Mean ± sem)	Day 8 N (Mean ± sem)	Day 9 N (Mean ± sem)
BSA	1228	1106 (87.7 ± 2.0)	198 (15.7 ± 1.8)[a]	381 (31.1 ± 2.5)	426 (34.4 ± 2.3)
BSA^{EV}	1281	1144 (89.1 ± 1.2)	201 (15.7 ± 2.0) [a]	371 (28.8 ± 2.4)	410 (31.6 ± 2.5)
dFCS	1261	1102 (87.3 ± 2.3)	361 (28.1 ± 2.2)[b]	495 (38.5 ± 2.9)	511 (39.7 ± 3.0)
$dFCS^{EV}$	1253	1075 (87.2 ± 2.2)	390 (30.1 ± 2.4)[b]	500 (39.0 ± 3.2)	523 (40.8 ± 3.2)

n: Total number of presumptive zygotes placed un culture.

[a,b] Values in the same column with different superscripts differ significantly ($p < 0.05$).

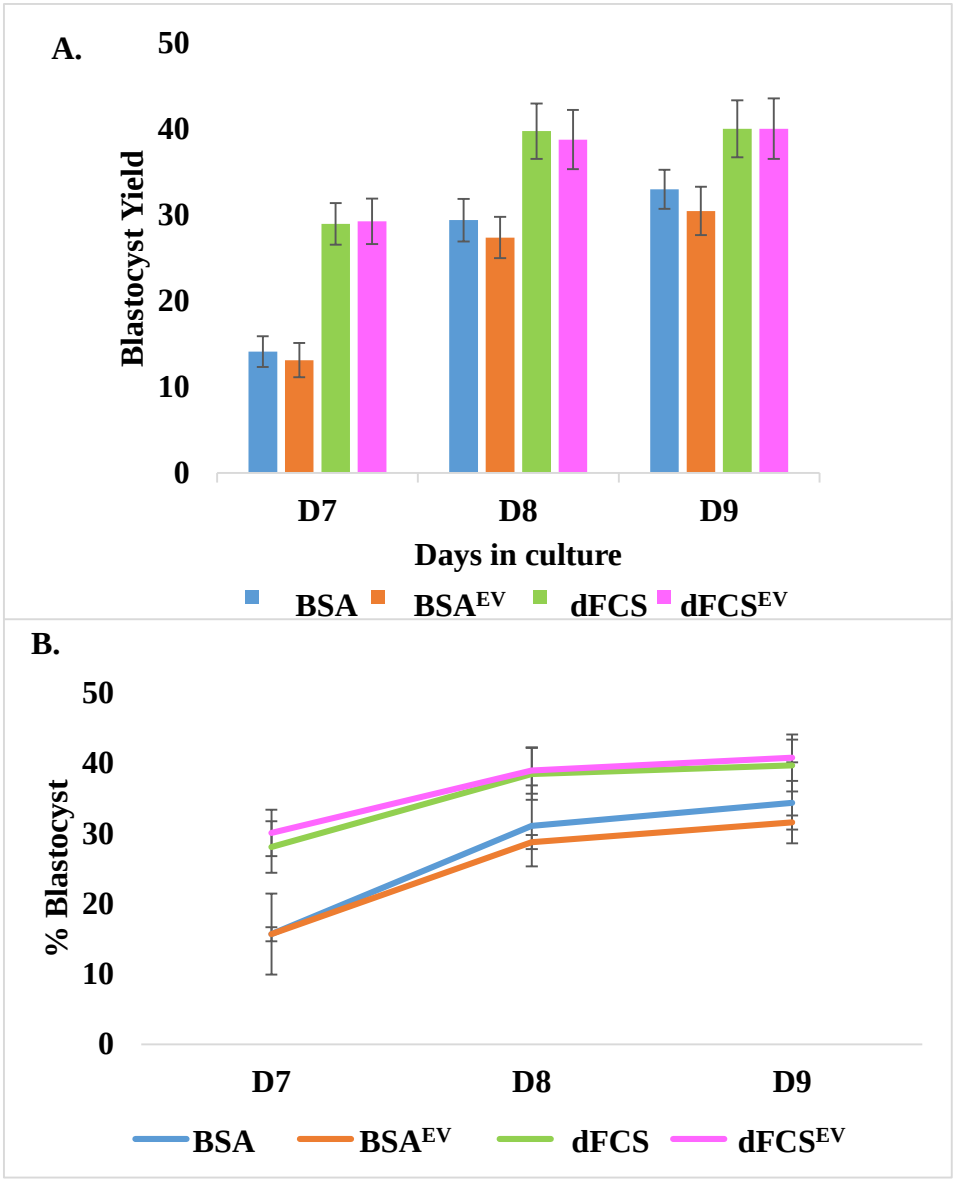


Figure 12. Graphical representation of the A. blastocyst yield (D7, 8 and 9) and B. progression of the blastocyst development rate from days 7 to 9.

3. Embryo quality assessment

3. Vitrification

Blastocyst survival after vitrification/warming was high in all groups up to 72h (range: 80.8±3.8 –100%, $p>0.05$) as shown in Table 3 and figure 13. However, needs to be pointed out that no differences observed were maybe due to the low number of embryos and replicates in some groups.

Table 3. Vitrification survival rates at 4, 24, 48 and 72 h of post-devitrification cultivation (3 replicates).

Vitrificación	4h N (Mean ± sem)	24 h N (Mean ± sem)	48 h N (Mean ± sem)	72 h N (Mean ± sem)
BSA	36 (100.0 ± 0.0)	34 (90.5 ± 9.5)	34 (90.5 ± 9.5)	32 (87.0 ± 8.3)
BSA^{EV}	20 (100.0 ± 0.0)	20 (100.0 ± 0.0)	20 (100.0 ± 0.0)	20 (100.0 ± 0.0)
dFCS	26 (100.0 ± 0.0)	25 (96.2 ± 3.8)	25 (96.2 ± 3.8)	21 (80.8 ± 3.8)
$dFCS^{EV}$	46 (100.0 ± 0.0)	44 (94.9 ± 3.2)	41 (90.7 ± 4.9)	39 (85.6 ± 1,7)

n: Total number of blastocysts placed un culture after vitrification/warming.

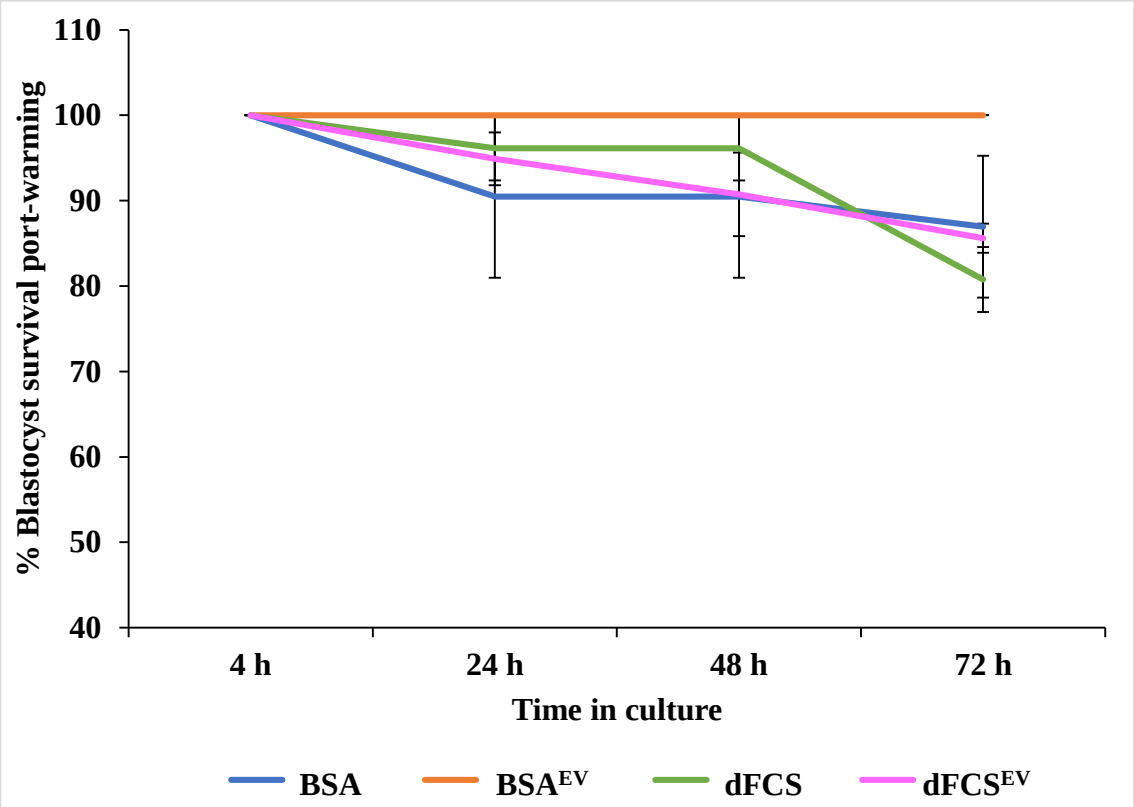


Figure 13. Graphical representation of the progression of blastocysts survival after vitrification/warming at 4, 24, 48 and 72 h of in vitro culture.

1. Total cell number

In relation to blastocysts total cell number (Figures 14 and 15, Table 4), the addition of EVs had a positive effect. EVs significantly increased ($p<0.05$) blastocysts total cell number in $dFCS^{EV}$ (152.47±2.87) and BSA^{EV} (140.47±1.47) compared to dFCS (117.88±1.97) and BSA groups (122.39±1.07). Nevertheless, $dFCS^{EV}$ group had the highest cell number compared to all other groups ($p<0.05$).

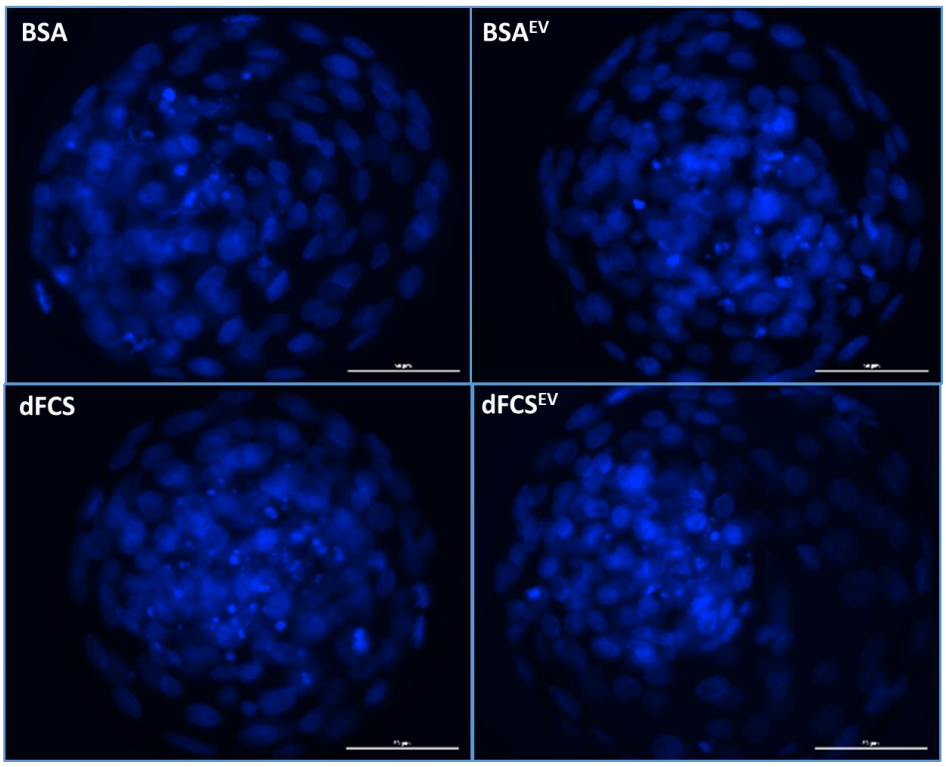


Figure 14. Representative images of D7-8 blastocysts cultured in vitro in the different treatments: A) BSA, B) BSA^{EV}, C) dFCS and D) $dFCS^{EV}$; embryos were assessed by epifluorescence microscopy for cell counting (nuclei stained with Hoechst 33342) (magnification 40x).

Table 4. Effects of in vitro embryo culture with EVs from bovine oviductal fluid (Days 1–4) and from uterine fluid (Days 4–9) on blastocyst total cell number.

Group	N	Total nuclei (Mean ± SE)
BSA	18	(122.4 ± 1.1) [b]
BSA^{EV}	15	(140.5 ± 1.5) [c]
dFCS	8	(117.9 ± 2.0) [b]
dFCS^{EV}	17	(152.5 ± 2.9) [a]

n: Number of blastocysts stained for total cell count

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

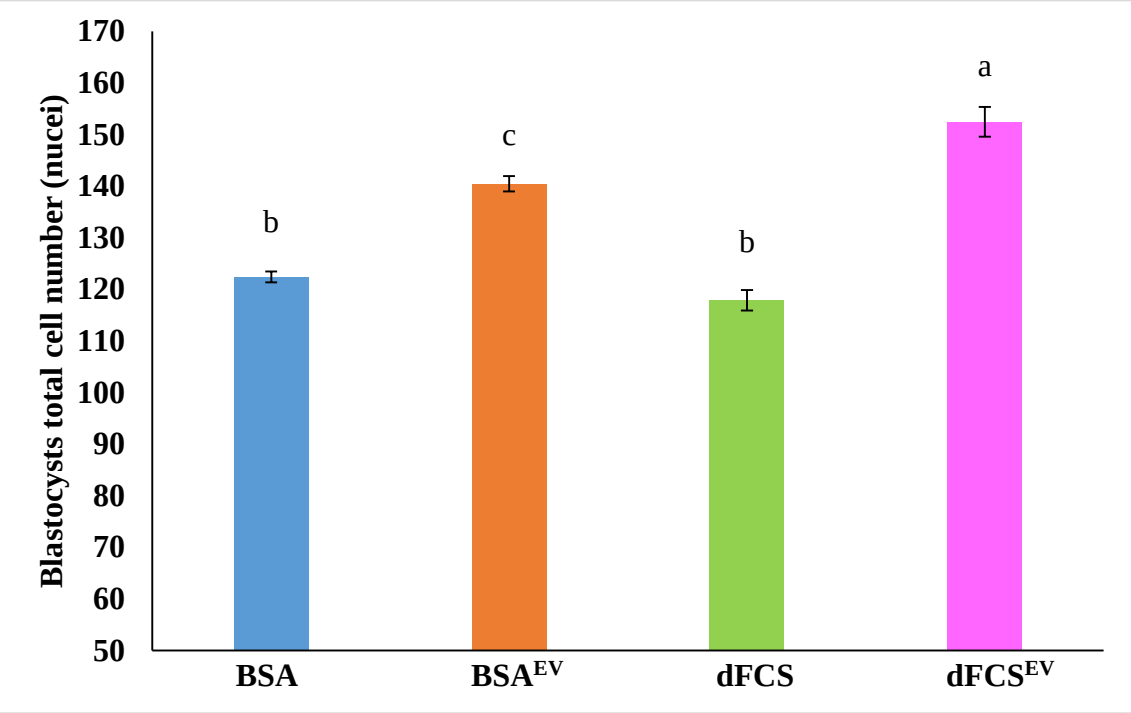


Figure 15. Mean number of total number of cells per blastocyst from days 7-8 cultured in vitro with of without EVs from bovine oviductal fluid (Days 1–4) and uterine fluid (Days 4–9).

2. Lipid contents

In relation to lipid contents (Figures 16 and 27, Table 5), the addition of EVs only had effect in the dFCS^{EV} group by decreasing significantly the lipid content (0.23±0.05µm2) ($p<0.05$), compared to BSA (0.39±0.03µm2) and BSA^{EV} (0.38±0.03µm2) groups, with the dFCS group no had differences (0.37±0.05µm2, $p>0.05$). Lipid content was measured by image J software (NIH, USA) and expressed as the area of lipid droplets (µm2) relative to the total area of the blastocyst without cavity.

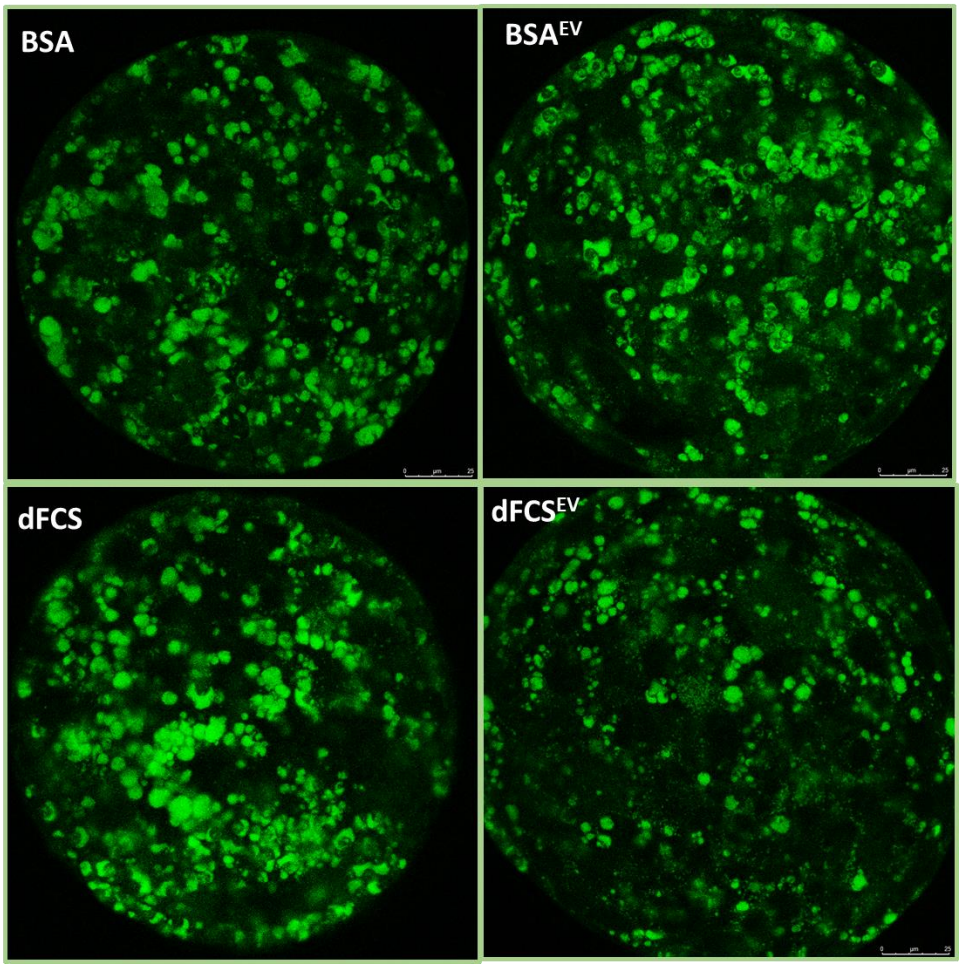


Figure 16. Representative images of D7-8 blastocysts cultured in vitro in the different treatments: A) BSA, B) BSA^{EV}, C) dFCS and D) dFCS^{EV}); embryos were assessed by confocal laser scanning microscopy for lipid contents by staining with BODIPY 493/503 (magnification 63 X).

Table 5. Effects of in vitro embryo culture with EVs from bovine oviductal fluid (Days 1–4) and uterine fluid (Days 4–9) on lipid area (µm2) related to the blastocyst cell area.

Group	N	Total lipids (Mean ± SE)
BSA	18	(0.39 ± 0.03) [a]
BSA^{EV}	15	(0.38 ± 0.03) [a]
Dfcs	8	(0.37 ± 0.05) [ab]
dFCS^{EV}	17	(0.23 ± 0.05) [b]

n: Number of blastocysts stained for lipid content analysis

[a,b] Values in the same column with different superscripts differ significantly (p< 0.05).

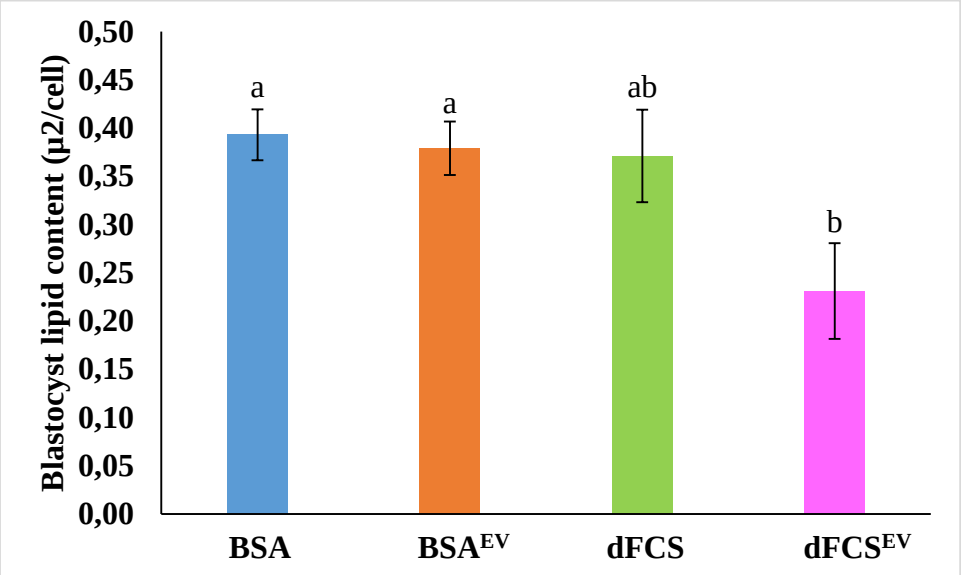


Figure 17. Graphical representation of the effects of in vitro embryo culture with EVs from bovine oviductal fluid (Days 1–4) and uterine fluid (Days 4–9) on lipid area (µm2) related to the blastocyst cell area.

3. Mitochondrial activity

In relation to mitochondrial activity (Figures 18 and 19, and Table 6), it was observed that protein supplementation (BSA x dFCS) had no effect (5663989.0 ± 754754.3 x 6048279.2 ± 818824.2 in arbitrary units of fluorescence intensity = fiau, respectively, P>

0.05). On the other hand, the addition of EVs did not affect the embryos in relation to their respective control for the BSA^{EV} group (5753764.4 ± 646748.0 fiau, P> 0.05), but in the $dFCS^{EV}$ group there was an increase in mitochondrial activity in comparison with all other groups (8570133.6 ± 1050744.3 fiau; P <0.05).

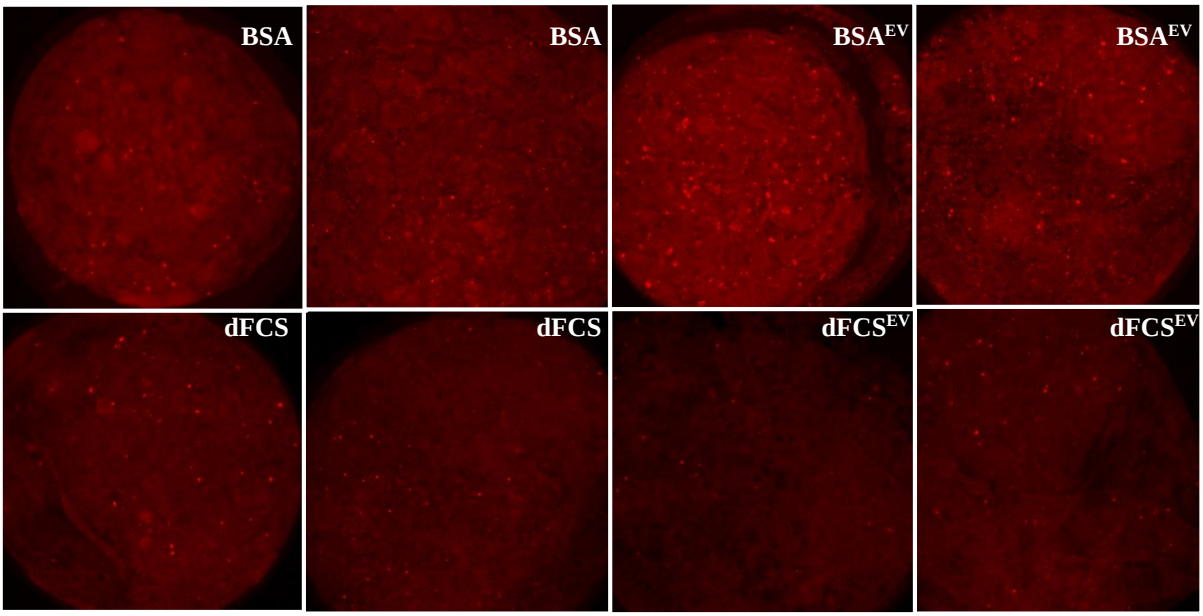


Figure 18. Representative images of D7-8 blastocysts cultured in vitro in the different treatment; embryos were assessed by confocal laser scanning microscopy for mitochondrial activity by staining with MitoTracker Deep Red. (magnification 63 X)

Table 6. Effects of in vitro embryo culture with EVs from bovine oviductal fluid (Days 1–4) and from uterine fluid (Days 4–9) on mitochondrial activity (fiau).

Group	**n**	**mitochondrial activity (fiau) (Mean ± SE)**
BSA	29	(5663989.0 ± 754754.3) [a]
BSA^{EV}	29	(5753764.4 ± 646748.0) [a]
dFCS	33	(6048279.2 ± 818824.1) [a]
$dFCS^{EV}$	27	(8570133.6 ± 1050744.3) [b]

n: Number of blastocysts stained for mitochondrial activity analysis

[a,b] Values in the same column with different superscripts differ significantly (p< 0.05).

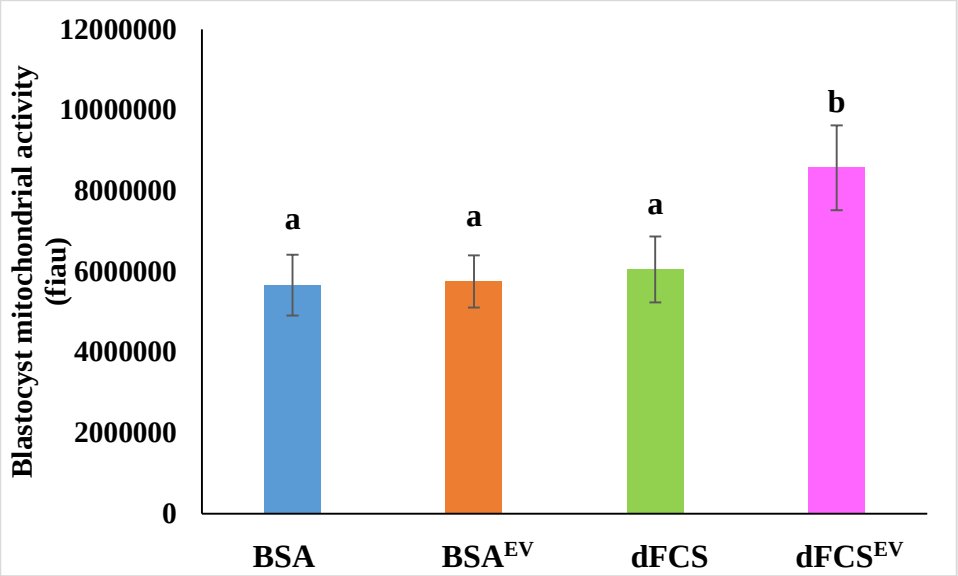


Figure 19. Graphical representation of the effects of in vitro embryo culture with EVs from bovine oviductal fluid (Days 1–4) and from uterine fluid (Days 4–9) on mitochondrial activity (fiau).

DISCUSSION

The oviduct and uterus are the anatomical parts of the reproductive tract where fertilisation and early embryo development take place (Hunter, 2012). In vitro fertilisation and embryo culture try to mimic as closely as possible the conditions that occur in vivo to provide high quality embryos capable of continued development and implantation, resulting in viable births (Menezo *et al*., 1998).

Embryos can be cultured with oviductal and uterine fluid or their extracellular vesicles (Maillo *et al*., 2016). Oviductal fluid (OF) and uterine fluid (UF) contain carbohydrates, ions, lipids, phospholipids and proteins (Aguilar and Reyley 2005; Avilés *et al*., 2010), as well as other unknown components. Some molecules identified have been used in vitro to improve embryo production. The possible role of extracellular vesicles as a mean for nanomaterial-mediated delivery into gametes and embryos under in vitro conditions recently gained more interest (Pavani, *et al*., 2019).

To elucidate the role of bovine oviductal and uterine fluid EVs on early embryo development and embryo quality the in vitro culture was used as a tool. In our study, exosomes were successfully isolated by Size Exclusion Chromatography (commercial kit: PURE-EVs from Hansa Biomed) from oviductal and uterine fluids collected from oviducts and uterine horns ipsilateral to the corpus luteum at the early and middle luteal phase, respectively. EVs number and concentration were identified through Nanosight®, while morphologic examination was performed by electronic microscopy and EVs marker detection by Western blot.

In the study by Almiñana *et al.* (2018) the concentration of EVs from OF at stage 1 was slightly lower (5 $x10^8$ particles / ml) than the one observed here, while their mean size was 135 nm, similar to our observations. Previous studies with stage 1 OF reported EVs <200 nm and a concentration of 9-11 x 10^8 / ml (Lopera-vasquez *et al.*, 2017a). The fact that we obtained a higher concentration (2.97 x 10^{10}) is probably due to the technique of EVs isolation used, in which we used SEC and the other studies used ultracentrifugation. This is in accordance with clear evidence that different isolation methods can affect EVs concentration (Gudbergsson *et al.*, 2016).

Studies in uterine EVs from stage 2 (Qiao *et al.*, 2018) or from different days of pregnancy (17 to 22 days, Kusama *et al.*, 2018) reported EVs sizes from 50-150 nm and

concentrations in the range of 3 x 10^5 and 5 x 10^6 / ml. Same as mentioned above, the processing of the samples (form of collection and isolation of the EVs) was different in each study, which possibly resulted in the difference of EVs concentration between them.

Transmission electron microscopy confirmed the NTA data in relation to the presence and mean size of EVs in the samples studied. The morphology was similar to that described for bovine OF and UF (Lópera-Vázquez *et al.*, 2017a, Almiñana *et al.*, 2018, Kusama *et al.*, 2018 and Qiao *et al.*, 2018).

Furthermore, our western blot results confirmed the presence of 3 major exosomal marker proteins (CD9, CD63 and HSP70), existed in the EVs from oviductal and uterine fluids as has been also identified the in other studies in the ovuductal EVs (Almiñana *et al.*, 2018; Lópera-Vázquez *et al.*, 2017a) and uterine EVs (Kusama *et al.*, 2018, Qiao *et al.*, 2018).

It has been shown uterine secreted exosomes at the early luteal phase might play an important role in the development of somatic cell nuclear transfer (SCNT) bovine embryos (Qiao *et al,*. 2018). Similarly, Lopera-Vasquez *et al*. (2017) showed that oviductal EVs improve the development rate and the quality of *in vitro* produced embryos. While another study from the same group showed that the addiction of EVs from BOEC conditioned media does not affect the embryo development but only embryo quality (Lopera-Vásquez *et al.*, 2016). However, to our knowledge, this is the first report where EVs from bovine oviductal and uterine fluids have been successfully isolated, morphologically characterized and used in in vitro embryo culture, somehow mimicking in vivo conditions. Our results showed that supplementation of EVs from OF and UF in a sequential culture didn´t exhibited an improvement on embryo development (apart from Day 7) but the quality of the produced blastocysts was improved in terms of total cell number, amount of lipid droplets and mitochondrial activity.

The differences observed in the development of the blastocyst on Day 7 may be due to the type of protein supplementation used in the culture media, in which the depleted FCS showed better development support than the BSA. This is in agreement with results from Rizos *et al* (2003) when FCS was affecting the kinetics of development compared to BSA only at the beginning of blastocysts formation (Day 6) without any differences afterwards up to day 8. Similar, resent studies from our laboratory have shown a lower developmental rate of BSA compared to serum on Day 7 (Lópera-Vázquez *et al.*, 2017a,

Hamdi *et al.*, 2018). Consistent the above, could be concluded that presence of serum in the medium has a stimulatory effect on the speed of development, with more blastocysts appearing early by Day 7 of culture than in its absence and that the difference in development rate is no longer apparent.

To evaluate the quality of the embryos produced in the different groups, one of the parameters commonly used is cryotolerance and determination of survival after thawing and culture in vitro between 24 and 72 h. Under our experimental conditions, we found the survival rates to be high for all experimental groups up to the end of culture at 72 h, with proportion of live blastocysts between 80 to 100%. This could be due to the low number of embryos and replicates used so far, which may result in differences not being significant, even though numerically, the survival rate in both EVs supplemented groups was higher. Thus, further investigation on embryo cryotolerance is necessary.

The survival of embryos after cryopreservation is a multifactorial process influenced by lipid content, lipid composition, embryo metabolism, apoptosis rates, and the overall gene expression (Amstislavsky *et al*., 2018). Furthermore, there are clear indications that lipid abundance aggravates the cryopreservation of oocytes and preimplantation embryos (Sudano *et al*., 2011). Considering these statements, we can infer that producing embryos with fewer lipids could improve their ability to resist freezing.

The addition of EVs from oviductal and uterine fluids to embryo culture in a serum-free and EVs serum-depleted media, had a positive effect on embryo quality. The total number of cells in embryos cultured with EVs was significantly higher than the control groups. It has been shown that embryo quality determined by blastocyst total cell number (Fleming *et al*., 2004). In corroboration with our results, the increase number of cells in embryos has also been reported with the use of EVs from BOEC conditioned media (Lópera-Vázquez *et al.*, 2016), with EVs from stage 2 UF (Qiao *et al.*, 2018), and also EVs form stage 1 OF (Almiñana *et al.*, 2017).

Moreover, the trophoblast cells play a crucial role in pregnancy establishment when intense trophoblast proliferation begins together with increased trophoblast secretion of the pregnancy recognition factor interferon-t (Ealy and Yang 2009). Therefore, trophoblast cells have an essential role in implantation and placentation. The increased proliferation of trophoblast cells can be translated into a positive effect on pregnancy rate as reported by Hung *et al.* (2017), who observed a proliferative effect of follicular fluid

EVs on bovine granulosa cells in vitro. A similar effect could be occurring with the use of EVs of OF and UF on the cell division of embryos.

Regarding the amount of lipids, in our experiment we showed that EVs supplementation in presence of BSA does not affect the amount of lipids. This maybe explained based on the fact that BSA alone reduce the lipids produced in embryos (Abe *et al.*, 2002), while the addition of EVs did not favor an additional reduction. On the other hand, embryos cultured with serum usually have a higher lipid content (Rizos *et al.*, 2003). However, in the present study, the use of EVs-depleted serum showed a significant reduction of lipids which could be reflecting in better cryotolerance similar to embryos produced with BSA.

Similar to the lipids, our results indicated that the mitochondrial activity could be stimulated in blastocysts produced with EVs but this effect can be influenced by protein supplementation in the medium, with BSA having no effect. The fact that media with depleted FCS and EVs showed the highest mitochondrial activity and the lowest lipid content, could be a due to the beta-oxidation in the embryo's mitochondria as has been evidenced in bovine granulosa cells exposed to EVs from cells exposed to oxidative stress, (Saeed-Zidane *et al.*, 2017). Therefore, EVs from OF and / or UF affecting embryo mitochondrial activity. To our knowledge, this is the first study to indicate a relationship between reproductive tract EVs and mitochondrial activity in bovine embryos.

Conclusively, supplementation of in vitro culture media with oviductal and uterine EVs affect some mechanism related to embryo quality as it increases the total cell numbers, decreases the amount of lipids and increases mitochondria activity. The positive effect of EVs on embryo quality could be attributed to the oviductal/uterine embryotropic factors that are absent in defined and serum-supplemented media.

CONCLUSION

EVs from oviductal and uterine fluids from early and medium luteal phase, respectively, can be isolated, characterized and used successfully in in vitro bovine embryo culture.

The use of stage 1 oviductal and stage 2 uterine EVs in a sequential in vitro culture system for bovine embryos in order to mimic the in vivo condition does not affect embryo development, but improves embryo quality by increasing the total number of nuclei, reduced lipid content and increasing mitochondrial activity.

The effects of oviductal/uterine EVs on embryo quality maybe depend on the protein supplementation used in culture medium (BSA or EVs depleted-serum).

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APPENDIX

All reagents were purchased from Sigma (Madrid, Spain), except those where something else is indicated. Every media has to be filtrated by a 22 µm filter.

1. Phosphate buffer solution (PBS) composition

Table 2.1. Stock A

Compound	Reference	g/500 mL
NaCl	Merck 6404	**4.000**
KCl	Merck 4936	**0.100**
KH_2PO_4	Merck 4873	**0.100**
$Na_2 HPO_4 *H_2O$	Merck 6580	**0.715**

Dissolved in 500 mL of Sigma Water (W-1503)

Table 2.2. Stock B:

Compound	Reference	g/100 mL
$CaCl_2 *2H_2O$	Merck 2383	**0.070**
$MgCl_2*6H_2O$	Merck 5833	**0.100**

Dissolved in 100 mL of Sigma Water (W-1503)

Table 2.3. Mix both Stock and add:

Compound	Reference	g/500 mL
D-glucose	G-8270	**0.500**
Pyruvate	P-3662	**0.018**
Gentamicin	G-1272	**2.5 mL**

Dissolved in 500 mL of Sigma

Water (W-1503) Can be conserved for a month at 4 °C

Osmolarity 280 mOsm, pH 7.2-7.4

2. **Maturation media: TCM-199 (Sigma) supplemented with 10 % foetal calf serum (FCS) and 10 ng/mL epidermal growth factor (EGF)**

First day of use, add 0.5 mL of gentamicin (Sigma G1272) to 1 bottle of 100 mL of M199 (Sigma M4530). Use for 2 weeks.

Tube I Stock: add 100 µL of EGF (Sigma E4127) to 10 mL of M199.

Tube II Wash (+E): add 1 mL of tube I Stock to 9 mL of M199.

Tube III Culture(+E+FCS): add 1 mL of tube I Stock to 9 mL of M199 and mix well. Replace 1 mL of this solution with 1 mL of FCS (Sigma F2442-heat treated post purchase).

3. **Fertilisation media: Tyrode´s medium**

Table 3.1. Tyrode´s media composition:

Compound	Reference	mM
NaCl	Merck 106404	**100**
KCl	Merck 104936	**3.1**
NaH_2PO_4*H2O	Merck 106346	**0.3**
$CaCl_2$*$2H_2O$	Merck 102382	**2.1**
$MgCl_2$*$6H_2O$	Merck 105833	**0.4**
Red Fenol	Merck 107241	**-**

Can be conserved for a month at 4 °C Supplemented with:

2.16 g/L NaHCO2

1 mM pyruvate

1 mM Na-Lactate

6 g/L BSA without fatty acids.

5 mL/L gentamicin

10 µg/mL heparin (Calbiochem 37505)

4. Synthetic oviductal fluid (SOF).

Table 3.1. Stock A:

Compound	Reference	g/50 mL
NaCl	Merck 106404	**3.145**
KCl	Merck 104936	**0.267**
KH_2PO_4	Merck 104873	**0.081**
$MgSO_4*7H_2O$	Merck 105886	**0.091**
H_2O	W-1503	**49.70 mL**
Na-Lactate	L-4263	**0.3 mL**

Can be conserved for a month at 4 ℃ Table 4.2.

Stock B:

Compound	Reference	g/50 mL
$NaHCO_3$	S-5761	**1.05**
Red fenol	Merck 107241	**0.005**
H_2O	W-1503	**50 mL**

Can be conserved for a month at 4 ℃ Table 4.3.

Stock C:

Compound	Reference	g/10 mL
Na-pyruvate	P-3662	**0.080**
H_2O	W-1503	**10 mL**

Can be conserved for a month at 4 ℃ Table 4.4.

Stock D:

Compound	Reference	g/10 mL
$CaCl_2*2H_2O$	P-3662	**0.262**
H_2O	W-1503	**10 mL**

Can be conserved for a month at 4 °C

Table 4.5. Synthetic oviductal fluid (SOF) preparation:

Compound	Reference	g/100 mL
H_2O	W-1503	**78 mL**
Tri-sodium Citrate	S-4641	**0.010**
Myo-inositol	I-7508	**0.050**
Stock A		**10 mL**
Stock C		**1 mL**
Stock D		**1 mL**
***BME 50X**	B-6766	**3 mL**
****MEM 100X**	M-7145	**1 mL**
Glutamine	G-6392	**100 µL**
Gentamicin	G-1272	**500µL**
*****BSA**	A-9647	**0.3**
FCS	F-2442	**5 %**
Stock B		**10mL**

*BME 50X aminoacids solution

**MEM 100X non-esencial aminoacids solution

***can be used before filtration Osmolarity 275-285 mOsm, pH 7.2-7.3

Aliquote in 9.5 mL, can be conserved for 1 -2 weeks at 4 °C.

5. Vitrification and Warming solutions.

Table 7.1. Maintenance medium (MM):

Compound	Reference	Volume
TCM199	M7528	**100 mL**
FCS	F2442	**20 %**

Table 7.2. Sucrose medium (SM):

Compound	Reference	Volume
TCM199	M7528	**100 mL**
Sucrose		**34.2 g**

Adjust to 75mL. Add 25 % FCS