UNIVERSITY OF TERAMO

FACULTY OF VETERINARY MEDICINE

PhD in

"Veterinary Medical Sciences, Public Health and Animal Welfare"

XXXII CYCLE

MICROTUBULAR NUCLEATING CENTER DYNAMICS IN ASSISTED REPRODUCTION TECHNOLOGY (ICSI, SHEEP MODEL)

SDS (scientific disciplinary sector): VET02 Veterinary physiology

PhD Student	Tutor	Co-tutor
Yosra Ressaissi	Prof. Pasqualino Loi	Dr. Marta Czernik

PhD Coordinator
Prof. Fluvio Marsilio

REP-EAT Project Coordinator
Prof. Barbara Barboni

Academic year 2020-2021

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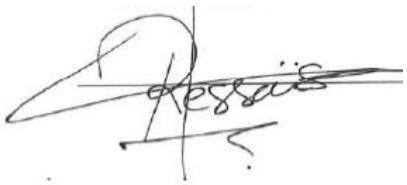

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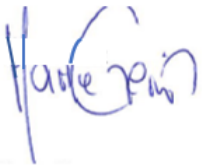

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ESR 12. Yosra Ressaissi

Valorization of alternative, healthier and environmentally compatible food production chain on typical sheep breed in Abruzzo region

MICROTUBULAR NUCLEATING CENTER DYNAMICS IN ASSISTED REPRODUCTION TECHNOLOGY (ICSI, SHEEP MODEL)

Tutor
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Co-Tutor
DR. MARTA CZERNIK

Università Degli Studi di Teramo (UNITE)

Declaration of authority

1. I declare that this thesis has been composed by myself with the assistance of the seniors of my laboratory team, along with the supervisor directivities.

2. I declare that this thesis has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

3. I confirm that this presented thesis for the degree of Veterinary Medical Sciences, Public Health and animal welfare has:

i) been composed by myself with the assistance of the senior of my laboratory team and the directive of my supervisor;

ii) been the result of my own work within the laboratory research group

iii) not been submitted for any other degree or professional qualification

I declare that this thesis is an original report of my research, has been written by me and has not been submitted for any previous degree. The experimental work is almost entirely my own work; the collaborative contributions have been indicated clearly and acknowledged. Due references have been provided on all supporting literatures and resources.

The data presented in the appendix of this thesis was obtained in an experiment carried out during the planned secondment in the Institute of Biomedicine and Translational Medicine, the ERA chair of Translational Genomics TRANSGENO, University of Tartu (Estonia), and the Institute of Veterinary Medicine and Animal Sciences, Tartu, Estonian University of life sciences (Estonia). I played a role in the preparation and execution of the experiment.

I am aware of and I understand the university's policy on plagiarism and I certify that the work presented in it has not been submitted in support of another degree or qualification from this or any other university or institute of learning.

Certificate by the Research Tutor

Here I certify that the PhD's students claim and statements are honest and true, and the work described in the thesis have been carried out by Mrs. Yosra Ressaissi, previous proper training by my senior staff members.

Approval of Thesis

Myself, Prof. Pasqualino Loi, supervisor of Yosra Ressaissi, here identified as ERS 12, approve the thesis in all its parts

Yours Faithfully

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Acknowledgments

I would like to express my gratitude to each person who has supported me through this journey and helped me to develop as a scientist.

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University of Teramo

Abruzzo Region

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List of abbreviations

AI = Artificial insemination

ART = Assisted Reproductive Technologies

AV = Artificial Vagina

BME = Basal Medium Eagle (BME)

BSA = Bovine Serum Albumin

Ca^{2+} = Calcium

COCs = Cumulus-Oocytes-Complexes

CO_2 = Carbon dioxide

cs-FBS = charcoal stripped FBS

ddH20 = bi-distilled water

EAA = Essential Amino Acids

EGTA = Calbiochem, Ethyleneglycol- *bis*(β-aminoethyl)-N,N,N′,N′-tetraacetic Acid

ET = *In Vitro* Embryo Transfer

FAO = Food and Agriculture Organization

FBS = Fetal Bovine Serum

FD = freeze-dried

FITC = Fluorescein isothiocyanate isomer I

FT = frozen/thawed

G2/M = cell cycle checkpoint

HEPES = 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)

ICSI = Intracytoplasmic sperm injection

IgG = Immunoglobulin G

IVC = *In Vitro* Culture

IVF = *In vitro* fertilization

IVM = *In Vitro* Maturation

IVEP = *In Vitro* Embryo Production

LH = Luteinizing Hormone

LN = Liquid Nitrogen

MII = mature oocytes

MEM = Minimum Essential Medium (MEM)

MgSO 4 ·7H 2 O = Magnesium sulfate heptahydrate

NEAA = nonessential amino acids

NT = Nuclear Transfer

O_2 = Dioxygen

pa = post-activation

pf = post-fertilization

PBS = Phosphate buffered saline

PFA = paraformaldehyde

PHEM = Microtubule stabilizing buffer

PIPES = 1,4-Piperazinediethanesulfonic acid

PN = pronuclear stage

PVA = polyvinyl alcohol

PVP = PolyVinylPyrrolidone

SOF = Synthetic Oviduct Fluid

SOF+ = Synthetic Oviduct Fluid supplemented with glucose

TCM-199 = medium 199

TRIS = 2-amino-2-hydroxyméthylpropane-1,3-diol

RT = Room Temperature

Microtubular Nucleating Centre Dynamics in Assisted Reproduction technology

(ICSI sheep model)

Yosra RESSAISSI

Dissertation Advisors:

Prof. Pasqualino LOI
Dr. Marta CZERNIK

ABSTRACT

The scope of this thesis is to investigate abnormal sperm centriole function during the early stages of Intra Cytoplasmic Sperm Injection (ICSI) embryo development using sheep as an animal model. The main focus was on evaluating the timing and dynamics of the sperm microtubular aster nucleation and organization as a possible factor that undermines early embryonic development following ICSI. The main finding was that ICSI derived embryos using freeze-dried spermatozoa displayed a delay in their aster nucleation and a noticeable hampered embryo development. We also noticed that ICSI-derived embryos failed to undergo subsequent development and were blocked at the pronuclear stage. In this work, we demonstrated that embryo development failure following ICSI in sheep is not actually related to a centriole dysfunction; rather, the major problem recorded is the lack of syngamy. Thus, besides our objective data ruling out centriole dysfunction as a cause of developmental failure in sheep/ruminant embryos, we have opened a worth theme to investigation, that is the perfecting of artificial protocols in sheep oocytes fertilized by ICSI.

Chapter I

Introduction

I. The current state of sheep genetic resources biodiversity

Genetic resources variability between species and within an ecosystem define biodiversity. Agricultural biodiversity, in terms of plants and livestock, ensures human survival and economic development through contributing with important services in terms of employment, community belonging, tourism, religion and sustainability. Livestock contributes with 40% to the global agriculture production and small ruminants, mostly sheep, are a significant element in meat and wool production. Currently, the sheep industry counts more than 1 billion head worldwide which is satisfying a big part of the global demanded animal products, leading to classify the species as one of the most important genetic resources. Unfortunately, the importance of biodiversity eco-services is still not well assimilated and livestock genetic resources remain undergoing serious alarming genetic erosion threats due to technical, biotechnological, financial, socioeconomic and even cultural developments. Up to now, different sheep breeds have disappeared at a speed that exceeds thousand times the natural estimated rate and some scientists guarantee that this rate would be higher in the future and expect a sixth wave of extinction after the five massive extinctions that the earth knew during the last five hundred million years. As a matter of fact, according to the Second Report on the State of the World's Animal Genetic Resources for Food and Agriculture (FAO, 2019), a total of 647 authentic mammalian species breeds have extinct, among which 160 sheep breeds. The report also highlighted that 352 mammalian breeds are in a critical risk of extension worldwide and sheep is one of the species in the highest risk counting 53 breeds in a critical status of erosion, 86 breeds as endangered and a registered increase of 24%, between 2007 and 2015, in the percentage of disappeared sheep breeds. Such diversity loss trend is a result of multiple factors namely the viability declines of traditional livestock production systems, the ecosystem extermination of indigenous breeds, the substitution of native genetic resources with highly productive breeds and the expansion of economic globalization (Zhang et al., 2018). The country-report analysis of the Food and Agriculture Organization (FAO) (2015) has revealed that several countries are concerned about the intensive international effects of gene flows on the diversity of their native livestock populations and are actually facing a serious challenge in the management of their local animal genetic resources. In this context, they have expressed the urgent necessity of some governmental interventions to implement some conservation priorities to ensure that the ongoing agricultural

development suits sustainable use of local livestock diversity, since the loss of any species would interrupt the normal functioning of a whole ecosystem. For instance, Pagliarola is an ancient sheep breed widely distributed over the Italian central regions, especially in Abruzzo mountains. Statistics made by the Italian Sheep Association have demonstrated that Pagliarola was the most prevailing sheep breed in Abruzzo by 1971 but, since then, the number of animals has severely decreased over years. The remarkable decline was explained, firstly, by the deficient productive performances of the breed comparing to highly specialized breeds and secondly by the strong urbanization. Consequently, Pagliarola is currently rare and only some small flocks, owned by old shepherds, are barely found through extreme environmental conditions. These findings suggest that this typical breed is actually endangered and the establishment of a conservation plan is highly recommended in order to preserve the historical background, the authentic morphology and the traditional products of the population (Ceccobelli et al., 2016).

II. Assisted Reproductive Technologies (ART) in the management of livestock genetic resources

The conservation of animal genetic biodiversity requires a wide knowledge of the reproductive physiology in and within the different existing species. Infertility related issues are one of the major and significant impediments that hamper successful natural mating leading to genetic heritage loss. It is here that Assisted Reproductive Technologies (ART) were found to be a relevant tool to overcome mating difficulties and to conserve genetic biodiversity of small, rare, isolated and threated animal populations. ART application in a conservation perspective was mainly introduced in the 1990s. In fact, over 30 years ago, ART in livestock have experienced several advances in terms of *In Vitro* Embryo Production (IVEP) including Artificial insemination (AI), *In Vitro* fertilization (IVF), *In Vitro* Embryo Transfer (ET), embryo sexing, sperm selection, embryo splitting, cloning and Intracytoplasmic sperm injection (ICSI). Mainly, ART were intensively used to overcome infertility but interestingly they have been proved very efficient in accelerating, achieving and diffusing significant valuable genetic merit and in improving productivity in different breeds of interest. Giving the advantage of choosing and selecting male and female gametes, these methodologies led to reduce risks associated with transporting

animals and thereby facilitated propagating particular traits from genetically superior animals or safeguarding valuable germplasm, i.e. genes, from isolated and endangered mammalian species or a breed (Singh Parmar et al., 2013; Paulson and Comizzoli, 2018). Small ruminants, i.e sheep and goat, are considered the best animal model for ART development as these species stand out a huge ability to be genetically modified for meat and milk productivity. As a matter of fact, the first successful experience in term of cloning in livestock was a cloned sheep by somatic cell nuclear transfer (NT) (Wilmut et al., 1997). During the last years, considerable qualified protocols were established to improve IVEP technologies efficiency and to meet requirements of some specific cases (Herrick, 2019). In sheep, for instance, IVEP protocols were developed on the basis of *in vivo* conditions to mimic as much as possible the embryo development processes in the female reproductive tract. Several protocols were developed between laboratories and experiments mainly to better standardize the required conditions for the three major steps in IVP namely *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). Also, numerous methodologies were tested within the species which the most used are *In vitro* production of embryos by IVM/IVF, intra-cytoplasmic sperm injection (ICSI), as reported by Paramio and Izquierdo (2016). IVP has been then proved to be efficient in sheep reproduction system since results have demonstrated embryo rates of 62% and 67%, respectively from ovulated and IVM eggs after IVF and IVC (Crozet et al., 1987) and embryo transfer that lead to 50% of pregnancies and birth of 26 normal healthy lamb from 124 zygotes from IVF ovulated eggs (Cognie et al., 1999). However, despite the released achievements so far and the efforts made to improve blastocyst yield and quality, IVEP outputs in sheep are still low and not enough satisfactory. Such an evident proves that the technique is still considered at the development stage and that the achieved progress is facing critical limitation converging towards bottlenecks, mostly for the preservation of engendered breeds. One highly relevant reason is the lack of knowledge about male and female gamete physiology which seems to compromise the success of even the most basic and simple methods (Herrick, 2019). On the other hand, fertility preservation techniques were also developed in parallel to IVEP and were found of significant help to face breed extermination. The practice defines long term storage of biomaterials and refers to biobanking. It evolves cryopreservation of fertile oocytes, sperm, and even embryos to create reservoirs of the existing diverse gene pool from young, adult, or aged individuals, to protect the pre-existing genetic diversity and its sustainability over the future

generations. Basically, male fertility preservation has been always made through semen freezing and storing which is no longer an innovation. Believing in cryopreservation and biobanking benefits and following the advanced research on spermatozoa sensitivity to cryopreservation procedure, consistent progress was achieved and other alternatives have been proposed for sperm preservation (Comizzoli, 2015). The most recent interesting technique for sperm preservation is lyophilization or freeze-drying which is an ecological non-cryogenic storage solution that facilitated the storage and the distribution of genetic resources in different animal species. The main advantage of the technique is the long-time storage option at 4°C or even at ambient temperature. Freeze-drying implies water removal and the conservation of the sample under an anhydrous state (Loi et al., 2013; Kaneko at al., 2014; Gil et al, 2014). Freeze-dried spermatozoa retain their entire fertilizing capacity and have proven their efficacity in many mammalian species since viable live offspring were successfully reported in mouse (Wakayama and Yanagimachi, 1998), rabbits (Liu et al., 2004), rats (Hirabayashi et al., 2005) and horses (Choi et al., 2011) through intracytoplasmic sperm injection (ICSI) of lyophilized spermatozoa. In sheep, Anzalone et al. (2018) have demonstrated that sperm lyophilization is a relevant low-cost storage option for conserving ram spermatozoa from the endangered typical sheep breed of the Italian Abruzzo region, Pagliarola. In their study, they have established a sperm bank for a seriously endangered sheep breed and they have reported that the sperm lyophilization process requires further development to be more efficient for sperm storage.

III. Intracytoplasmic Sperm Injection (ICSI)

Intracytoplasmic sperm injection (ICSI) implies the microsurgical injection of a single spermatozoon into the cytoplasm of a mature egg. The technique was proposed in 1992 to mainly circumvent severe male fertility disorders related to the sperm failure to fuse with the oocyte or to cross the egg barriers and was found very successful. Currently, ICSI is the best micromanipulation technique, for both animals and humans, for treating male factor infertility (Yanagimachi, 2004; Terada et al., 2010). In livestock, ICSI has been extensively introduced to realize different achievements such as producing high-valuable genetics animals, conserving endangered and valuable breeds, producing transgenic animals and overcoming fertilization issues encountered in IVF systems. Thus, it is presented as a worth assisted reproductive tool in *in*

vitro monospermic zygote production with no exclusive focus on male infertility factors (García-Roselló et al., 2009). Since its introduction in farm animals, ICSI has been extended to various species, including rabbits (Iritani and Hosoi, 1989), cattle (Goto et al., 1990), sheep (Catt et al., 1996), goats (Keskintepe et al., 1997), horses (Cochran et al.,1998) and pigs (Kolbe and Holtz, 2000). The technique has been mostly implemented as an experimental device to investigate fundamental biological question related to molecular mechanisms of oocyte activation, ooplasmic factors responsible for male pronucleus transformation as well as early embryo development events (Singh Parmar et al., 2013). In ruminants, ICSI was highly efficient in overcoming polyspermic fertilization which has been identified the main factor associated with IVF system failure. However, in comparison to human, it has been noticed that the technique is quite less successful in some species and ICSI embryos were found to display low developmental rate compared to IVF embryos (Salamone et al., 2017). Investigations have identified that the sperm oocyte-activating factor as well as the sperm introduction into the ooplasm are the major handicaps that interrupt normal embryo development (Chung et al., 1999; Devito et al. 2010; Arias et al. 2014). For instance, in cattle, despite the very significant rates of oocyte maturation and fertilization, around 90% and 80% respectively, low blastocyst yield, from 30% to 40%, has been always reported. In small ruminants, sheep and goats, the number of studies is quite limited, particularly on the latter one, and have also highlighted a percentage of blastocyst ranging from 20% to 30%, thus, there is clearly room for improvement. Up to now, various issues are hampering the practical use of ICSI in these species although several studies have been conducted endlessly to improve the efficiency of the technique. *In vitro* embryo micromanipulation in small ruminants is an outstanding source of low-cost embryos for the implementation of basic research on developmental biology and physiology as well as for the commercial application of the newest biotechnologies such as nuclear transfer and transgenesis. Hence, future works must focus deeply on factors and molecular mechanisms which are limiting embryos developmental competence following ICSI (Cognié et al., 2003; Paramio and Izquierdo, 2016).

IV. ICSI development in sheep

In sheep embryos, it has been noticed that commercial activity worldwide is suffering a lack of proper care comparing to cattle and other farm animals (Paramio and Izquierdo, 2016). Catt and Rhodes (1995) have published the first report in sheep ICSI following a study in which they explored ICSI potential in fertilizing. Embryonic division was observed when they cultivated the injected oocytes *in vitro* using Synthetic Oviduct Fluid (SOF) medium. By making ICSI available in sheep, Catt et al. (1996) performed a prompt ICSI assay using sexed sperm and by transferring the presumptive embryo immediately after the injection, they obtained the first offspring which was male, according to the type of sexed sperm used. ICSI application in sheep has been widespread and several studies were carried out. The obtained results have showed remarkable variability and have led to conclude that oocyte activation after injection is an essential factor that significantly affect the blastocyst development rate in ICSI sheep embryos. As a matter of fact, studies developed by Gómez et al. (1997, 1998) showed lower cleavage rates of 30% and 39% when no activation was carried out. However, they have observed a remarkable improvement of 75% in their next assay in which they attempted to activate the oocytes using calcium ionophore. Catalá et al. (2012) and Hosseini et al. (2012) have tested ionomycin as activation treatment for the injected oocytes and they have reported acceptable cleavage rate respectively about 78% and 64%. While the most relevant cleavage rate improvements were found by Shirazi et al. (2009, 2011) who assessed a combination of ionomycin plus 6-dimethylaminopurine for the activation process reporting a better cleavage rate of 86.50% and 78%. In the same context, Anzalone et al. (2016) performed a study to assess the need for artificial activation of sheep oocytes following ICSI using ionomycin on damaged ram sperm plasma membrane and acrosome vesicle. They have found that oocyte activation only did not improve the embryonic development while damaging the ram sperm plasma membrane and acrosome vesicle together with ionomycin activation substantially enhanced the zygotes and blastocysts rates respectively from 45% to 84% and from 1% to 15% in ICSI sheep embryos.

V. Implication of sperm factors in ICSI failure

Despite the successful achievements realized with ICSI and also the better fertilization rates obtained comparing to IVF, many cases of failure have been reported where ICSI derived embryos did not develop in term, besides pregnancy rates related to these embryos have been always poor, not more than 20%. A concern which is probably due to the lack of knowledge in comprehending and identifying some disorders at the cellular and molecular levels within to the different post-fertilization events and which has stimulated research to investigate about the latest (Manipalviratn et al., 2009; Schatten and Sun, 2015). Briefly, fertilization consists of the several stages that lead to male and female gametes fusion into a single cell called a zygote. The process begins by the contact between both gametes and the fusion of their plasma membranes which enables the sperm to incorporate into the oocyte. As soon as the sperm is incorporated, the oocyte begins a whole series of molecular modifications grouped under the term activation and which are triggered by oscillations series of calcium concentration (Ca^{2+}) from the sperm. Once activated, the egg completes the division cycle and two haploid pronuclei or nuclei appear then fuse which mark the start of embryonic development. Gamete movement and fusion is promoted by the sperm centrosome, which nucleates the sperm aster microtubules (Manandhar et al., 2005). Figure 1 (Schatten and Sun, 2009) summarize fertilization events and the implication of sperm centrosome. In fact, ICSI assists sperm-oocyte fusion handicaps but does not address defects related to genome fusion events. Most human infertility diagnosis have shown that unfertilized zygotes were arrested at a point of post-ICSI events which was found to define the survival of the newly formed embryo (Terada et al., 2010). This explain ICSI fertilization disorders are mainly due to the several molecular mediators evolved in post-ICSI mechanisms, mostly related to sperm factors.

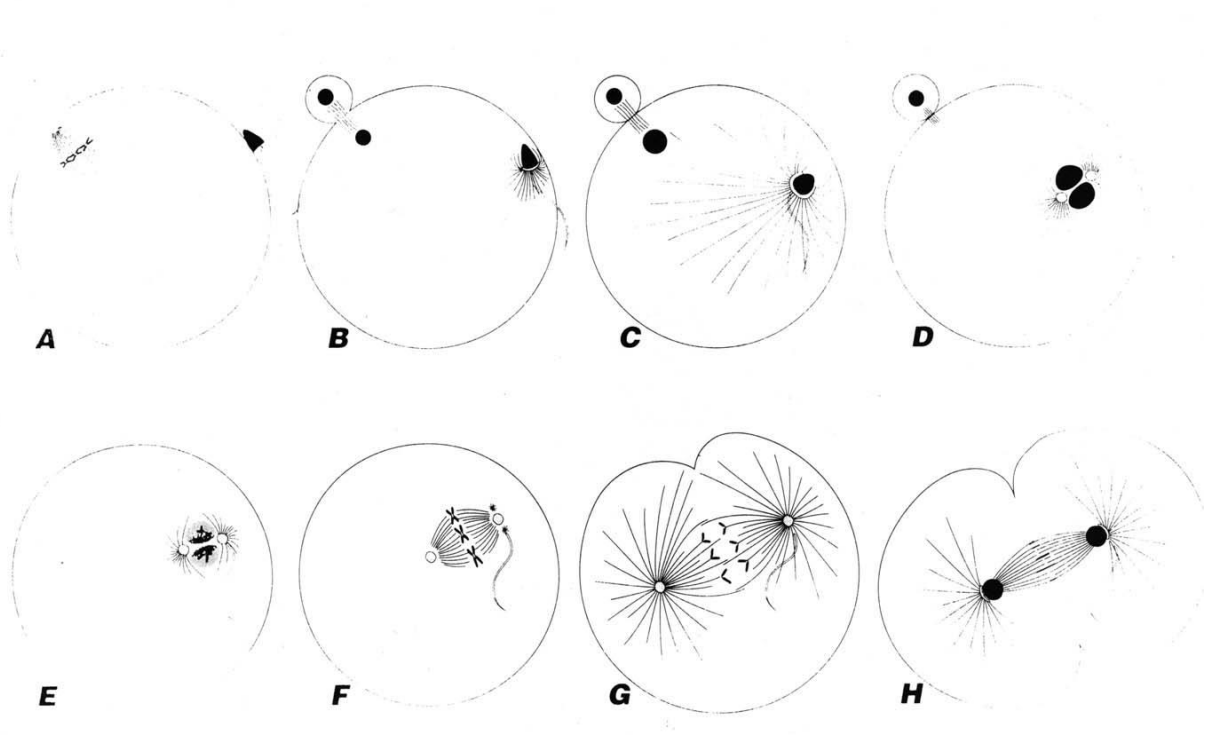

Figure 1. Schematic diagram of fertilization events and the implication of centriole–centrosome complex. In (A), prior fertilization, the sperm shows a proximal and a distal centriole and the MII stage oocyte contains acentriolar centrosomes. In (B), shortly after fertilization, the sperm proximal centriole initiates sperm aster nucleation. In (C), sperm aster microtubules elongate and bring both pronuclei in the center, pronuclear stage. In (D), after syngamy, the duplicated centriole–centrosome complex migrates around the zygote nucleus and relocates to opposite poles to form the centers of the mitotic spindle poles. (E) Mitosis of the first cell cycle (Schatten and Sun, 2009).

The major sperm issues responsible for abnormal development of ICSI derived embryos are related to the sperm ability to activate oocyte, the introduction of sperm membranes into the oocyte and centrosome dysfunction in early embryo development (García-Roselló et al., 2009). In ICSI procedure, spermatozoa are injected with acrosome and plasma membrane into the oocytes, unlike the usual conditions of fertilization. It was found that spermatozoa membrane and acrosome persistency lead to atypical and incomplete Ca^{2+} releasing machinery, supporting the hypothesis that sperm is in charge of signaling oocyte activation. Hence, ineffective sperm oocyte-activation compromise early embryo development, as reported by Meerschaut et al. (2014) and Salamone et al. (2017). To overcome these limitations, and to partially mimic natural fertilization, spermatozoa were treated to remove the membranes prior ICSI, which was found to regulate the onset of Ca^{2+} waves and induce proper activation of the injected oocyte and pronuclear formation upon ICSI (Moisyadi et al., 2009). In ruminants as well, low development

ability of ICSI embryos was found to be due to the same factors leading to low blastocyst rates in bovine (Chung et al., 1999; Devito et al. 2010; Arias et al. 2014; Morrell and Humblot, 2016) as well as in sheep (Shirazi et al. 2018). However, besides these expedients, embryonic development following ICSI remains low in sheep and other ruminant embryos (Shirazi at al., 2018). Along this line, Anzalone et al. (2018) have revealed an asymmetry between the activation, judged on the pronuclear detection (80% in average), and the cleavage rate, lower (30% in average) in sheep ICSI embryos. This findings and published data pointed out that further research which focus on relevant processes in fertilization such as centriole dynamics, would provide helpful insights on the defective steps that hamper development in sheep ICSI. As a matter of fact, Schatten et al. (1999) have reported that calcium oscillations during oocyte activation phosphorylate the spermatozoa centrosome, which become able to accumulate additional proteins from the oocyte cytoplasm leading to sperm aster organization and microtubules nucleation. Various cases of incomplete fertilization following ICSI were associated to sperm aster abnormalities due to irregular microtubule organization (Rawe et al, 2000; Nakamura et al, 2001). Sperm aster nucleation and microtubule dynamics are crucial post-ICSI events (Palermo et al., 1997; Hochi, 2016); however, their involvement in ICSI failure is insufficiently documented, especially in ruminants. In bovine, some data are available on the implication of sperm centriole in male fertility; but still, the missing centrosomal component of the infertile spermatozoa is not identified yet (Terada et al., 2010; Conduit et al., 2015).

VI. Centrosome dynamics and microtubular organization in early embryonic development: the role of the sperm aster

Centrosome is a permanent cell structure which has particular features and represents the unique active division center that establishes spindle bipolarity and assembles microtubules for embryonic development (Conduit et al., 2015).

1. Centrosome composition and function

An ordinary mammalian centrosome is the principal microtubule organizing center (MTOC) of the cell which is represented by a perpendicularly arranged pair of centrioles composed of radial array of microtubules associated by interconnecting fibers (Manandhar et al., 2005; Hoyer-Fender, 2012). A typical centrosome stands out for its duplication cycle in accordance with DNA replication. Thus, once per cell cycle, each single centrosome duplicates, simultaneously with the S-phase of the cell cycle, to ensure the assembly of a newly formed centriole, the daughter centriole, orthogonally disposed to the pre-existing centriole, the mature or the mother centriole, generating two functional centrosomes (Wilson, 2008). Centriole production cycle is influenced by substantial cell cycle regulators, signaling molecules and different types of proteins which together organize the pericentriolar material (PCM) components. The assembly and accumulating mechanisms of this electron dense matrix of proteins around centrioles is still a mystery; however, it is a primary condition for centrosome as the dominant MTOC (Chavali et al., 2015). Centriolar microtubules are composed α/β-tubulin protofilaments anchored on the ring of γ-tubulin subunits by their minus ends. They are highly spatial and temporal dynamic filaments which grow and to extend into the cytoplasm through the polymerization of their α/β-tubulin heterodimers; defining the process of microtubule nucleation (Petry and Vale, 2015; Akhmanova and Steinmetz, 2015; Gunes et al., 2020). A schematic representation of a typical mammalian centrosome is on figure 2.

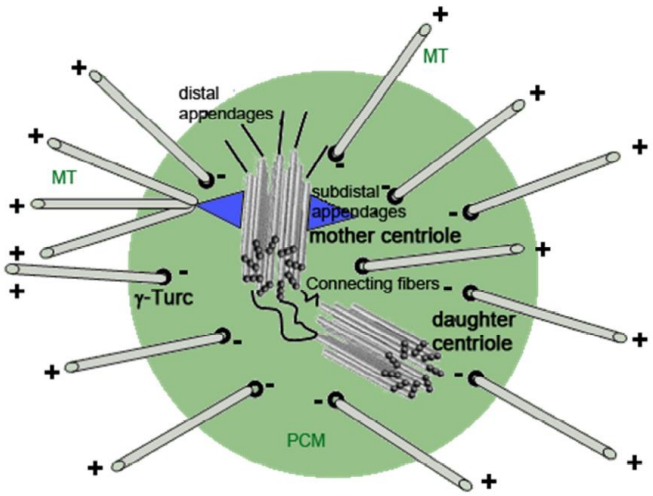

Figure 2. Schematic representation of a typical mammalian centrosome. It consists of mother and daughter centrioles enclosed by the pericentriolar matrix (PCM). The mother centriole contains distal and subdistal appendages (not all are shown) and both centrioles are associated by interconnecting fibers. In the PCM, γ-TuRCs are located that anchor MT minus ends. (Hoyer-Fender, 2012).

2. Centrosome inheritance during fertilization

Normal embryo development requires a single centrosome duplication resulting in two centrosomes that allow the zygote to enter cell division through ensuring bipolar spindle formation and equal segregation of the genetic material. Thus, centrosome number regulation in early embryogenesis has to be carefully regulated in tight coordination with DNA replication and cell division cycle (Bennabi et al., 2016). In mammalian reproductive systems, if each parental gamete contributes with one full centrosome, which will undergo a canonical duplication cycle; then the zygote would present four centrosomes at the first mitosis and thereby an abnormal division cycle (Navara, 1995). Thereby, even if centrosomes reconstitution and mechanisms governing centrioles biogenesis and controlling centrosome number are still not well understood, it was believed that organisms are able to down regulate the gamete centrosome by restoring only one copy of during fertilization (Crozet el al., 2000). As reported by Sathananthan et al. (1996), Boveri (1887, 1901) have revealed over a century ago typical paternal model of centrosome inheritance showing that the active division-center is provided by the spermatozoa, except in rodents. According to his theory, male and female gametes are partial complementary entities; each is contributing differentially to provide one complete functional centrosome to the zygote. As a matter of fact, the ripe egg lacks centrioles but provides the whole accessory elements such

as proteins, mRNAs, metabolites and others elements which are mandatory to support early embryo development. Elimination of the major microtubule organizing centers in most oocytes is still based on some conjectures rather than knowledge. However, it has been proved that PCM is maternally contributed to the one-cell stage embryo and that sperm centrosome duplicates and ensures fundamental tasks for all embryo development stages through rapid organization of astral microtubules (Delattre and Gönczy, 2004; Chatzimeletiou, 2008; Bennabi et al., 2016). In parthenogenetic embryos from different species, except rodents, the absence of functional centrioles in the early stages of development was reported. In fact, after parthenogenetic activation, it was found that the oocyte acquires the capacity to nucleate microtubule, to organize a functional bipolar spindle and thereby to enter mitosis; but at the morula or blastocyst stages. However, the observed spindles were "anastral"; meaning that no centrioles were spotted in the mitotic spindle of these eggs. Such evidences were also reported in sheep (Crozet et al., 2000) and have confirmed that the absence of centrioles in sheep mature oocytes is a general feature. Sperm centriole–centrosome complex has been studied to investigate about its functionality upon fertilization. It has been found that the spermatozoa count two separated centriolar structures within the connecting piece namely the proximal centriole, situated besides the sperm head basal plate, and the distal centriole, perpendicularly disposed to the proximal centriole and aligned with sperm tail (Schatten and Sun, 2009). Shortly after sperm incorporation into the egg, it was found that the distal centriole degenerates while the proximal centriole was observed to be surrounded by pericentriolar components and nucleating a monopolar array of microtubules, the sperm aster. The flexible structure of the sperm centrosome, its plasticity, together with its molecular reorganizations as well as its microtubule dynamics have made the sperm aster a highly dynamic structure which is able to undergo rapid growth and modifications. As the prominent microtubule structure within the zygote, the sperm centriole accumulates maternal γ-tubulin leading to radial microtubule nucleation and growth of the sperm aster. Aster microtubules elongation ensures male and female pronuclear movement, gamete union, adequate genetic information inheritance and thereby normal embryo development (Simerly et al., 1995; Sathananthan et al., 1996; Manandhar et al., 2005; Hoyer-Fender, 2012; Gunes et al., 2020). The same sperm behavior has been reported by Crozet (1990) in sheep zygotes. A schematic representation of the sperm centriole–centrosome organization and duplication are on figure 3.

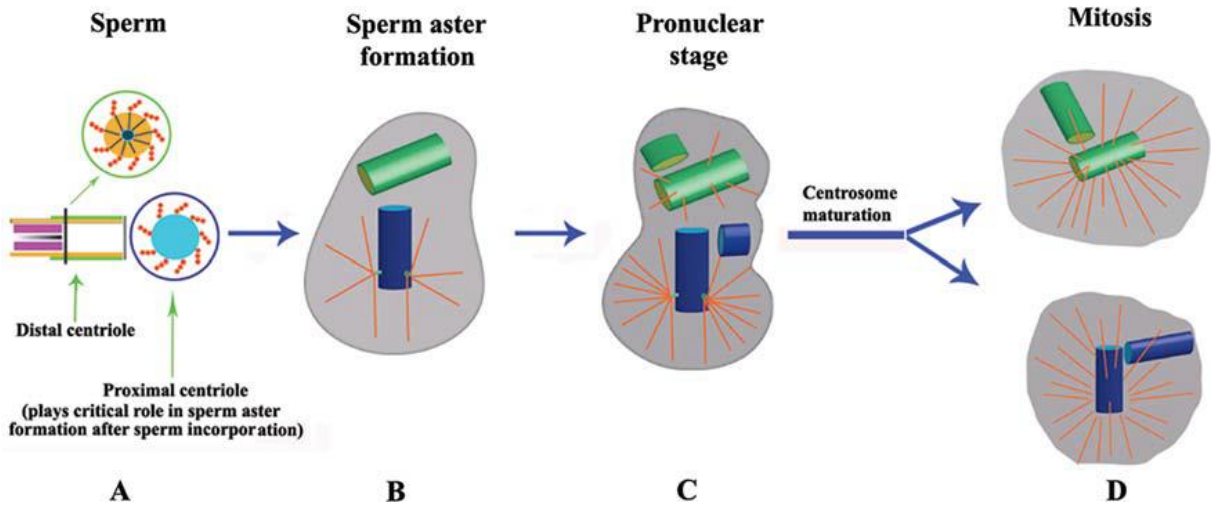

Figure 3. Schematic representation of centriole–centrosome organization and duplication. (A) before fertilization, the spermatozoon displays the distal centriole aligned with the sperm tail and the proximal centriole located within the connecting piece next to the basal plate of the sperm head. (B) Shortly after sperm incorporation, the sperm aster is nucleated from the proximal centriole allowing pronuclear apposition. (C) After pronuclear apposition, the sperm centrioles duplicate during the pronuclear stage. (D) The duplicated centrioles separate and migrate around the zygote nucleus to form the opposite poles of the first mitotic spindle (Schatten and Sun, 2009).

3. Centrosome dysfunction and implication in male infertility

The sperm centrosome intervenes at the ultimate stage of the fertilization process; thus, sperm centrosomal defects would interrupt the development of post-fertilization events at the pronuclear stage. Several pathologies of human male infertility were related to abnormalities within the sperm centriole–centrosome complex and sperm aster organization disorders (Chatzimeletiou et al., 2008). As a matter of fact, investigations on centriole/centrosome implication in male infertility were initially established by Sathananthan (1994) and results led to postulate sperm with defective centriole/centrosome causes abnormal cleavage and produce aberrant embryos. In fact, abnormal pronuclear morphology and apposition as well as unusual aster formation were observed to disrupt fertilization through generating a slow cleavage trend, blastomere irregularities and embryonic development arrest (Tesarik, 2002, 2005). Other cases have revealed, that centrosome dysfunction is responsible for polyploidy and failure of post-zygotic chromosomal division which lead to delay or arrest the cleavage-stage development. Sathananthan et al. (1996) have highlighted that as long as the sperm centrosome and the surrounding

PCM constitute an unabridged complex, then centriolar dysfunctionality might be resulting from defects of either or both of these components. As a matter of fact, some authors have pointed out that several proteins, such as centrin, pericentrin, γ-tubulin and speriolin, were recognized surrounding the sperm centrosome and the connecting piece and might be correlated with centriole–centrosome complex dysfunctionality (Manandhar et al., 2000). Also, some sperm proteasomes have been identified in the neck region of the human spermatozoa by Wojcik et al. (2000) while reporting their possible involvement in the releasement of the sperm centriole after fertilization. However, there is a huge gap in the investigations about their possible involvement. According to Schatten et al. (1994), calcium oscillations during oocyte activation lead to phosphorylate the sperm centrosome which become able to accumulate additional γ-tubulin from the oocyte cytoplasm and form a centriole. The accumulated γ-tubulin lead to microtubules nucleation and sperm aster organization. Other proteins, such as centrin and dynein, might also be involved in microtubule nucleation. Thus, insufficient protein supply impact the sperm ability to fertilize and diminish sperm aster organization upon fertilization which affects the fertilization success and interrupts normal embryonic development (Hinduja et al., 2010). In bovine fertilization, sperm aster nucleation and size have been studied by Navara et al. (1996) and Rawe et al. (2002), that have shown that sperm centrosome and aster organization is an individual feature which varies from one bull to another while affecting male fertility and early development. More detailed molecular analyses are needed about requirements for centrosome growth after fertilization, as mentioned by Schatten et al. (2009). In the same context, Nakamura et al. (2002) and Manandhar et al. (2005) have point out that evaluating sperm centrosomal functionalities would be very useful to understand new insights of infertility that would lead to immediate medical benefit.

VI. Aim of the thesis

Up to now, timing and the dynamics of sperm aster organization, in early sheep zygote derived by ICSI has not been thoroughly investigated. In this work, we set up to investigate centriole/micro-tubular and nuclear dynamics in zygotes obtained by ICSI-fertilized sheep eggs with fresh and freeze-dry spermatozoa and *In Vitro* Fertilization (IVF) as control.

RESEARCH QUESTIONS

1. Starting from the moment of fertilization, how does centrosome and centrioles behave in early development of sheep embryos?

2. Are there any differences in microtubular nucleating centers between ICSI and normal IVF embryos?

3. Does spermatozoa lyophilization affects the microtubular nucleating potential of the centrosome?

4. Does sperm centrosome dysfunction is the main factor behind ICSI failure in sheep zygotes?

VII. Experimental design

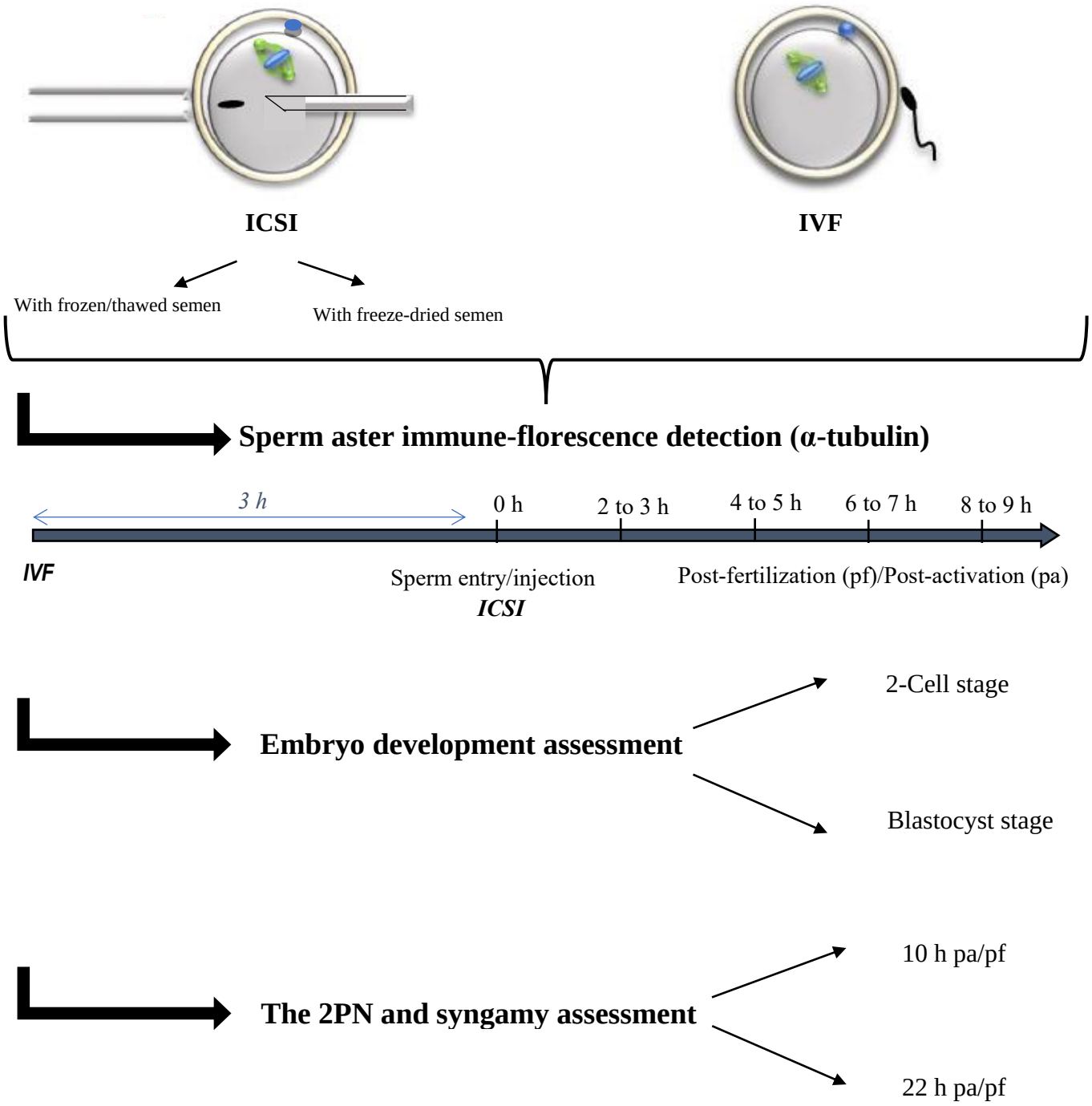

Figure 4. Experimental design

Chapter II

Material and Methods

Unless otherwise stated, all materials used were purchased from Sigma-Aldrich, Darmstadt, Germany.

I. Oocyte recovery and in vitro maturation (IVM)

Sheep ovaries were collected from local slaughterhouses and transferred within 1 to 2 hours at 37 °C to the laboratory. Ovaries were washed twice in Phosphate buffered saline (PBS) solution (1X) (figure 5A). Aspiration of the oocytes from the follicles was done in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered TCM-199 medium with 0.005 % heparin using 21 G needles (figure 5B). Then oocytes were selected under the microscope (figure 6A) and only the oocytes with at least 2 or 3 layers of compact cumulus cells were considered for maturation (figure 5B). *In vitro* maturation (IVM) medium is composed by MEM-199 containing 2 mM glutamine, 0.3 mM sodium pyruvate, 100 µM cysteamine, 10 % fetal bovine serum (FBS), 5 µg/mL follicle stimulating hormone FSH, 5 µg/mL luteinizing hormone (LH), and 1 µg/mL estradiol. After 2 washes in the IVM medium (figure 6C) oocytes underwent maturation in 4-well dish in 0.5 mL of IVM medium (figure 6D) in a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 24 h.

Figure 5. Oocyte collection: Ovaries wash in PBS (A); Oocyte aspiration from the follicles using 21G needle (B).

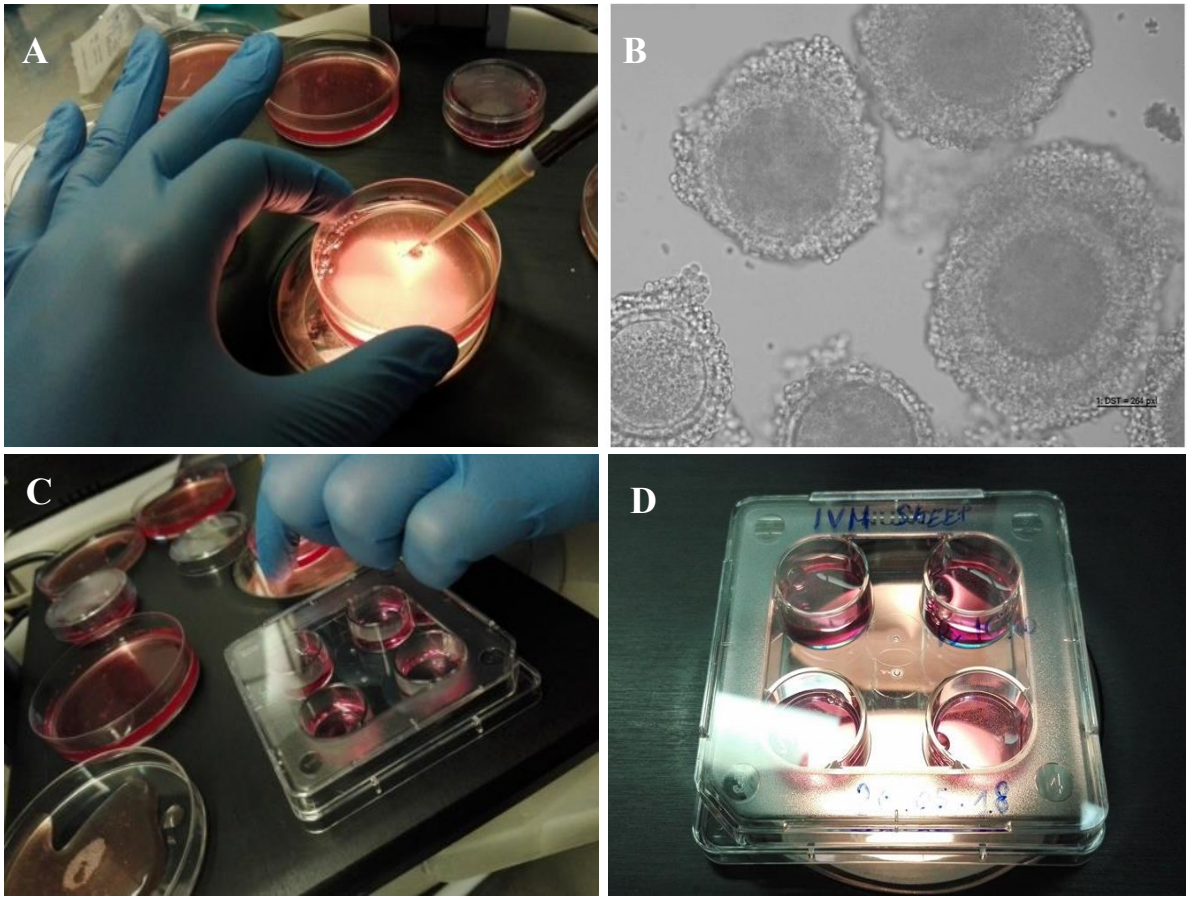

Figure 6. *In vitro* maturation of sheep oocytes. Selection of oocytes under the microscope (A); Oocytes with at least 2 or 3 layers of compact cumulus (B); Wash of the oocyte in the IVF medium (C), Oocytes underwent *In vitro* maturation in 4 well dish (D).

II. Semen preparation

1. Semen collection

Semen collection was made from adult fertile Sardinian rams by the mean of an Artificial Vagina (AV) filled with warm water (40–44°C) and connected to a 15ml tube. Sperm motility was checked right after collection by a stereomicroscope. Sperm concentration was also checked using a Burker chamber. Sperm selection to be used for the experiment was based on the concentration and the motility; thus, ejaculates that count $\geq 1.8\times10^9$ spermatozoa/ml and showing a motility of ≥70%, were selected.

2. Semen cryopreservation

First, a basic medium was prepared by dissolving 2.42 g TRIS base, 1.36 g citric acid, 1 g fructose, 100.000 IU penicillin G, 0.1 g streptomycin in 67.20 ml of bi-distilled water (ddH20) (pH = 6.7–6.8). The medium was equally divided into 2 volumes of 33.60 ml each. Each volume served to prepare Medium A (30°C medium) and Medium B (4°C medium) separately in 15 ml tubes. For medium A, 10 ml of egg yolk and 6.40 ml of ddH20 were added and for Medium B, 10 ml of egg yolk plus 6.40 ml of glycerol. Each tube was kept at 30 and 4 °C, respectively, before use. Shortly after sperm collection, Medium A was poured to the ejaculate and transferred immediately to a cold room (4 °C) for 2 hours to enable a controlled cooling - from 30 to 4 °C. Then, Medium B was gently poured to the suspension which was kept for additional 2 hours in the cold room. Equal volumes of both mediums were added to ensure a final dilution of the ejaculate to a concentration of 400×10^6 spermatozoa/ml. Tubes were gently mixed each 30 minutes by rotating the plugged flask 180 degrees. The suspension was divided into 250 µl plastic straws which were sealed with polyvinyl alcohol (PVA) and placed on a metallic grid to stabilize for 2 hours at 4 °C. Finally, the sealed straws were exposed to Liquid Nitrogen (LN) vapors (−80°C) in a Dewar flask initially for 6 min, then plunged into LN to be stored in filled tanks with LN until use.

3. Semen freeze-drying

Freeze-drying spermatozoa was performed as previously described by Anzalone et al. (2018). Briefly, semen was transferred into a polypropylene tube which contained 1 ml of CZB medium supplemented with 10% fetal bovine serum. Semen was incubated for 30 min at 37.5°C, then the upper 0.3- 0.5 ml of the sperm suspension was discarded. Semen was diluted in freeze-drying medium [1 ml 0.5 M TRIS (in water), 5ml 0.5MEGTA (in water), 2.5 ml 1MNaCl (in water)], after that an aliquot of 100 µL of the sperm suspension was put inside a 2 ml ampule which was directly plunged into liquid nitrogen for 10 min. After semen freezing, the vials were placed into Freeze-Dry apparatus (SP Scientific-VirTis, 2.0 BenchTop) with the condenser at a temperature of -58°C and the freeze-drying chamber at -12°C. After 12 h approximatively, each ampule was sealed under vacuum condition (pressure 15 mBar). Vials were individually wrapped with aluminum foil and stored at room temperature until use (figure 7).

Figure 7. Freeze-dried spermatozoa stored at room temperature in glass vials.

III. In vitro fertilization (IVF)

Twenty-four hours post IVM, only mature oocytes (MII) with expanded cumulus and normal morphology were selected for *in vitro* fertilization (IVF) (figure 8). Expanded Cumulus-Oocytes-Complexes (COCs) were mechanically deprived by the majority of cumulus cells by repeated pipetting in 300 U/mL hyaluronidase dissolved in Hepes buffered M199. A total of 287 oocytes were used for IVF, 220 oocytes were dedicated for the sperm aster immunofluorescence and 67 oocytes were maintained in culture for embryo development assessment. Groups of 10 oocytes were put in 50 µL drops of IVF medium composed by Synthetic Oviductal Fluid (SOF) medium, plus 20% estrus sheep serum and 16 µM isoproterenol and covered by mineral oil (figure 9A). In parallel, frozen ram semen was rapidly thawed in 35°C water and centrifuged in bicarbonate-buffered SOF containing 0.4% BSA at 112 × g for 5 min. Then, supernatant was discarded and 1.3 to 2 µL of pellet (± 5× 10^6 spermatozoa/ml) were aspirated and transferred into oocytes containing drops (figure 9B). Gametes were co-incubated in a humidified atmosphere at 38.5 °C, 5% CO_2, and 7% O_2, at least for 3 hours and a half to allow fertilization (figure 9C). Then to check the status of microtubule formation, IVF has been stopped at different time points and processed for immune-detection of α-tubulin, in line with the experimental design.

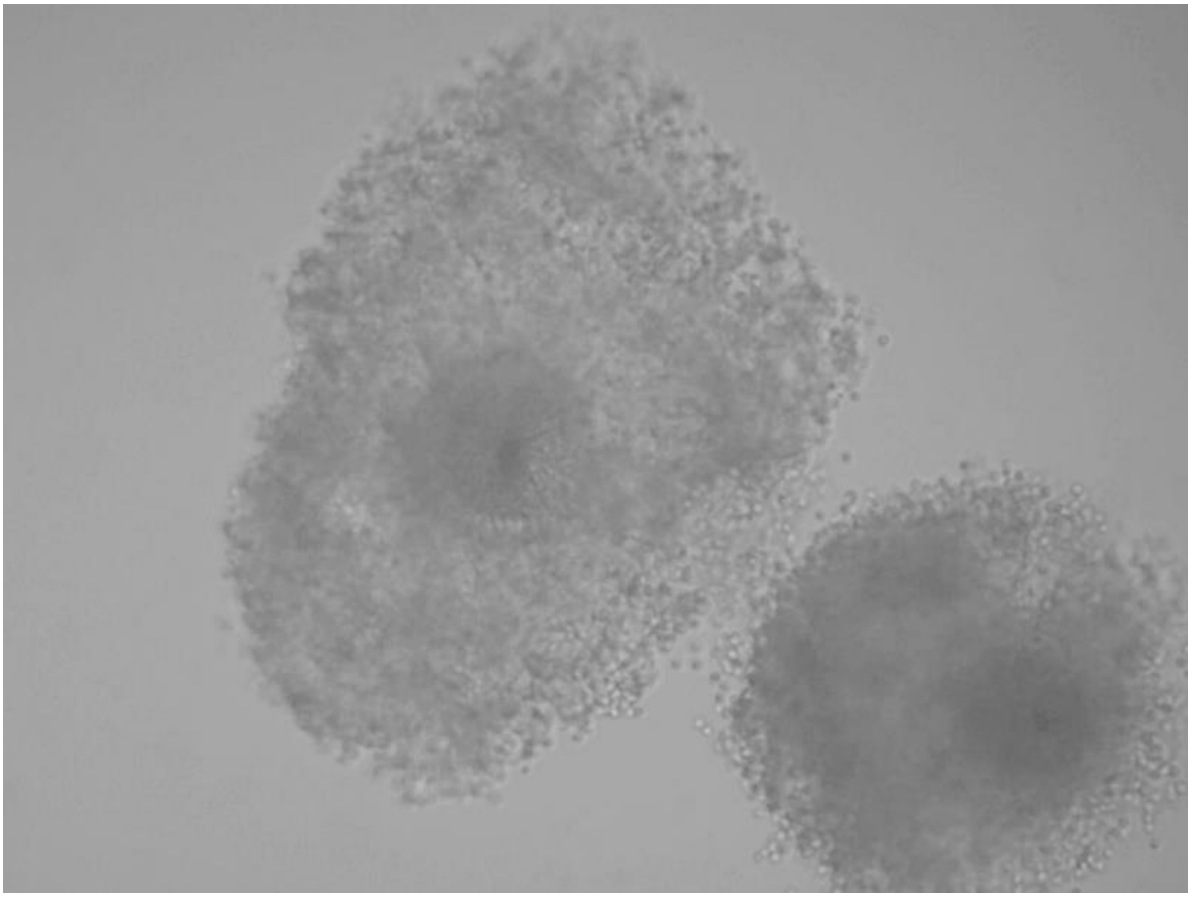

Figure 8. Matured sheep oocytes, with an expanded cumulus

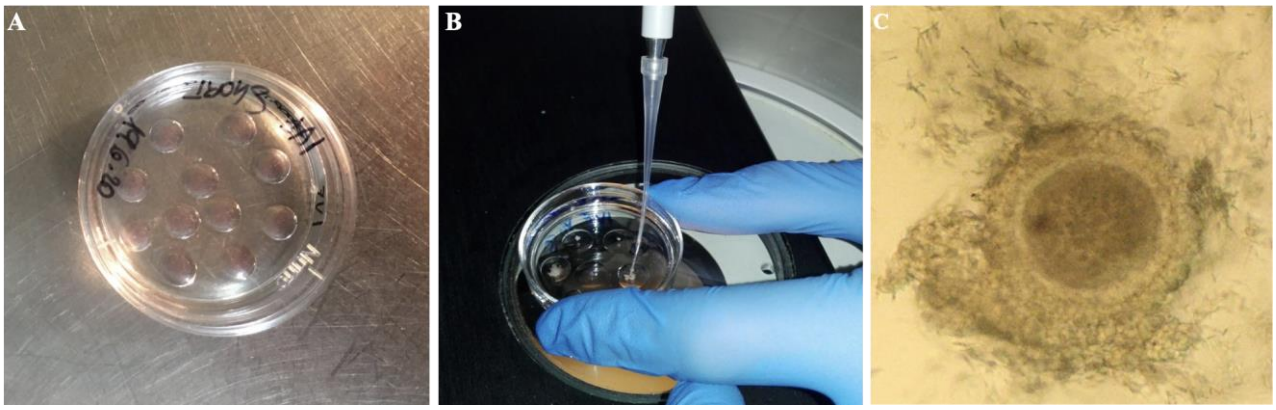

Figure 9. *In vitro* fertilization (IVF). Oocytes containing drops of IVF medium (A) into which washed semen were transferred (B), incubation of the oocytes with spermatozoa (C).

IV. Intracytoplasmic sperm injection (ICSI)

At 24 hours from IVM, a total of 290 matured (MII) oocytes were completely denuded from cumulus cells by pipetting in 300 U/mL hyaluronidase dissolved in Hepes buffered M199. One part of the oocytes (157 oocytes), were injected with FT (87 oocytes) and FD semen (70 oocytes) to allocated to sperm aster immunofluorescence detection. Second part of the oocytes (133 oocytes) were allocated to embryo development as described below. ICSI procedure with frozen/thawed and freeze-dried semen was performed as described by Anzalone et al. (2016-2018) and below.

1. Sperm preparation

1.1.For frozen/thawed sperm

Frozen/thawed semen (FT) was fast-thawed by immersing the straw in 35°C water for a few seconds. A volume of 5 µl of semen was diluted in 100 µl of H199 medium, then the mixture was diluted 1:1 with 12% PolyVinylPyrrolidone (PVP, 360 kDa) and 10 µl drop of this final dilution was placed on the lid of a Petri dish, on a warmed microscope stage covered by mineral oil.

1.2.For freeze-dried sperm

Freeze-dried spermatozoa (FD), were rehydrated by adding 100 µl of bi-distilled water then 5 µl aliquot was suspended in 100 µl of H199 medium and diluted with PVP as described previously for frozen spermatozoa.

2. ICSI procedure

A 50 µl drops containing 10 oocytes, and two drops of PVP with FT and FD spermatozoa suspensions were prepared for ICSI. Spermatozoa were loaded into a suitable injection pipette and submitted to few piezo pulses before ICSI to dissect the tail from the head; only the head was injected into the oocytes. The injection was performed under an inverted microscope (Nikon Eclipse E-800) connected to a micromanipulation system (Narishige, Tokyo, Japan), using a piezo-driven system (PiezoXpert, Eppendorf, Milan, Italy). After ICSI, oocytes underwent a

recovery of 5 minutes in H199, then were artificially activated by culturing them in 5 µM ionomycin dissolved in H199 plus 0.4% BSA for 5 minutes; then washed 5 minutes and placed in embryo culture dish. A schematic representation (figure 10) summarizes ICSI procedure using only the sperm head.

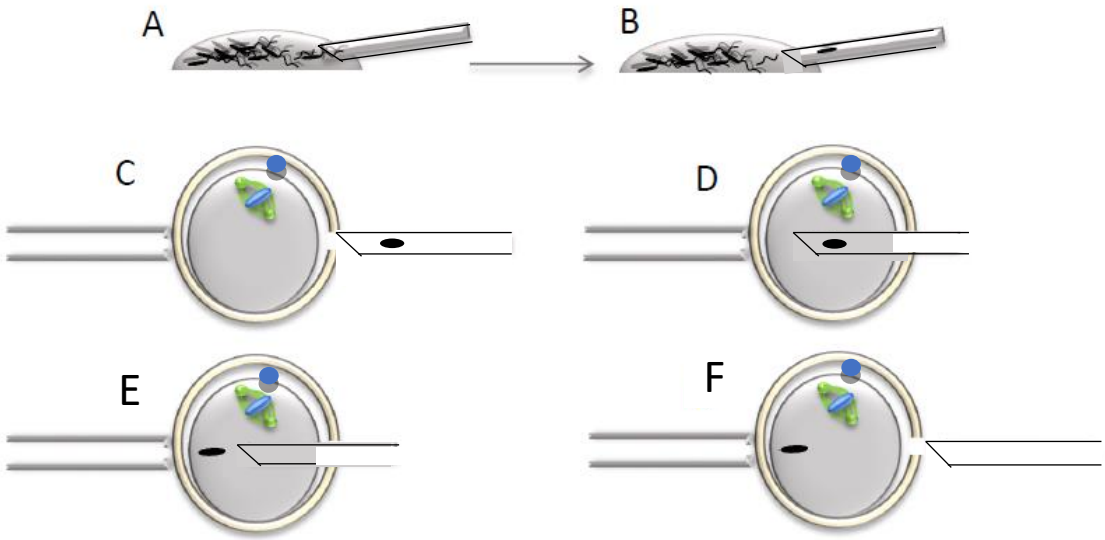

Figure 10. Schematic representation of ICSI procedure. The sperm in suspension with PVP drop is pulled into the injection from the tail until the neck level (A). The application of 1 to 2 piezo pulses enables the head detachment (B). The sperm head is aspirated into the injection pipet then brought near the oocyte and the application of 2 to 3 piezo pulses creates an opening into the zone pellucida first (C) and through the plasmatic membrane after (D). The sperm head is then released in the ooplasm (E) and the injection pipet is removed (F).

V. Embryo culture

Presumptive zygotes, resulting from ICSI with FT (ICSI-FT), from ICSI with FD (ICSI-FD) and from IVF, were cultured in groups of 5 in 20 µl drops of SOF medium with 2 % (v:v) basal medium Eagle (BME)-essential amino acids (EAA), 1 % (v:v) minimum essential medium (MEM)-nonessential amino acids (NEAA) (Gibco), 1 mM glutamine, and 8 mg/mL fatty acid-free BSA, covered by mineral oil in a humidified atmosphere at 38.5 °C, 5 % CO_2, and 7 % O_2.

For ICSI-FT, ICSI-FD and IVF presumptive zygotes which were maintained in culture, the medium was refreshed at day 3 (SOF- supplemented with 0.27 mg/mL glucose (SOF+), 2% EAA, 1% NEAA), day 5 (SOF+ with 10% of charcoal stripped FBS (cs-FBS), 2% EAA, 1% NEAA); and day 6 (1:1 MEM/M199 enriched with 10% cs-FBS, 2.5 µg/mL gentamicin and 1% sodium pyruvate) until day 7 or 8 of culture. Embryo development was assessed 24 hours post-culture for the cleavage (2 cell-stage) and at day 7 or 8 post-culture for blastocyst formation.

VI. Immunofluorescence detection of tubulin

Presumptive zygotes were fixed at different time points after sperm-egg co-culture starting from 1 to 2 hours till 9 hours post-fertilization (*pf*), for IVF derived embryos, and post-activation (*pa*) for IVF derived embryos, or post-activation (pa), for ICSI derived zygotes. Zygotes were treated with acid Tyrode's solution (pH 2.4) for 30 sec followed by 30 sec incubation in 0.5% Pronase to dissolve the ZP. Zone free zygotes were fixed in 4 % paraformaldehyde (PFA) in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM MgSO 4 ·7H 2 O, pH 6.9) (Riris et al., 2013) for 20 min then washed in PBS with 0.4% PVP, and next permeabilized for 15 min with 0.5% Triton X-100 in PHEM. After four washes (5 min each) in PHEM with 0.05% Tween 20, embryos were blocked in 1% BSA in PHEM with 100 mM glycine at room temperature for 1 h. Zygotes were incubated in mouse monoclonal IgG anti-a-tubulin from Santa Cruz (sc-23949 Lot #C0718) (1:200 in 1% BSA/PHEM with 100 mMglycine) overnight at 4°C. After four washes in PHEM with 0.05% Tween 20, the zygotes were incubated with FITC-conjugated goat-anti-mouse IgG (Sigma F5262 Lot # 057M4883V) and/or rabbit anti-goat IgG-PF from Santa Cruz (sc-3755, Lot #I2503) (1:200 in 1% BSA/PHEM with 100 mM glycine) for 45 min

at room temperature. DNA was counterstained for 5 min in 1 mg Hoechst 33,342 in 10 ml of PBS at room temperature. Then slides were mounted, and images were captured using a confocal microscope (Nikon Eclipse Ti-E).

VII. Pronuclear detection

The formation of 2 pronuclei (2PN) was checked in all experimental groups (ICSI-FT, ICSI-FD and IVF). IVF group was considered as control, which is supposed to show 2 PN at 10 h pf the syngamy at 22 h pf. To this extend, a total of additional 90 fertilized oocytes (30 ICSI-FT, 30 ICSI-FD and 30 IVF) were carefully cleaned from cumulus cells and incubated in 5 min 1 µg Hoechst 33,342 in 10 mL of embryo culture medium. Oocytes were observed one by one under inverted microscope connected with a micromanipulator system, at 10 hours *pf*/*pa* first, for the 2PN assessment, and later at 22 hours *pf/pa*, for syngamy stage assessment.

VIII. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6.0 software. Kruskal-Wallis test was applied to compare in vitro embryo development on a minimum of 5 replicates for each group (ICSI-FT, ICSI-FD and IVF). The considered data in each group were the number of the cleaved embryos and the blastocyst number in each group, a multiple comparison was done between groups.

Statistical significance threshold has been set with $P < 0.05$. Chisquare test was applied to compare 2 PN formation and early syngamy event. The accurate number of oocytes showing 2 PN and syngamy events were the considered data. ICSI groups were compared to IVF. ICSI-FT and ICSI-FD groups were also compared between each other. Statistical significance threshold has been set with $P < 0.01$.

Chapter III

Results

1. Sperm evaluation

After rehydration, freeze-dried spermatozoa exhibited the 58.5% of external membrane damaged (valuated by Pisum sativum agglutinin-PSA). The percentage of intact DNA was similar as reported in our previously work (Palazzese et al., 2018) with an estimation of 9.9%, analyzed by Comet-assay.

2. Sperm aster nucleation and microtubular dynamics

Research on microtubular dynamic in sheep fertilization are missing and data regarding the timing of aster nucleation are not available yet. In some preliminary experiments, we wet a series of conventional IVF assays according to which we have observed that the spermatozoon takes between 3 to 4 hours following sperm-egg co-incubation to get in contact with the oocyte, fuse with its plasma membrane and finally incorporate into the ooplasm. Considering our observations, we initiated evaluating the sperm aster nucleation between 4 to 5 hours from sperm-egg co-culture, which corresponds to 1 to 2 hours *pf* in IVF embryos. For ICSI embryos, giving the fact that the spermatozoon is directly injected into the ooplasm, we started assessing aster nucleation from the moment of the egg activation.

2.1.ICSI presumptive zygotes

2.1.1. ICSI with frozen/thawed semen

In ICSI-FT zygotes, we started assessing the sperm aster activities from 1 to 2 hours *pa*. We have observed that zygotes completed the II meiosis by 3h *pa* and simultaneously the proximal centriole initiated aster nucleation (figure 11A, arrow; figure 12A). A further microtubular growth was observed from 4 to 5-hour *pa* (figure 11B, arrow; figure 12B) with a marked increase by 8 to 9-hour *pa*, with microtubules radiating from the decondensed male pronucleus (figure 11C, arrow; figure 12C, figure 11D, arrow; figure 12D).

2.1.2. CSI with freeze-dried semen

In ICSI-FD zygotes, like in ICSI-FT, we started assessing the sperm aster activities from 1 to 2 hours *pa*. Unlike the previous observations, aster formation was delayed 1 hour (figure 11E,

arrow; figure 12E). In fact, aster nucleation starting only by 4h *pa* (figure 11F, arrow; figure 12F). From the 6th hour onward and once the nucleation started, the sperm aster followed the same dynamics for the upcoming hours, as previously described, marked by the microtubule elongation from 6 to 7 hours *pa* and microtubule enlargement from 8 to 9 hours *pa* (figure 11G-H, arrow; figure 12G-H).

2.2.IVF presumptive zygotes

In IVF zygotes, the observed trend overlapped with ICSI-FT. As a matter of fact, within 3h *pf* the oocytes were observed to complete II meiosis with a simultaneous nucleation of the proximal centriole aster (figure 11I, arrow; figure 12I). Further microtubular growth was observed from 4 to 5-hour *pf* (figure 11J, arrow; figure 12J) with an increased microtubular growth 8 to 9 hours *pf*, (figure 11K, arrow; figure 12K, figure 11L, arrow; figure 12L).

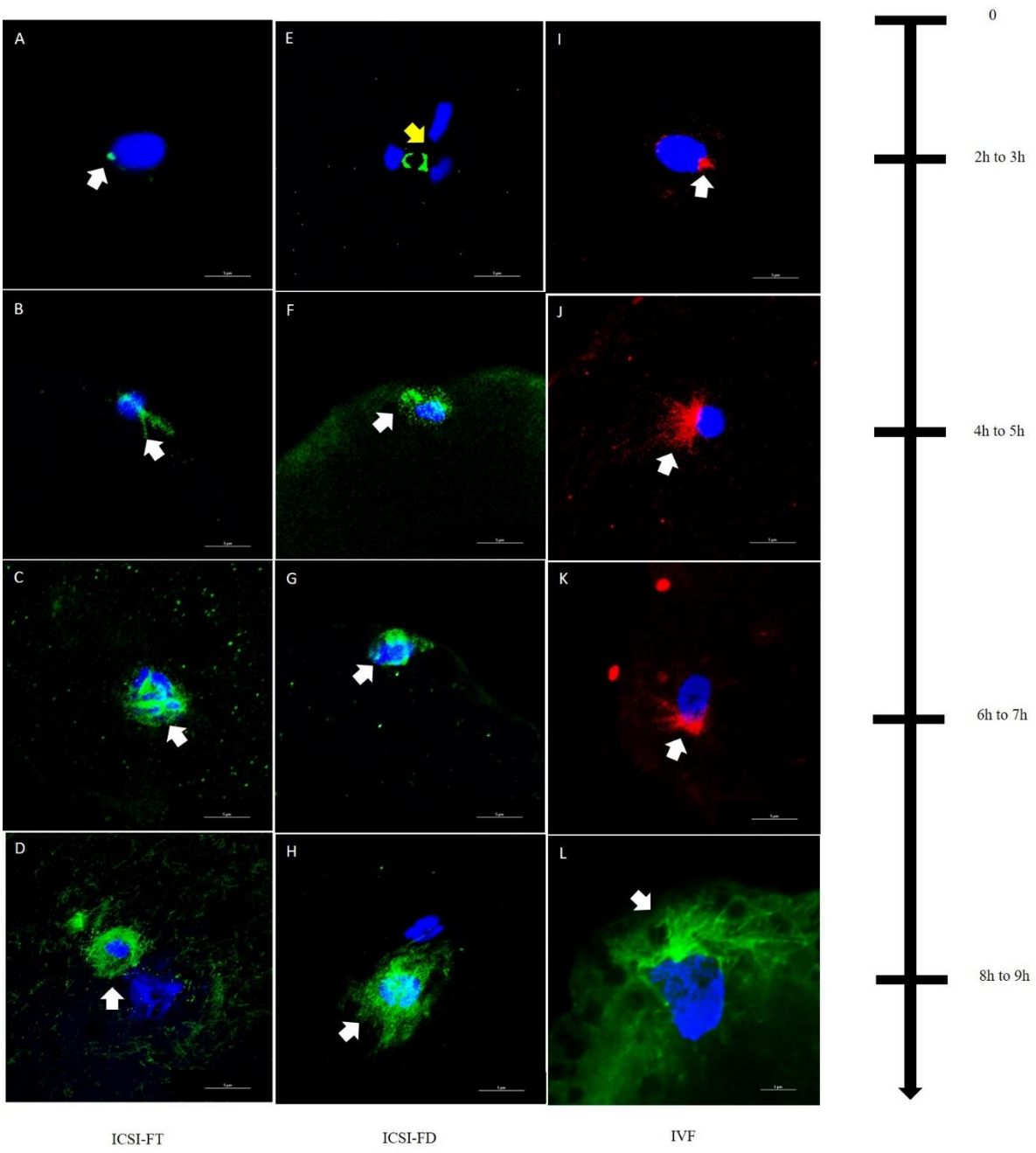

Figure 11. Sperm aster nucleation dynamics in ICSI-FT (A – D); ICSI-FD (E – H) and IVF (I – L) presumptive zygotes after 2-3 hours *pf/pa* (A-I), 4-5 hours *pf/pa* (B-J), 6-7 hours *pf/pa* (C-K), 8-9 hours *pf/pa* (D-L). Arrows: sperm aster nucleation and microtubule elongation; yellow arrow: absence of sperm aster nucleation. Red/green:α– tubulin; blue – nucleus/Hoechst. Red color: zygotes were incubated with rabbit anti-goat IgG-PF from Santa Cruz (sc-3755, Lot #I2503). Green color: zygotes were incubated with FITC-conjugated goat-anti-mouse IgG (Sigma F5262 Lot # 057M4883V).

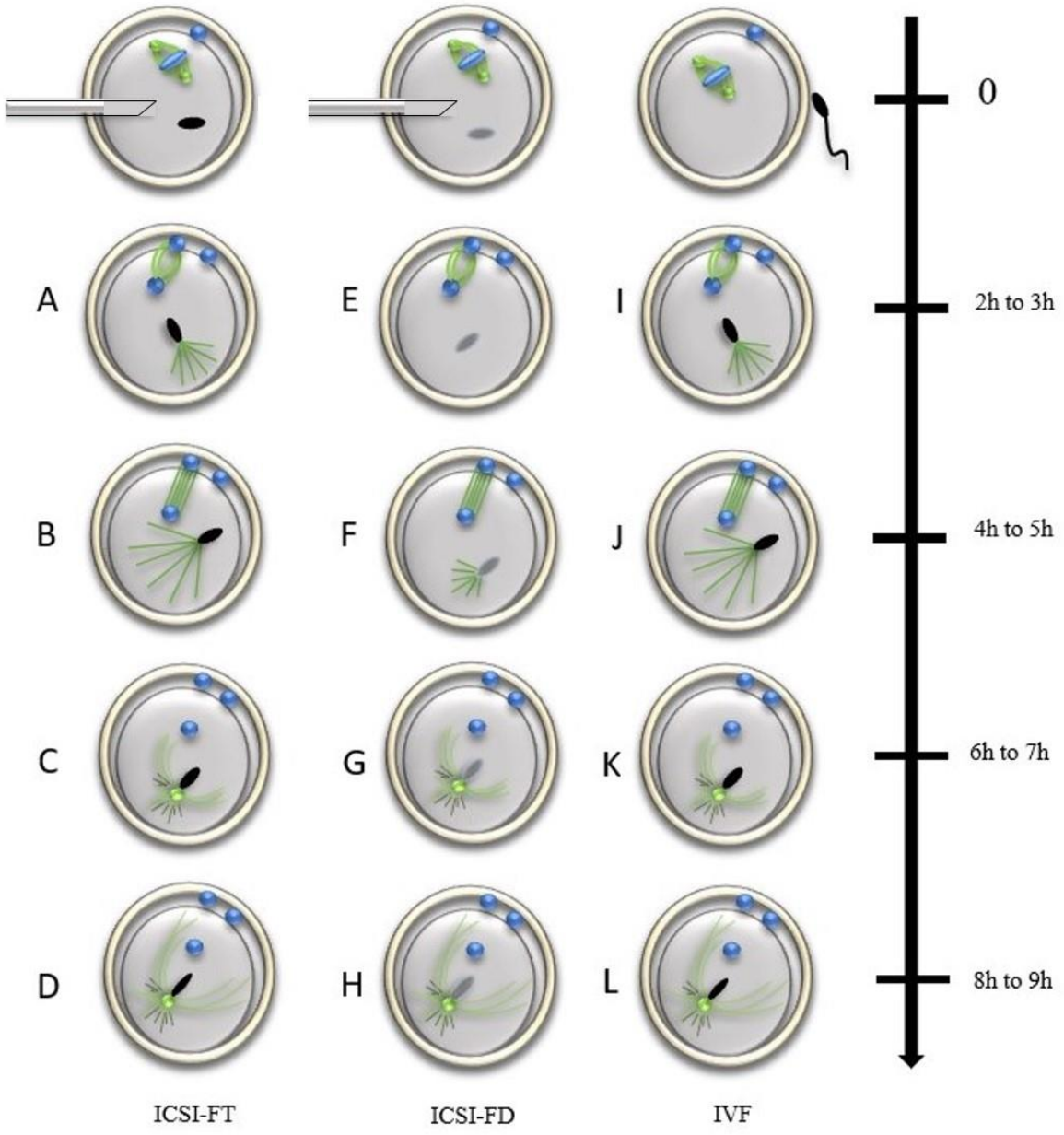

Figure 12. Schematic representation of the sperm aster nucleation dynamics in ICSI-FT, ICIS-FD and IVF zygotes when fertilization beginning (time 0), at 2-3h, 4 -5h and 6 -9 h post-fertilization.

3. Pronuclear stage and syngamy assessment

Subsequent embryo development was predicted in accordance to the 2PN stage and syngamy event at 10 hours *pf*/*pa* and 22 hours *pf/pa*, respectively. At 10 hours *pf*/*pa*, we have found that the percentage of embryos which were at the 2PN was not different in ICSI-FT and ICSI-FD, respectively 47% (14/30 zygotes) and 53% (16/30 zygotes) while73% (22/30) were at the 2PN stage in IVF zygotes (Table 1) (IVF vs ICSI-FT, P<0.001).

Around 22 hours *pa*, syngamy was detected only in 28.5% (4/14) of ICSI-FT and 12.5% (2/16) of ICSI-FD zygotes while a higher proportion of ICSI zygotes, respectively 71.5 % (10/14) for ICSI-FT and 87.5% (14/16) of ICSI-FD were found blocked at the pronuclear stage.

The picture changed radically in IVF zygotes, where half 50% (11/22) completed syngamy, and half 50% (11/22) were found blocked at 2PN stage (IVF vs ICSI-FD, P<0.001).

Table 1. Pronuclei stage and syngamy assessment in ICSI-FT, ICSI-FD and IVF embryos.

Groups	No. oocytes	PN (%)	Syngamy (%)
ICSI-FT	30	14/30 (47%)[a]	4/14 (28.5%)
ICSI-FD	30	16/30 (53%)	2/16 (12.5%)[b]
IVF	30	22/30 (73%)	11/22 (50%)

[a]IVF vs ICSI-FT, P<0.001
[b]IVF vs ICSI-FD, P<0.001

4. Embryo development assessment

Embryo development was followed and recorded for the different groups which is summarized in Table 2.

Table 2. Embryo development outcomes from ICSI-FT and ICSI-FD and IVF.

Groups	No. oocytes	2-Cells (%)	Blastocysts (%)
ICSI-FT	56	19/56 (34%)	11/56 (19%)[a]
ICSI-FD	77	24/77 (31%)	8/77 (10%)[b]
IVF	67	30/67 (44%)	22/67 (33%)

[a]IVF vs ICSI-FT, $P<0.05$
[b]IVF vs ICSI-FD, $P<0.005$

No significant differences were found in the cleavage rate between the different groups (31% vs 34% and 44%, respectively).

Regarding the blastocyst rate, the percentage of the ICSI-FT and ICSI-FD were statically lower than the IVF group (19% and 10% vs 33%, respectively; IVF vs ICSI-FT, $P<0.05$; IVF vs ICSI-FD, $P<0.005$). Moreover, the lowest blastocyst rate corresponded to ICSI-FD zygotes. The obtained trend explains that blastocyst development is more impaired in ICSI-FD derived embryos.

Chapter IV

Discussion

In human, various ICSI failure were associated to sperm aster abnormalities and irregular microtubule organization (Rawe et al., 2000; Nakamura et al., 2002). Sperm aster nucleation and microtubule dynamics are crucial post-ICSI events but their involvement in ICSI failure is insufficiently documented, especially in ruminants (Hochi, 2016). This background prompted us to investigate whether sperm centrosome dysfunction could be the main factor undermining the development of ICSI derived, sheep embryos.

Our findings on the control IVF and ICSI injected oocytes with frozen/thawed spermatozoa have demonstrated that aster nucleation and microtubular dynamics are practically synchronous, and the delay recorded in ICSI FD ones is promptly recovered. It seems likely that the structural modifications induced by the de-hydration might have rendered the centrosome less accessible to the oocyte's reducing cytoplasm, a fundamental requirement for the acquisition of microtubule nucleating activity (Simerly et al., 2000). These findings differ from a similar study performed by Hara et al. (2011) on ICSI derived bovine embryos injected with freeze-dried bull spermatozoa. In their experiments, they have noticed that the sperm aster nucleation activity is significantly lower in ICSI derived embryos using both frozen/thawed or freeze-dried spermatozoa. Thus, they have concluded that the freeze-drying process for bull spermatozoa has no negative effects on sperm aster formation and microtubule network assembly. They also observed a significant difference in microtubule network function between ICSI and IVF embryos, suggesting that this might be responsible for the lower blastocyst yield in ICSI derived embryos compared with IVF ones.

Our data indicate instead that microtubule nucleation is retarded only in ICSI FD zygotes, but once started, it takes place normally, just like the IVF and ICSI FT ones. Therefore, we have objectively demonstrated that aster formation and microtubular dynamics are normal in sheep ICSI zygotes, which means that other factors are responsible for the reduced development of sheep ICSI-derived embryos.

Thus, while we rule out centrosome dysfunction as major determinant in ICSI's low efficiency, highlighting observations can be drown from our assessments of pronuclear stage and the syngamy in ICSI-produced zygotes. The formation of 2 pronuclei is a good prognostic for further

embryo development, because studies have shown that the morphological status of the 2 pronuclei is correlated with the subsequent cleavage of the embryo, blastocyst development and quality (Balaban, 2004).

Our findings have revealed that an enormous proportion of ICSI zygotes, 71% and 87.5% respectively in ICSI-FT and ICSI-FD, failed to achieve syngamy, despite the formation of the two pronuclei in 47% and 53% of ICSI-FT and ICSI-FD zygotes, respectively, and remained blocked at the pronuclear stage. The picture instead changed radically in IVF zygotes, where half of the zygotes successfully undergo syngamy. These data confirm unpublished observation in our laboratory that have indicated an asymmetry between the activation, judged on the pronuclear detection (80% in average), and the cleavage rate, lower (30% in average, Anzalone personal communication).

Pronuclear apposition and fusion of their nuclear envelope is a key event for embryo development. However, the double membrane layer of the two pronuclei makes this phenomenon a complicated event, which is poorly understood. Recent data have indicated that vesicles structures, localized at the pronuclear interface are the initial steps that triggers nuclear membrane fusion by pronuclear apposition in preparation of the first mitosis (Rahman et al. 2020). Thus, according to this new model, nuclear membrane fusion, promoted by Ca^{2+} ions, promotes pronuclear apposition (Ma and Starr, 2020). A series of Ca^{2+} spikes induced by sperm soluble factor, identified as PLCzeta (zeta) (Swann et al., 2016) but known also as “sperm factor” or “sperm-borne oocyte-activating factor”.

This protein is responsible for switching on an oscillatory calcium release within the oocyte, that is fundamental for oocyte activation. Once activated, the oocyte, in turn, displays a series of calcium waves that trigger the sperm centrosome phosphorylation, resulting in its activation, as reported by Simerly et al. (2000) and Schatten et al. (1994). Even though we did not visualize the early Ca^{2+} spikes (our laboratory is not equipped for that), the early centrosome dynamics indirectly suggest that they are in place. What appear missing in our ICSI zygotes are the late Ca^{2+} spikes, preparatory for pronuclei apposition/fusion. Of interest for our observations, recent data demonstrated that prolonged Ca^{2+} oscillations might be due to the presence of a secondary source/mechanism for late Ca^{2+} oscillations, important also for pronuclear fusion

(Swann, 2020). It is likely that the late Ca^{2+} spiking is missing in ICSI embryos Jones (2007; 2018); if proven correct, this might explain the dramatically reduced syngamy in ICSI embryos, an event that might activate the G2/M phase arrest, and thereby lack of mitosis, with early embryo development arrest, as reported by Wang et al. (2003).

This work is a part of our efforts undertaken to optimize ICSI in sheep. The main message our data conveys is that, while centriole and microtubular dynamics are normal in sheep ICSI zygotes, ruling out previous reports (Manandhar et al., 2005; Schatten and Sun, 2009; Hoyer-Fender, 2012), we have identified the major roadblock that hampers ICSI embryo development: a dramatic reduction of syngamy. These findings call for a radical revision of the activation procedures of sheep oocytes. The current procedures rely on ionophores, a rather dated approach (Loi et al., 1998) that our and published data has found unfit for sheep ICSI derived embryos. A freshly published approach by leading scientist working on mice (Hirose and Wakayama 2020) has demonstrated that activation with the co-injection of PLCζ-cRNA of oocytes injected with round spermatid or somatic cell nuclear transfer resulted in higher frequencies of development, comparing with the controls. We believe that time is ready to challenge the activation protocols in sheep oocytes, and we will embark on this endeavour in our further research.

Chapter V

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Chapter VI

Annexes

The aim focus of the thesis was dedicated to explore the functionality of the sperm centrosomal material in the *in vitro* produced embryos by Assisted reproduction (ART) techniques.

The research activities of the main focus of the thesis led to the successive scientific publication:

- **Yosra Ressaissi**, Debora Agata Anzalone, Luca Palazzese, Marta Czernik, Pasqualino Loi. 2021. The impaired development of sheep ICSI derived embryos is not related to centriole dysfunction. Theriogenology 159: 7-12. doi.org/10.1016/j.theriogenology.2020.10.008

Although the majority of the period of the doctoral program was dedicated to the previously mentioned theme, I had the opportunity to work in collaboration with the partner institutes designated in my Career Development Plan (CDP) and to explore other scientific "nuances" always linked to reproductive medicine.

The idea was to investigate alternative procedures that could be useful to optimise *In vitro* embryo production procedure and which might be of interest for further implementation in *In vitro* sheep assisted reproduction technologies. The scope was on studying and characterizing Extracellular Vesicles (EVs) produced by *in vitro* cultured preimplantation bovine embryos. The main studied topic was to assess the effect of supplementation of bovine embryo-derived EVs on the early development of *In vitro* cultured bovine embryos.

The research activities within this collaboration, in which I actively contributed in the preparation and execution of the experiment led to the successive scientific publications:

- Keerthie Dissanayake, Monika Nõmm, Freddy Lättekivi, **Yosra Ressaissi**, Kasun Godakumara, Arina Lavrits, Getnet Midekessa, Janeli Viil, Rikke Bæk, Malene Møller Jørgensen, Sourav Bhattacharjee, Aneta Andronowska, Andres Salumets, Ülle Jaakma, Alireza Fazeli. 2020. Individually cultured bovine embryos produce extracellular vesicles that have the potential to be used as non-invasive embryo quality markers. Theriogenology 149: 104-116. doi.org/10.1016/j.theriogenology.2020.03.008
- **Yosra Ressaissi**, Keerthie Dissanayake, Monika Nõmm, Janeli Viil1, Andres Salumets, Ülle Jaakma, Aneta Andronowska, Pasqualino Loi, Alireza Fazeli. Assessing the effects of bovine embryo-derived extracellular vesicles on the development of individually

cultured bovine embryos. Poster presentation. CellFit annual meeting, Athens, Greece (10-12 October 2019).

Annex I

The impaired development of sheep ICSI derived embryos is not related to centriole dysfunction

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The impaired development of sheep ICSI derived embryos is not related to centriole dysfunction

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ABSTRACT

While intracytoplasmic sperm injection (ICSI) is an asset in human Assisted Reproduction Technologies (ART), its outcomes, in terms of blastocyst, is still unacceptably low in ruminants. The picture typically found in ICSI derived bovine and ovine embryos is an asymmetry between a high activation rate, marked by a pronuclear development, and a low first cleavage rate. Abnormal centriole function has been indicated as a possible factor which undermines embryonic development following ICSI, especially when Freeze Dried spermatozoa (FD) are used. In order to verify the hypothesis that centriole dysfunction might be responsible for low ICSI outcomes in sheep, we have investigated micro-tubular dynamics, markedly aster nucleation, in fertilized sheep zygotes by ICSI with frozen/thawed (FT) and FD spermatozoa; In Vitro Fertilized (IVF) sheep oocytes were used as control. The spermatozoa aster nucleation was assessed at different time points following ICSI and IVF by immune-detection of α-tubulin. Pronuclear stage, syngamy and embryo development were assessed. No difference was noticed in the timing of aster nucleation and microtubule elongation in ICSI-FT derived embryos with control IVF ones, while a delay was recorded in ICSI-FD ones. The proportion of 2-pronudear stage zygotes was similar in ICSI-FT and ICSI-FD (47% and 53%, respectively), both much lower comparing the IVF ones (73%). Likewise, syngamy was observed in a minority of both ICSI groups (28.5% vs 12.5% in ICSI-FT/FD respectively) comparing to IVF controls (50%), with a high number of zygotes blocked at the 2-pronuclear stage (71.5% vs 87.5% respectively). While no significant differences were noticed in the cleavage rate between ICSI-FD, ICSI-FT and IVF groups (31%, 34% and 44%) respectively, development to blastocyst stage was markedly compromised in both ICSI groups, especially with FD spermatozoa (10% in ICIS-FD and 19% in ICSI-FT vs 33% in IVF ($P < 0.005$, ICSI-FD vs IVF and $P < 0.05$, IVF vs ICSI-FT, respectively). Hence, here we have demonstrated that the reduced cleavage, and the ensuing impaired development to blastocysts stage of ICSI derived sheep embryos is not related to centriole dysfunction, as suggested by other authors. The major recorded problem is the lack of syngamy in ICSI derived zygotes, an issue that should be addressed in further studies to improve ICSI procedure in sheep embryos.

1. Introduction

ICSI was introduced to reduce *in vitro* fertilization failures and mainly to treat severe male infertility. The technique was originally mastered in human [1] and has been successfully applied in other species such as cow [2], sheep [3], horse [4] and goat [5]. ICSI has been widely used as a standard fertilization technique, but also as a relevant tool to understand fundamental biological questions like cell cycle control, molecular mechanisms of oocyte activation, male pronucleus transformation as well as early embryo development events [6]. Despite the increased use of the technique and its potential advantages, severe limitations are still encountered in terms of its application in many species, particularly in ruminants. As a matter of fact, ICSI derived embryos in ruminants display a considerably lower developmental rate compared to IVF ones; and several factors need to be taken into account in order to justify this failure. For instance, the persistence of the spermatozoa membrane, and consequently the acrosome, are thought to be the major determinants in compromising embryo development, as they were found to cause ineffective sperm oocyte-activation [7,8]. Likewise, atypical and incomplete calcium (Ca^{2+}) oscillations were described in ICSI bovine embryos using frozen bull semen [9–12]. The same

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findings have been reported in sheep as well [13–15]. To overcome these limitations, while partially mimicking natural fertilization, chemical treatments using some detergents were proposed to remove the spermatozoa membranes prior ICSI. Along with this line, it has been also found that mechanically damaged ram spermatozoa prior ICSI enhance embryonic development until the blastocyst stage [15]. However, despite these expedients, *in vitro* embryonic development following ICSI remains still low in sheep and others ruminants [13]. Previous studies in our laboratory have revealed an asymmetry between the activation, based on the pronuclear detection (80% in average), and the cleavage rate, lower (30% in average). In the same study, freeze-dried spermatozoa were suggested to be used in ICSI procedure as an eventual procedure for fertility preservation [17]. In human, most ICSI failures were associated to post-ICSI events and the related molecular mediators [18]. Sperm aster abnormalities and irregular microtubule organization, in particular, were identified as causal factors of developmental restrain following ICSI [19,20]. In fact, sperm aster nucleation and microtubule dynamics are crucial post-ICSI events [21,22], however, their involvement in ICSI failure in ruminant embryos is insufficiently documented [18]. In this work we set up to investigate centriole, microtubule dynamics and developmental potential in zygotes obtained by ICSI-fertilized sheep eggs. Given that ICSI outcomes are particularly negative using freeze-dried spermatozoa, we decided to use them to exacerbate the problem, rendering it easier to detect.

2. Material and methods

All chemicals were purchased from Sigma (Milano, Italy), unless otherwise stated.

2.1. Sperm preparation

Semen was collected from adult fertile Sardinian rams using an Artificial Vagina. Ejaculates were selected for further processing based on their concentration and motility.

2.1.1. Spermatozoa cryopreservation

Initially, basic medium was prepared by dissolving 2.42 g TRIS base, 1.36 g citric acid, 1 g fructose, 100.000 IU penicillin G, 0.1 g streptomycin in 67.20 ml of bi-distilled water (ddH2O) (pH = 6.7–6.8), then equally divided into 2 vol of 33.60 ml each. Medium A (30 °C medium) and Medium B (4 °C medium) were prepared separately in each tube by respectively adding 10 ml of egg yolk and 6.40 ml of ddH2O and 10 ml of egg yolk plus 6.40 ml of glycerol. Each tube was kept at 30 and 4 °C, respectively, before use. Shortly after sperm collection, Medium A was poured to the ejaculate and transferred immediately to a cold room (4 °C) for 2 h to enable a controlled cooling - from 30 to 4 °C. Then, Medium B was gently poured to the suspension which was kept for additional 2 h in the cold room. Equal volumes of both mediums were added to ensure a final dilution of the ejaculate to a concentration of 400×10^6 spermatozoa/ml. Tubes were gently mixed each 30 min by rotating the plugged flask 180°. The suspension was divided into 250 μl plastic straws which were sealed with polyvinyl alcohol (PVA) and placed on a metallic grid to stabilize for 2 h at 4 °C. Finally, the sealed straws were exposed to Liquid Nitrogen (LN) vapors (−80 °C) in a Dewar flask initially for 6 min, then plunged into LN to be stored in filled tanks with LN until use.

2.1.2. Sperm freeze-drying

Freeze-dying spermatozoa was performed as previously described [17]. Briefly, semen was transferred into a polypropylene tube which contained 1 ml of CZB medium supplemented with 10% fetal bovine serum. Semen was incubated for 30 min at 37.5 °C, then the upper 0.3–0.5 ml of the sperm suspension was discarded. Semen was diluted in freeze-drying medium [1 ml 0.5 M TRIS (in water), 5 ml 0.5 M EGTA (in water), 2.5 ml 1 M NaCl (in water)], after that an aliquot of 100 μL of the sperm suspension was put inside a 2 ml ampule which was directly plunged into liquid nitrogen for 10 min. After semen freezing, the vials were placed into Freeze-Dry apparatus (SP Scientific-VirTis, 2.0 BenchTop) with the condenser at a temperature of −58 °C and the freeze-drying chamber at −12 °C.

After 12 h approximatively, each ampule was sealed under vacuum condition (pressure 15 μBar). Vials were individually wrapped with aluminum foil and stored at room temperature until use.

2.2. Oocyte recovery and in vitro maturation (IVM)

Sheep ovaries were collected from local slaughterhouses and transferred within 1–2 h at 37 °C to the laboratory. Aspiration of the oocytes from the follicles was done in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered TCM-199 medium with 0.005% heparin using 21 G needles. In Vitro Maturation (IVM) medium is composed by MEM-199 containing 2 mM glutamine, 0.3 mM sodium pyruvate, 100 μM cysteamine, 10% fetal bovine serum (FBS), 5 μg/ml follicle stimulating hormone FSH, 5 μg/ml luteinizing hormone (LH), and 1 μg/ml estradiol. Only oocytes with at least 2 or 3 layers of compact cumulus cells were selected for maturation. After 2 washes in the IVM medium, oocytes underwent maturation in 0.5 ml of IVM medium in a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 24 h.

2.3. In vitro fertilization (IVF)

Twenty-four hours post IVM, only mature oocytes (MII) with expanded cumulus and normal morphology were selected for *in vitro* fertilization (IVF). Expanded Cumulus-Oocytes-Complexes (COCs) were mechanically deprived from the majority of the cumulus cells by repeated pipetting in 300 U/mL hyaluronidase dissolved in Hepes buffered M199. A total of 287 oocytes were used for IVF, 220 oocytes were dedicated for the sperm aster immunofluorescence and 67 oocytes were maintained in culture for embryo development assessment. Groups of 10 oocytes were put in 50 μL drops of IVF medium composed by Synthetic Oviductal Fluid (SOF) medium, plus 20% estrus sheep serum and 16 μM isoproterenol and covered by mineral oil. In parallel, frozen ram semen was rapidly thawed in 35 °C water and centrifuged in bicarbonate-buffered SOF containing 0.4% BSA at 112 × g for 5 min. Then, supernatant was discarded and 1.3–2 μL of pellet (±5× 10^6 spermatozoa/ml) were aspirated and transferred into oocytes containing drops. Gametes were co-incubated in a humidified atmosphere at 38.5 °C, 5% CO_2, and 7% O_2, at least for 3.5 h to allow fertilization. IVF has been stopped at different time points and processed for immune-detection of α-tubulin, in line with the experimental design.

2.4. Intracytoplasmic sperm injection (ICSI)

At 24 h from IVM, a total of 290 matured (MII) oocytes were completely denuded from the cumulus cells by pipetting in 300 U/mL hyaluronidase dissolved in Hepes buffered M199. One part of the oocytes (157 oocytes), were injected with FT and FD semen (87 oocytes and 70 oocytes, respectively) and allocated to sperm aster immunofluorescence detection. Second part of the oocytes (133 oocytes) were allocated to embryo development as described below. ICSI with FT (ICSI-FT) and ICIS with FD spermatozoa (ICSI-FD) were performed as described by Ref. [15–17] and briefly below. FT were fast-thawed by immersing the straw in 35 °C water for a

few seconds and FD were rehydrated by adding 100 μL of bi-distilled water. For ICSI-FT and ICSI-FD, a volume of 5 μL of semen was diluted in 100 μL of M199 medium, then the mixture was diluted 1:1 with 12% PolyVinylPyrrolidone (PVP, 360 kDa). The injection was performed under an inverted microscope (Nikon Eclipse E−800) connected to a micromanipulation system (Narishige, Tokyo, Japan), using a piezo-driven device (PiezoXpert, Eppendorf, Milan, Italy. Immediately after ICSI, oocytes were artificially activated by culturing them in 5 μM ionomycin dissolved in M199 plus 0.4% BSA for 5 min; then washed 5 min and placed in embryo culture dish.

2.5. Embryo culture

Presumptive zygotes, resulting from ICSI and IVF, were cultured in groups of 5 in 20 μL drops of SOF medium with 2% basal medium Eagle (BME)-essential amino acids (EAA), 1% minimum essential medium (MEM)-nonessential amino acids (NEAA) (Gibco), 1 mM glutamine, and 8 mg/ml fatty acid-free BSA, covered by mineral oil in a humidified atmosphere at 38.5 °C, 5% CO_2, and 7% O_2.

For ICSI-FT, ICSI-FD and IVF derived embryos which were maintained in culture, the medium was refreshed at day 3 (SOF-supplemented with 0.27 mg/ml glucose (SOF+), 2% EAA, 1% NEAA), day 5 (SOF+ with 10% of charcoal stripped FBS (cs-FBS), 2% EAA, 1% NEAA); and day 6 (1:1 MEM/M199 enriched with 10% cs-FBS, 2.5 μg/ml gentamicin and 1% sodium pyruvate) until day 7 or 8 of culture. Embryo development was assessed 24-h post-culture for the cleavage (2 cell-stage) and at day 7 or 8 post-culture for blastocyst formation.

2.6. Immunofluorescent detection of tubulin

Presumptive zygotes were fixed at different time points after sperm-egg co-culture starting from 1 to 2 h till 9-h post-fertilization (pf), for IVF derived embryos, or post-activation (pa), for ICSI derived zygotes. Zygotes were treated with acid Tyrode's solution (pH 2.4) for 30 s followed by 30 s incubation in 0.5% Pronase to dissolve the ZP. Zone free zygotes were fixed in 4% paraformaldehyde (PFA) in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM MgSO 4 ·7H 2 O, pH 6.9) [23] for 20 min then washed in PBS with 0.4% PVP, and next permeabilized for 15 min with 0.5% Triton X-100 in PHEM. After four washes (5 min each) in PHEM with 0.05% Tween 20, zygotes were blocked in 1% BSA in PHEM with 100 mM glycine at room temperature for 1 h. Zygotes were incubated in mouse monoclonal IgG anti-α-tubulin from Santa Cruz (sc-23949 Lot #C0718) (1:200 in 1% BSA/PHEM with 100 mM glycine) overnight at 4 °C. After four washes in PHEM with 0.05% Tween 20, the zygotes were incubated with FITC-conjugated goat-anti-mouse IgG (Sigma F5262 Lot # 057M4883V) and/or rabbit anti-goat IgG-PF from Santa Cruz (sc-3755, Lot #I2503) (1:200 in 1% BSA/PHEM with 100 mM glycine) for 45 min at room temperature. DNA was counterstained for 5 min in 1 μg Hoechst 33,342 in 10 ml of PBS at room temperature. Then slides were mounted, and images were captured using a confocal microscope (Nikon Eclipse Ti-E).

2.7. Pronuclear detection

The formation of 2 pronuclei (2 PN) was assessed in all experimental groups (ICSI-FT, ICSI-FD and IVF). IVF group was considered as control, which is supposed to show 2 PN at 10 h *pf* the syngamy at 22 h *pf*. To this extend, a total of additional 90 fertilized oocytes (30 ICSI-FT, 30 ICSI-FD and 30 IVF) were carefully cleaned from cumulus cells and incubated in 5 min 1 μg Hoechst 33,342 in 10 ml of embryo culture medium. Oocytes were observed one by one under inverted microscope connected with a micromanipulator system, at 10 h pf/pa first, for the 2 PN assessment, then later at 22 h pf/pa, for syngamy stage assessment.

2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6.0 software. Kruskal-Wallis test was applied to compare *in vitro* embryo development on a minimum of 5 replicates for each group (ICSI-FT, ICSI-FD and IVF). The considered data in each group were the number of the cleaved embryos and the blastocyst number in each group, a multiple comparison was done between groups. Statistical significance threshold has been set with $P < 0.05$. Chi-square test was applied to compare 2 PN formation and early syngamy event. The accurate number of oocytes showing 2 PN and syngamy events were the considered data. ICSI groups were compared to IVF. ICSI-FT and ICSI-FD groups were also compared between each other. Statistical significance threshold has been set with $P < 0.01$.

3. Results

3.1. Sperm evaluation

After rehydration, freeze-dried spermatozoa exhibited the 58.5% of external membrane damaged (valuated by Pisum sativum agglutinin-PSA). The percentage of intact DNA was similar as reported in our previously work [24] with an estimation of 9.9%, analyzed by Comet-assay.

3.2. Sperm aster nucleation and microtubular dynamics

ICSI-FT zygotes completed the II meiosis by 3 h pa and simultaneously the proximal centriole initiated aster nucleation (Fig. 1A, arrow; Fig. 2A). A further microtubular growth was observed from 4 to 5-h pa (Fig. 1B, arrow; Fig. 2B) with a marked increase by 8 to 9-h pa, with microtubules radiating from the decondensed male pronucleus (Fig. 1C, arrow; Fig. 2C; Fig. 1D, arrow; Fig. 2D). Aster formation was delayed 1 h in the ICSI-FD derived zygotes (Fig. 1E, arrow; Fig. 2E) with nucleation starting only by 4 h pa (Fig. 1F, arrow; Fig. 2F). However, once the nucleation started, the sperm aster followed the same dynamics from the 5 h onward, as previously described (Fig. 1G–H, arrow; Fig. 2G–H).

Overall, the aster formation and microtubular dynamics in ICSI-FT overlapped those detected in IVF control zygotes (Fig. 1I-J-K-L, arrows; Fig. 2I-J-K-L).

3.3. Pronuclear and syngamy assessment

Zygotes were checked for the 2 PN and syngamy at 10-h pf/pa and 22-h pf/pa respectively. At 10-h pf/pa, the percentage of pronuclear stage embryos was quite similar in ICSI-FT and ICSI-FD, respectively 47% (14/30 zygotes) and 53% (16/30 zygotes) while the majority of IVF zygotes, 73% (22/30) were at the 2 PN stage (Table 1) (IVF vs ICSI-FT, $P < 0.001$). Syngamy was detected at 22-h pa in only 28.5% (4/14) of ICSI-FT and 12.5% (2/16) of ICSI-FD zygotes, while most of them were arrested at 2 PN stage (71.5% (10/14) and 87.5% (14/16), respectively). The picture radically changed in IVF zygotes, where half 50% (11/22) completed syngamy, and half 50% (11/22) were found blocked at 2 PN stage (IVF vs ICSI-FD, $P < 0.001$).

3.4. Embryo development

Embryo development is summarized in Table 2. No significant differences were found in the cleavage rate between the different

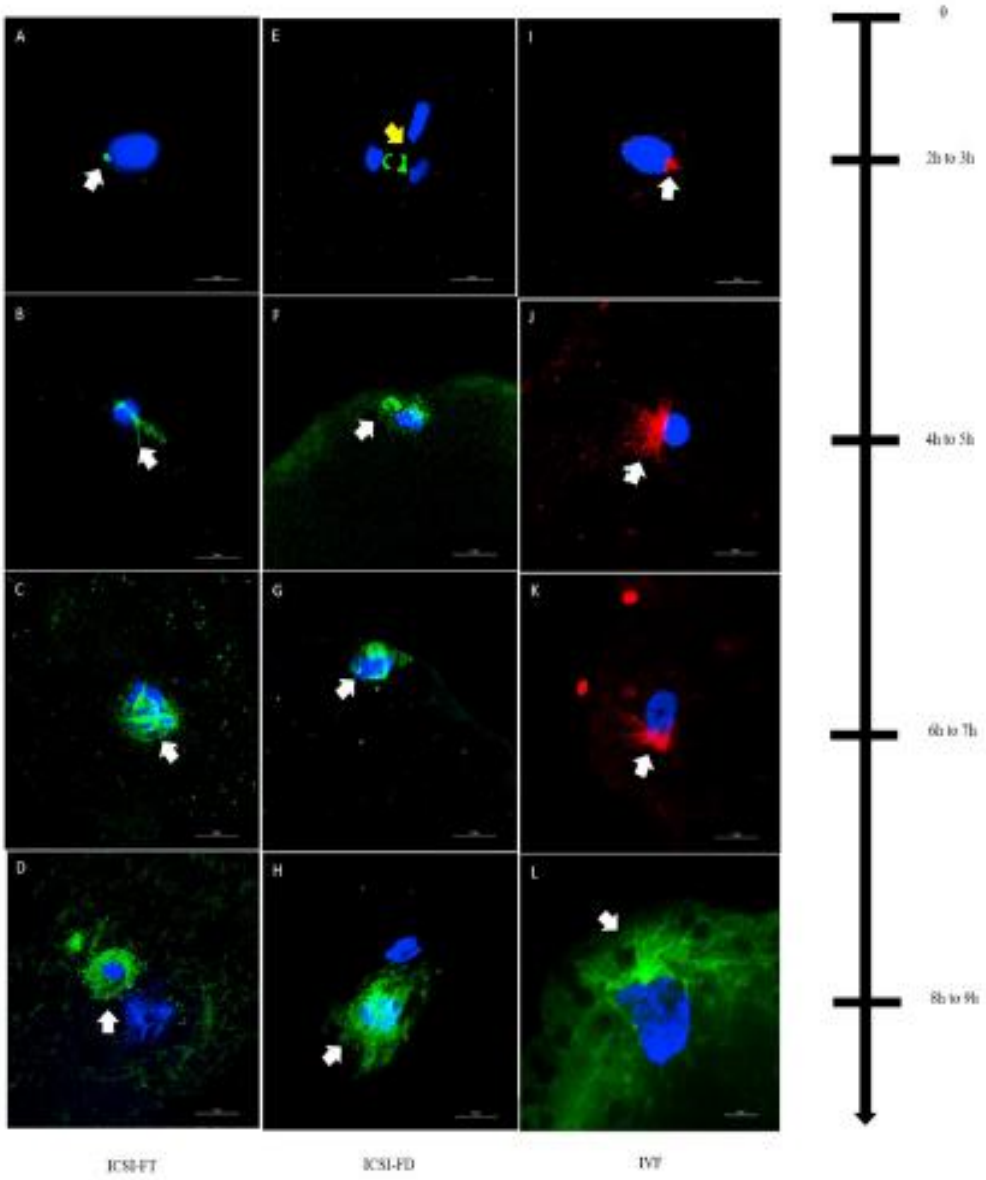

Fig. 1. Sperm aster nucleation dynamics in ICSI-FT (A-B-C-D); ICSI-FD (E-F-G-H) and IVF (I-J-K-L) presumptive zygotes; 2 to 3-h *pf/pa* (A-E-I), 4 to 5-h *pf/pa* (B–F-J), 6 to 7-h *pf/pa* (C-G-K), 8 to 9-h *pf/pa* (D-H-L). Arrows: sperm aster nucleation and microtubule elongation; yellow arrow: absence of sperm aster nucleation. Red/green: α− tubulin; blue − nucleus/Hoechst.

groups, while blastocyst rate significantly differed between ICSI embryos and IVF control ones (IV[a] vs ICSI-FT, $P < 0.05$; IVF vs ICSI-F[b], $P < 0.005$).

4. Discussion

The current low efficiency of ICSI assisted fertilization in ruminants, and the puzzling asymmetry between the activation and the cleavage rates, prompted us to investigate whether centriole dysfunction might be the cause. Our findings on spermatozoa aster nucleation and microtubule organization in normal IVF and ICSI sheep oocytes using frozen/thawed and freeze-dried ram spermatozoa ruled out this hypothesis. We have found that aster nucleation and microtubular dynamics in control IVF and ICSI with FT spermatozoa take place almost synchronously, while a delay in aster organization was instead noticed when FD spermatozoa were used for ICSI. In these latter zygotes, the aster started to assemble around 4-h pa, means 1 h later than the previous groups. In fact, the sperm centrosome must be exposed to the oocyte's reducing cytoplasm to be activated [25]. It is likely that the structural changes induced by drying in the peri-centrosomal area might have slowed down its "priming" to acquire microtubule nucleating capacity.

A similar study carried out in ICSI derived bovine embryos injected with FD bull spermatozoa partially disagrees with our findings [26]. The paper reports a reduced microtubular nucleating activity in ICSI embryos derived from FT and FD semen, without however any difference in centrosome function in FD spermatozoa. It is likely that differences in the drying technique, like the residual water, and length of de-hydration might account for the differences between our and their study. However, despite the recovery of microtubular nucleation in ICSI-FD embryos, their development to blastocyst stage was significantly lower comparing to the other groups, very likely owing to other factors, like DNA damage in freeze dry spermatozoa [24]. Therefore, our data have demonstrated that centriole dysfunction is not the major cause which hamper the development of ICSI derived embryos in sheep, as suggested by other authors [19,20]. The puzzling observation made in this study is that pronuclear formation in the majority of the two ICSI groups (71.5% and 87.5% respectively in ICSI-FT and ICSI-FD)

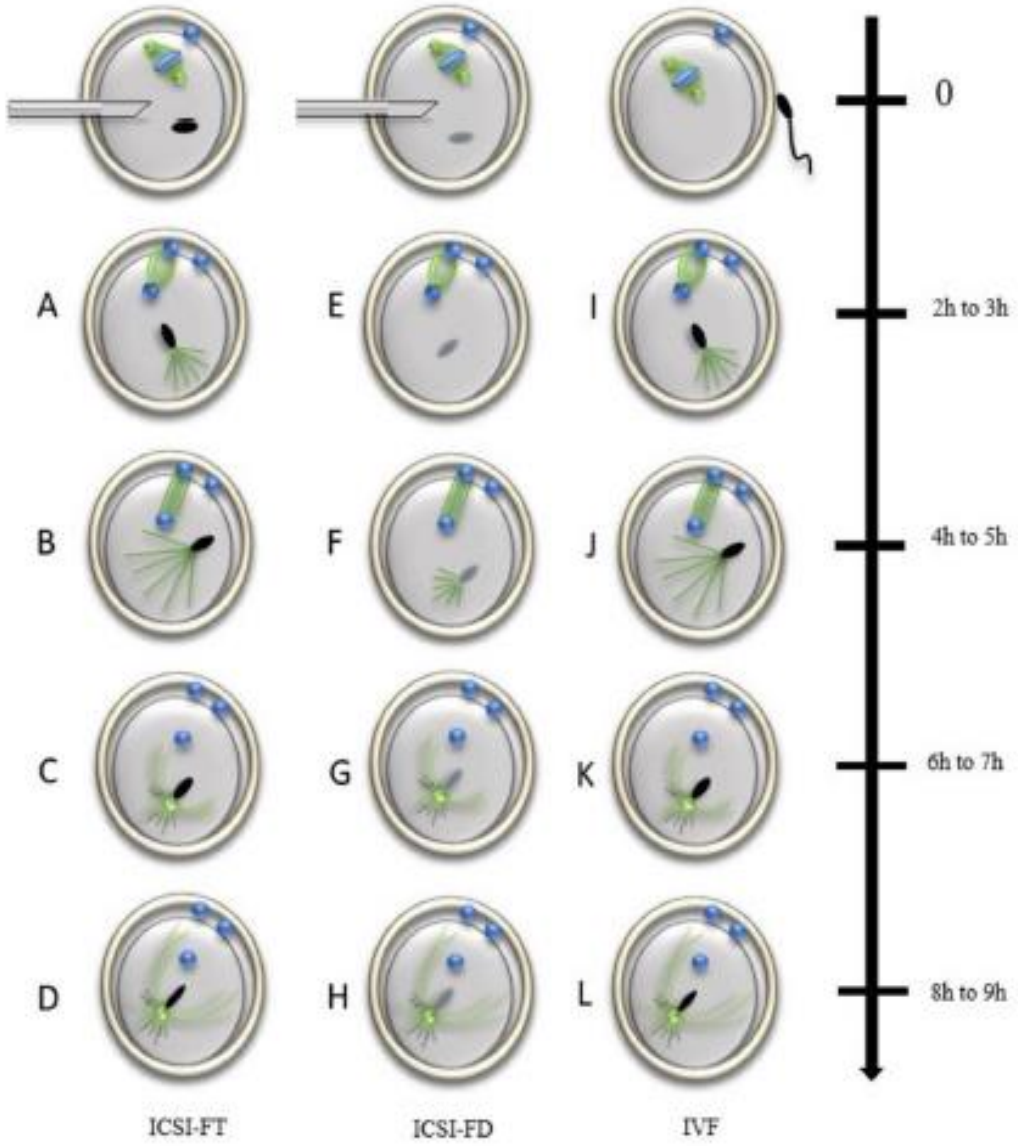

Fig. 2. Schematic representation of the sperm aster nucleation dynamics in ICSI-FT (A-B-C-D); ICSI-FD (E-F-G-H) and IVF (I-J-K-L) presumptive zygotes; Fertilization beginning (0), 2 to 3-h pf/pa (A-E-I), 4 to 5-h pf/pa (B–F-J), 6 to 7-h pf/pa (C-G-K), 8 to 9-h pf/pa (D-H-L).

was not followed by syngamy. It is thus likely that the absence of syngamy in ICSI derived zygotes might trigger the G2/M block, leading to the lack of mitosis [5]. Pronuclear apposition and the fusion of nuclear membrane in preparation for the first mitosis are poorly understood phenomenon. Apparently, nuclear membrane fusion promotes pronuclear apposition [27] and membrane fusion is typically promoted by Ca^{2+} ions. A series of Ca^{2+} spikes induced by sperm soluble factor, identified as PLCzeta (zeta) [28], affects several post-fertilization events [29] and very likely the pronuclear membrane fusion too. While we do not have elements to understand the absence of syngamy in ICSI derived embryos, the abnormal calcium spiking reported in ICSI zygotes, essentially a shorter duration of Ca^{2+} oscillations than IVF, might be responsible for the reduced pronuclear fusion [30,31]. The issue certainly needs further investigation in order to finally optimize embryonic development following ICSI in ruminant embryos.

CRediT authorship contribution statement

Yosra Ressaissi: Conceptualization, Methodology, Validation, Investigation, Data curation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Debora Agata**

Table 1
Pronuclei stage and syngamy assessment in IVF, ICSI-FT and ICSI-FD embryos.

Groups	No. oocytes	2 PN (%)	Syngamy (%)
ICSI-FT	30	14/30 (47%)[a]	4/14 (28.5%)
ICSI-FD	30	16/30 (53%)	2/16 (12.5%)[b]
IVF	30	22/30 (73%)	11/22 (50%)

[a] IVF vs ICSI-FT, $P < 0.001$.
[b] IVF vs ICSI-FD, $P < 0.001$.

Table 2
Embryo development outcomes from IVF, ICSI-FT and ICSI-FD.

Groups	No. oocytes	2-Cells (%)	Blastocysts (%)
ICSI-FT	56	19/56 (34%)	11/56 (19%)[a]
ICSI-FD	77	24/77 (31%)	8/77 (10%)[b]
IVF	67	30/67 (44%)	22/67 (33%)

[a] IVF vs ICSI-FT, $P < 0.05$.
[b] IVF vs ICSI-FD, $P < 0.005$.

Anzalone: Conceptualization, Methodology, Validation, Writing - review & editing. **Luca Palazzese:** Methodology, Data curation, Writing - review & editing. **Marta Czernik:** Conceptualization, Methodology, Validation, Writing - review & editing. **Pasqualino Loi:** Conceptualization, Methodology, Project administration, Writing - review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors have no conflicts of interest.

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Annex II

Individually cultured bovine embryos produce extracellular vesicles that have the potential to be used as non-invasive embryo quality markers

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ABSTRACT

Extracellular vesicles (EVs) are membrane-bound biological nanoparticles (NPs) and have gained wide attention as potential biomarkers. We aimed to isolate and characterize EVs from media conditioned by individually cultured preimplantation bovine embryos and to assess their relationship with embryo quality. Presumptive zygotes were cultured individually in 60 μl droplets of culture media, and 50 μl of media were collected from the droplets either on day 2, 5 or 8 post-fertilization. After sampling, the embryo cultures were continued in the remaining media until day 8, and the embryo development was evaluated at day 2 (cleavage), day 5 (morula stage) and day 8 (blastocyst stage). EVs were isolated using qEVsingle® columns and characterized. Based on EV Array, EVs isolated from embryo conditioned media were strongly positive for EV-markers CD9 and CD81 and weakly positive for CD63 and Alix among others. They had a cup-like shape typical to EVs as analyzed by transmission electron microscopy and spherical shape in scanning electron microscopy, and hence regarded as EVs. However, the NPs isolated from control media were negative for EV markers. Based on nanoparticle tracking analysis, at day 2, the mean concentration of EVs isolated from media conditioned by embryos that degenerated after cleaving (8.25×10^8/ml) was higher compared to that of embryos that prospectively developed to blastocysts (5.86×10^8/ml, $p < 0.05$). Moreover, at day 8, the concentration of EVs isolated from media conditioned by degenerating embryos (7.17×10^8/ml) was higher compared to that of blastocysts (5.68×10^8/ml, $p < 0.05$). Furthermore, at day 8, the mean diameter of EVs isolated from media conditioned by degenerating embryos (153.7 nm) was smaller than EVs from media conditioned by blastocysts (163.5 nm, $p < 0.05$). In conclusion, individually cultured preimplantation bovine embryos secrete EVs in the culture media and their concentration and size are influenced by embryo quality and may indicate their prospective development potential.

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1. Introduction

Nanomaterials, including nanoparticles (NPs), are conventionally defined as particles with a size of ≤100 nm in at least one dimension [1], and thus NPs with its conventional spherical shape are typically ≤100 nm in all dimensions. However, NPs vary based on many factors including source, size, and composition. Both synthetic and biological NPs have a wide range of applications in medicine [2]. Extracellular vesicles (EVs) are membrane-bound biological NPs that are secreted both *in vivo* and *in vitro* by many cell types under physiological and pathological conditions [3–5], while they are found in all biological fluids, such as blood, milk, synovial fluid, and also in conditioned cell culture media [6,7]. Their molecular cargo primarily includes proteins, nucleic acids (including messenger and non-coding RNA) and lipids [8]. The compartmentalization of the molecular cargo within EVs protect the labile cargo from inactivation and further degradation in the extracellular environment [9]. Secreted EVs are internalized by the target cells and thus, the molecular cargo of the EVs alters the activity and phenotype of the recipient cells and can also induce (epi) genetic modifications [10]. Moreover, EVs are gaining wider attention as potential biomarkers [11] and therapeutic DDSs (drug delivery systems) [12].

The production of embryos *in vitro* has multiple applications, such as using assisted reproductive technologies (ART) to treat human subfertility, animal breeding, and research in the preimplantation embryonic development [13,14]. Despite recent advances in embryo culturing protocols, the quality of embryos produced *in vitro* is lower compared to their *in vivo* counterparts [15]. Compared to individual embryo culture, higher blastocyst formation rates and better quality have also been reported when embryos were cultured in groups [16] or with other somatic cells, such as the oviductal epithelial cells [17]. This could be due to the release of various autocrine and paracrine factors by these cells, which support the development of embryos in groups rather than in individual culture.

Traditionally, the method of choice to evaluate the embryonic quality has been the microscopic evaluation of embryo morphology. The field has further been revolutionized with the introduction of time-lapse microscopic systems enabling continuous monitoring of the embryos [18]. However, ART based live birth rates have yet not lived up to the expectations despite the technological advances in the field [19]. The biggest hurdle to overcome is to develop a reliable method to evaluate the quality of the generated embryos in order to select the best candidate for transfer. As a result, research focusing on the identification of a non-invasive biomarker of embryonic quality has emerged as an active field. By now, different non-invasive *in vitro* methods based on the analysis of conditioned embryo culture media, within the areas of metabolomics, proteomics, and small non-coding RNA, have been tested [20]. However, so far, no biomarker clearly stands out as an early, consistent and sensitive way to predict the embryonic developmental or transfer success.

It has been shown that *in vitro* produced preimplantation embryos of several mammalian species, including bovine [21], porcine [22], murine [23] and humans [24] secrete EVs. Giacomini et al. (2017) demonstrated that the EVs secreted by human embryos were uptaken by the primary human endometrial epithelial and stromal cells. Pavani et al. (2018) further illustrated that when EVs isolated from the bovine embryo conditioned media were supplemented to the embryo culture systems, they were internalized by the embryos; thus, providing evidence of embryo-embryo interactions via EVs [25]. Both these findings support the role of EVs in intercellular communications in mammalian preimplantation embryogenesis.

Melisho et al. (2017) reported the release of EVs by individually cultured bovine blastocysts produced by *in vitro* fertilization (IVF) and parthenogenetic activation (PA), during the days 7–9 of *in vitro* development. They found out that the concentration of vesicles, released between day 7–9 of development, was higher in IVF blastocysts with arrested development between day 9–11 of *in vitro* culture compared to competent PA blastocysts [21]. This indicated that the quality of embryos influences the release of EVs during embryonic development. Moreover, the same group recently investigated the release of EVs from individually cultured embryos during blastulation [26]. As these studies evaluated the EVs derived from the bovine embryos at a later stage, i.e., blastocyst stage, of preimplantation embryo development, it is still unknown if EVs are released at earlier stages of bovine embryonic development.

Cell culture media, including embryo culture media, can contain EVs and other NPs deriving mainly from the supplemented serum or serum derivatives, such as bovine serum albumin (BSA). Therefore, the depletion of these EVs from culture media is recommended when used for EV research [27]. However, even under EV depleted culture media conditions, it is difficult to quantify the exact proportion of remaining EVs out of all NPs due to the limitations of the technology. Also, the effects of EV depletion on the viability of individually cultured zygotes are yet to be verified. Adding to this, Pavani et al. (2018) cultured bovine embryos in groups using culture media supplemented with EV-depleted BSA (by ultracentrifugation) and regular BSA [25]. Although the blastocyst rates were unchanged, a significant impairment of the embryo quality, as measured by the total and inner cell mass cell numbers of the blastocysts, grown with EV depleted media, was noted indicating that some of the vital factors derived from the BSA might have been lost during the ultracentrifugation procedure. Nevertheless, the effects of EV depletion of BSA on individual embryonic culture systems remain to be established.

EVs in the culture media conditioned by embryos may vary depending on the developmental stage and the quality of the embryos. These differences could be apparent in terms of the quantity or the size of the EVs secreted, or in terms of the molecular cargo, such as proteins and nucleic acids. This way, these EVs could serve as a potential biomarker of embryonic quality and their prospective development. As these EVs can reflect the functional and physiological status of the developing embryos, they could potentially complement the morphology-based assessment of the embryo quality through a non-invasive embryo quality assessment. Therefore, the aims of the current study were firstly, to study the effects of depletion of EVs from the culture media on the developmental potential of individually cultured bovine embryos and secondly, to isolate and characterize EVs from culture media conditioned by *in vitro* produced and individually cultured bovine embryos, based on their quality, developmental stage, and prospective development.

2. Materials and methods

2.1. In vitro embryo production (IVP)

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich/Merck (Germany or USA). Bovine embryos were produced as previously described by Nõmm et al. (2019) with modifications of the embryo culture system [28]. Abattoir-derived cattle ovaries (*Bos taurus*) were transported to the laboratory in 0.9% sterile NaCl solution within 4 h after the sacrifice at ~32–37 °C and washed twice in a freshly prepared 0.9% NaCl solution.

Cumulus-oocyte complexes (COCs) were aspirated from follicles with a diameter of 2–8 mm using a vacuum pump (Minitüb GmbH, Germany). Quality code 1 COCs [29] were washed and matured in groups of 50 in 500 μl of IVM-medium (supplemented with 0.8% fatty acid-free BSA fraction V in 4-well plates (Nunc, Roskilde, Denmark). The COCs were incubated at 38.5 °C with 5% CO_2 in humidified air for 22–24 h.

Frozen-thawed semen was used to fertilize the matured COCs. Washed spermatozoa were diluted to the final concentration of 1×10^6 motile sperms per ml. The COCs and sperms were co-incubated in groups of 50 in 500 μl of Fert-TALP media in 4-well plates at 38.5°C with 5% CO_2 in humidified air for 18–20 h.

Cumulus cells were removed from the presumptive zygotes by vortexing, and the denuded embryos were transferred individually into 60 μl droplets of modified Synthetic Oviduct Fluid with amino acids and myo-inositol (SOFaaci) containing 0.8% BSA under mineral oil [28], for single embryo culture, which allowed sampling at different time points. The presumptive zygotes were cultured at 38.5 °C, 5% CO_2 and 90% N_2 with humidified air for eight days. Embryos were morphologically evaluated at day 2, 5 and 8 post-fertilization, and the developmental stages and embryo quality were recorded [29]. The three distinct development stages were: cleavage, morula and blastocyst stage.

2.2. Collection, storage, and categorization of embryo conditioned media

Conditioned media samples (50 μl) were collected at day 2 (cleavage stage), day 5 (morula stage), and day 8 (blastocyst stage) post-fertilization from different batches of individually cultured bovine embryos. After collecting the conditioned media, the embryos at day 2 and 5 were continuously cultured in the remaining 10 μl culture media droplet until day 8. The collected conditioned and control media were stored at −80 °C until further experiments.

Based on the morphological evaluation of the embryos on days 2, 5 and 8 post-fertilization, the collected conditioned media samples were categorized, and the samples relevant to the study were defined as follows.

Conditioned media collected at day 2: media conditioned by embryos cleaved by day 2 and subsequently developed to blastocysts by day 8 (hereafter referred as "Day 2 good quality embryo media"), and media conditioned by embryos that cleaved by day 2 but subsequently degenerated (hereafter referred as "Day 2 bad quality embryo media").

Conditioned media collected at day 5: media conditioned by embryos that developed to morula by day 5 and subsequently developed to blastocysts by day 8 (hereafter referred as "Day 5 good quality embryo media"), and media conditioned by embryos developed to morula by day 5 but subsequently degenerated (hereafter referred as "Day 5 bad quality embryo media").

Conditioned media collected at day 8: media conditioned by embryos that developed to blastocysts by day 8 (hereafter referred as "Day 8 good quality embryo media"), and media conditioned by embryos developed to morula by day 5 but subsequently degenerated (hereafter referred as "Day 8 bad quality embryo media").

In parallel, culture media samples were incubated for 2, 5 and 8 days, without embryos and labeled as "Day 2 control", "Day 5 control" and "Day 8 control," respectively.

2.3. Benchmarking qEVsingle® size exclusion chromatography columns

Commercial size exclusion chromatography columns (qEVsingle/70 nm by Izon Sciences, UK, product code SP2), specially designed to isolate EVs, were used for isolation of EVs from embryo conditioned media and controls. Initially, a column was benchmarked using RPMI-1640 media supplemented with 10% FBS to verify the performance of the qEV columns and the fractions which contained the EVs and the ones that contained proteins. In brief, 2 ml of complete media (RPMI-1640 media supplemented with 10% FBS), which were known to contain EVs due to the supplementation of FBS, was subjected to sequential centrifugation at 400g for 10 min and at 2000 g for 10 min to remove any larger particles. Subsequently, the media was concentrated up to 150 μl using Amicon® Ultra-2 10 K centrifugal filters (Merck Millipore Ltd, Ireland, catalog number UFC201024) at 3200 g for 40 min. The concentrated sample was used for EV purification using a qEVsingle column, while each eluted fraction (200 μl) was collected separately. The concentration of the EVs in each fraction was measured using ZetaView® nanoparticle tracking analyzer (PMX 120 by Particle Metrix GmbH, Inning am Ammersee, Germany). Similarly, the protein concentrations in each of the eluted fractions were measured using the Modified Lowry Protein assay kit (Thermo Scientific, U.S.A, catalog number-23240) according to the manufacturer's protocol.

2.4. Isolation of EVs from the embryo conditioned media and controls

Even though embryos are unlikely to introduce dead cells or larger particles such as apoptotic bodies to the conditioned media due to the *zona pellucida* (ZP), sequential centrifugation was carried out to remove such potential cells or bigger particles as recommended as such particles could clog the qEVsingle® columns, and affect the purification process. Dulbecco's phosphate-buffered saline (DPBS, Sigma® Life Science, UK, catalog number D8537) was filtered using 0.2 μm Ministart® syringe filters and was used freshly for sample dilution and EV isolation. Firstly, the culture media samples stored at −80 °C, were thawed on ice, while 45 μl of media were diluted in sterile-filtered DPBS to a final volume of 150 μl. These samples were then subjected to double centrifugation steps. Initially, the diluted samples were centrifuged at 400 g for 10 min at 4 °C to remove any dead cells and debris, while 145 μl from the supernatant was transferred to another fresh tube. The collected supernatants were centrifuged at 2000 g for 10 min to remove any apoptotic bodies. After centrifugation, 140 μl of the supernatant was transferred to another new tube and kept in ice until the isolation of EVs.

A standard protocol recommended by the Izon Science Ltd. was followed during the whole process of EV-isolation using qEVsingle size exclusion columns. Briefly, the columns were mounted vertically in a holder and were equilibrated by running through 10 ml of fresh filtered (0.2 μm) elution buffer (DPBS). Then, the sample (140 μl of media) was added to the top of the column, and fraction (200 μl) collection was initiated immediately. When the sample leveled with the upper column filter, the column was topped up with the elution buffer. The first five fractions (total of 1000 μl), which was the void volume, were collected together and discarded. Fractions 6–9 (total of 800 μl) were collected and pooled as EVs elute in these fractions should there be any in the sample. The size and the concentrations of EVs in the pooled fractions were then determined using nanoparticle tracking analyzer-ZetaView®.

2.5. Nanoparticle tracking analysis (NTA)

The measurement of the concentration and size profile of NPs of the samples were carried out using NP tracking analyzer-Zetaview®. Zetaview® was calibrated using 100 nm particle size standards (Applied Microspheres BV, Netherlands. Catalogue no. 10100). Before sample measurements, the concentration of the

filtered DPBS was measured to confirm the purity (i.e. minimum amounts of NPs), of the DPBS used. The NP concentration and the size profiles of the samples were measured in scatter mode under the following settings: sensitivity: 75, shutter: 100, frame rate: 30 fps and number of cycles: 3. Each biological sample was measured in triplicates. In between measurements, the measurement cell of the instrument was thoroughly washed with Milli-Q® water and DPBS before the injection of the next sample in order to minimize the inter-sample contamination.

2.6. High throughput multiplexed phenotyping of EVs (EV Array)

Characterization of the EVs, based on the EV markers, was carried out using EV Array [30]. IVC media conditioned by individually cultured day 5 bovine embryos that developed to morula (n = 40, 2 ml when pooled) and IVC media incubated till day 5 without embryos as control (n = 40, 2 ml when pooled) were used. Each sample was subjected to sequential centrifugation, as previously described, to deplete potential larger particles. From both embryo conditioned and the control media, 100 μl fractions were transferred to two separate tubes and labeled as media before EV isolation (sample and control). The rest of the conditioned and the control samples were subjected to EV isolation using qEVsingle columns. Fractions 6–9 were collected, pooled and concentrated to a final volume of 100 μl using Amicon® ultra-2 10 K centrifugal filters at 3200 g for 30 min at 4 °C. All samples were stored at −80 °C till used for EV protein profiling by EV Array.

Microarray slides were produced for the EV Array using a sciFLEXARRAYER S12 (Scienion AG, DE). Shortly, antibodies were printed on epoxy-coated slides (SCHOTT Nexterion, Germany) with a coated PDC3 size 60 (Scienion AG). Positive and negative controls were biotinylated human IgG (100 mg/ml) and PBS with 5% glycerol, respectively. One anti-bovine antibody (CD63, clone CC25, BioRad, CA, USA) together with a total of 21 anti-human antibodies, were used for production of the EV Array and listed in the following with the corresponding clone, if available: EGFR (Antibodies-online.com, Germany); Hsp 90 (IGF1), p53 (pAb 240), Flotillin-1, TSG101 (Abcam); CD63 (AbD serotec, UK); CD9 and CD81 (Ancell, MN, USA); Alix (3A9, Biolegend, CA, USA); HLA-G (87G, Novus Biologicals, CO, USA); Annexin V, Cathepsin D, Tspan8 (458811), CD82 (423524), CD151 (210127), Hsp 70 (242707), and LAMP-1 (R&D Systems, MN, USA); EpCam (0.N.277), GRP78 (N-20) and AKAP (C-20, Santa Cruz Biotechnologies, TX, USA). Antibodies were diluted in PBS with 5% glycerol and printed in triplicates at 200 mg/ml.

The EV Array was performed as described by Jørgensen et al. (2013), with modifications. In short, the microarray slides were initially blocked (50 mM ethanolamine, 100 mM Tris, 0.1% SDS, pH 9.0) prior to incubation with 40 μl sample diluted in wash-buffer (PBS/0.05% Tween®20). The incubation was performed in Multi-Well Hybridization Cassettes (Arraylt Corporation) at RT for 2 h followed by overnight incubation at 4 °C. Biotinylated anti-bovine antibody against CD9 (Antibodies-Online.com, DE) was diluted 1:1500 and used to detect retained EVs using Cy5-labeled streptavidin (Life Technologies, MA, USA) diluted 1:1500. Scanning and spot detection was performed as previously described [31].

2.7. Transmission electron microscopy (TEM)

Embryo culture media conditioned by *in vitro* cultured single bovine embryos that developed to morula stage were collected at day 5 and pooled together (n = 60, 3 ml). As the control, embryo culture media (3 ml) incubated for 5 days without embryos were used. The pooled conditioned media and control media were subjected to sequential centrifugation at 400 g for 10 min and 2000 g for 10 min to remove any existing larger particles. Subsequently, the supernatants were concentrated to 150 μl using Amicon® Ultra-15 10 K centrifugal filters (Merck Millipore Ltd. Ireland, catalog number UFC901024) by centrifuging at 3200 g for 45 min. From the concentrated media, EVs were isolated using qEVsingle columns, as described previously. Fractions 6–9 were collected, pooled and subsequently, concentrated using Amicon® ultra-2 10 K centrifugal filters by centrifuging at 3200 g for 80 min to a final volume of 60 μl. A droplet of the purified EV samples from the conditioned media and control were placed on formvar/carbon-coated 200 mesh grids (Agar Scientific, Stansted, UK) and allowed to adsorb for 20 min. Next, samples were fixed in Karnovsky fixative (2% paraformaldehyde and 1% glutaraldehyde; Sigma-Aldrich, Germany; Polysciences, USA, respectively) before being contrasted in uranyl oxalate [mixture of 4% uranyl acetate (Polysciences, Warrington, USA) and 0,15 M oxalic acid (Sigma-Aldrich, Schnelldorf, Germany)] and embedded in a mixture of methylcellulose (Sigma-Aldrich, Schnelldorf, Germany) and uranyl acetate (Polysciences, Warrington, USA). Samples were observed with a JEM 1400 transmission electron microscope (JEOL Ltd. Tokyo, Japan) at 80 kV, and digital images were acquired with a numeric camera (Morada TEM CCD camera, Olympus, Germany).

2.8. Scanning electron microscopy (SEM)

Embryo culture media conditioned by *in vitro* cultured single bovine embryos that developed to the morula stage were collected on day 5 and pooled together (n = 30, 1.5 ml). The pooled conditioned media were then subjected to sequential centrifugation at 400 g for 10 min and 2000 g for 10 min to remove any existing dead cells or larger particles. Subsequently, the media was concentrated to 150 μl using Amicon® Ultra-2 10 K centrifugal filters by centrifuging at 3200 g for 30 min at 4 °C. From the concentrated media, EVs were isolated using qEVsingle columns, as described previously. Fraction 6–9 were collected together and subsequently concentrated using Amicon® ultra-2 10 K centrifugal filters (3200 g for 60 min) to a final volume of 60 μl. The concentrated sample was frozen at −80 °C and transferred to a scanning electron microscopic (SEM) facility. The sample was thawed and a drop of the isolated EV sample was left on an aluminum foil for overnight drying and was imaged the following day in a Hitachi S-4300 SEM microscope after sputter coating the samples with gold.

2.9. Statistical analysis

Log-rank test was used to compare the survival distributions of embryos in EV depleted and regular IVC media culture settings. Obtained p-values were corrected for multiple testing with Bonferroni correction. Linear mixed models (LMM) fit via residual maximum likelihood (REML) approach were used to test for statistically significant differences in the concentration, and the average size of NPs observed in the three experimental groups and developmental stages. The LMMs were fit being nested for the three developmental stages with experimental replicate (or batch) as the mixed effect to test for differences between experimental groups in their respective developmental stage. P-values were obtained from t-tests on the resulting estimated marginal means (EMMs) and were further subjected to Tukey adjustments. Statistical analysis was conducted in R [32] using packages lme4 [33] and emmeans [34]. The means of the 3 technical measurement replicates for each biological sample were used in these tests. In order to compare nanoparticle concentrations in specific size ranges (bins), the nanoparticle concentrations in each size bin were normalized beforehand by dividing by the sum concentration of all size bins for the given sample. This resulted in normalized concentration values, i.e. fraction of total concentration in each size bin for the given sample.

2.10. Experimental design

Two experiments were carried out to accomplish the objectives. In the first experiment, the effects of EV-depletion on individual and grouped embryonic development was assessed. During the preparation of IVC media, EVs in BSA were depleted by ultrafiltration. BSA was ultrafiltered, using Amicon® Ultra-15 10 K centrifugal filters, by centrifuging at 3600 *g* for an hour under sterile conditions. The flow-through of the filtration was used when supplementing BSA to the culture media. Such prepared EV-depleted IVC media was used to culture embryos individually and in groups for 8 days. As a control, embryos were cultured individually in regular IVC media (without EV-depletion) for 8 days.

In the second experiment, presumptive zygotes produced *in vitro* were cultured individually in regular IVC media to isolate and quantify the EVs from the conditioned media based on the development stage and prospective embryonic development. Presumptive zygotes were individually cultured in IVC media under mineral oil for 8 days, and their development was assessed at day 2 (cleavage stage), day 5 (morula stage) and day 8 (blastocyst stage) post-fertilization. Fifty μl of media, conditioned by embryos, were collected at day 2 ($n = 35$), day 5 ($n = 35$) and day 8 ($n = 35$) post-fertilization and stored at −80 °C. After collecting the conditioned media, the embryos were cultured in the remaining 10 μl of media until day 8. The collected conditioned media samples were categorized based on embryonic development up to day 8. This experiment was carried out in three replicates using oocytes collected on three different days. Similarly, all embryos were cultured individually to characterize the EVs isolated from conditioned media by EV Array, nanoparticle tracking analysis, transmission and scanning electron microscopy.

3. Results

3.1. Experiment 1: evaluation of individual and grouped embryo development in EV-depleted and regular IVC media

In this experiment, presumptive zygotes were cultured both individually and in groups in EV-depleted media and individually in regular IVC media to assess the impact of EV depletion on individual and grouped embryonic cultures. The results showed that although 54.5% of embryos reached the morula stage, only 3% reached the blastocyst stage during the individual embryo culture in EV-depleted IVC media (Table 1). In contrast, 39.3% of the individually cultured embryos in regular IVC media reached the blastocyst stage. The overall survival of individually cultured embryos was found to be significantly poorer ($p = 0.013$, log-rank test, Bonferroni correction) when cultured in the EV-depleted media compared to regular IVC media. Interestingly, the survival of embryos in the EV-depleted media improved significantly ($p = 0.014$, log-rank test, Bonferroni correction) when cultured in a group compared to single embryo cultures. Embryos cultured in groups in EV-depleted medium were able to develop to the blastocyst stage at a rate similar to individual embryos in regular IVC media.

3.2. Experiment 2: individual embryo culture for isolation of EVs and their characterization

3.2.1. Individual culture of bovine embryos

Presumptive zygotes were cultured individually in 60 μl of regular IVC media under mineral oil until day 8 post-fertilization. Subsequent to the collection of 50 μl of conditioned media at day 2 or day 5, those embryos were allowed to culture in the remaining 10 μl media till day 8. For each time point, a total of 105 presumptive zygotes were cultured individually (35 zygotes for each of the three replicates). Embryo development was assessed based on morphological parameters.

In the day 2 group, 25% of the zygotes had developed into blastocysts, and 27% had cleaved by day 2, but had subsequently degenerated (Table 2). In the day 5 group, 28% of the zygotes had developed into blastocysts, and 17% had degenerated after developing into a morula by day 5. In the case of embryos at the time-point of day 8 post-fertilization, while 23% of the zygotes had developed to blastocysts, 21% of the zygotes had degenerated after developing into a morula by day 5. Therefore, the blastocyst rate did not seem to be affected by the collection of 50 μl of media before day 8.

3.2.2. Benchmarking qEVsingle size exclusion chromatography columns

Before isolating the EVs from embryo conditioned media, a qEVsingle® column was benchmarked using cell culture media known to contain EVs. Benchmarking confirmed the clear separation of the EVs from soluble proteins in the culture media (Fig. 1) as the result of size exclusion chromatography. Based on this observation, it was decided to collect fractions 6–9 to isolate the EVs from embryo-conditioned media and controls.

3.2.3. Characterization of the EVs isolated from culture media conditioned by individually cultured bovine embryos

EV samples isolated from culture media conditioned by individually cultured bovine embryos were characterized by EV Array, NTA, TEM, and SEM.

The EV Array technology was used to phenotype EVs using EV markers. Twenty-two different antibodies against EV-, surface- or surface-associated markers were used to capture EVs onto a microarray (EV Array). Detection of the captured EVs was performed using an anti-bovine CD9 antibody. The EV Array experiment showed that NPs isolated from media conditioned by embryos were strongly positive for CD9 and CD81. Weak signals were also detected for CD63 (using both anti-human and anti-bovine antibodies) together with CD82, p53, Alix and HLA G (Fig. 2A and B). In contrast, particles isolated from control culture media (incubated for 5 days without embryos) were only very weakly positive for some of the markers such as CD9, CD81, CD82, p53, Alix and HLA G and therefore the presence of EVs in these samples cannot be confirmed. Moreover, the enrichment of EVs, as shown by the signal intensity for EV markers, following EV isolation by qEVsingle is evident when comparing sample I and sample II. Similarly, a weak increase in the fluorescent signal intensity was observed for control media samples when comparing the sample III and IV.

Fig. 3 illustrates the average size distributions of EVs isolated from embryo conditioned media and NPs isolated from control media of day 2, 5, and 8 samples. Most of the particles were distributed within the 30–330 nm size range.

Culture media conditioned by 60 individually cultured bovine embryos that developed to morula by day 5 (day 5 media) were pooled and the isolated EVs were used for TEM based characterization (Fig. 4A). TEM imaging visualized EVs and most of these particles were in the size range of 50–150 nm (supplementary materials, S1). Similarly, culture media conditioned by 30 individually cultured bovine embryos that developed to morula by day 5 (day 5 media) were used for SEM-based characterization and confirmed the spherical shape of the EVs (Fig. 4B).

3.2.4. Mean concentrations and sizes of EVs are associated with embryo quality

When presenting and comparing the mean concentrations and diameters of particles that were measured using NTA, particles

Table 1
Individual and group culture of embryos in EV-depleted and regular IVC media.

Individual and group culture of embryos in EV-depleted and regular IVC media.

	Individual embryos cultured in regular IVC media	Individual embryos cultured in EV-depleted IVC media	Group culture of embryos in EV-depleted IVC media
	n (%)	n (%)	n (%)
Oocytes	33 (100)	33 (100)	63 (100)
Zygotes	31(93.9)	27 (81.8)	53 (84.1)
Morula	20 (60.6)	18 (54.5)	37(58.7)
Blastocysts	13 (39.3)	1 (3.0)	25 (39.6)
p-value		0.013*	0.014**

Data are represented as numbers (n) or percentages (%). IVC- *in vitro* culture. IVC media consisted of modified synthetic oviductal fluid with amino acids, and myo-inositol (SOFsaci) supplemented with 0.8% BSA. For EV depletion, the dissolved BSA used to supplement IVC media was ultrafiltered using Amicon®- 15 10k filters by centrifuging at 3200 g. * p-value resulting from Log-rank test for the overall survival of individually cultured embryos in EV-depleted IVC media compared to regular IVC media. ** p-value resulting from Log-rank test for the overall survival of group cultured embryos in EV-depleted IVC media compared to individually cultured embryos in EV-depleted IVC media.

Data are represented as numbers (n) or percentages (%). IVC- *in vitro* culture. IVC media consisted of modified synthetic oviductal fluid with amino acids, and myo-inositol (SOFsaci) supplemented with 0.8% BSA. For EV depletion, the dissolved BSA used to supplement IVC media was ultrafiltered using Amicon®- 15 10 k filters by centrifuging at 3200 g * p-value resulting from Log-rank test for the overall survival of individually cultured embryos in EV-depleted IVC media compared to regular IVC media. ** p-value resulting from Log-rank test for the overall survival of group cultured embryos in EV-depleted IVC media compared to individually cultured embryos in EV-depleted IVC media.

isolated from embryo conditioned media were denoted as EVs and particles isolated from control media were denoted as NPs. This is because particles isolated from embryo conditioned media were proven to contain EVs and particles isolated from control media were not (based on the results of the EV Array).

At day 2, the mean concentration of EVs isolated from day 2 bad quality embryo media (8.25×10^8/ml) was 1.41-fold higher compared to that of day 2 good quality embryo media (5.86×10^8/ml, $p < 0.05$) (Fig. 5A and B). Moreover, at day 2, the mean concentration of the NPs isolated from the control media (8.65×10^8/ml) was as high as the mean EV concentration of day 2 bad quality embryo media. In contrast, at day 5, there was no difference in the mean EV concentrations between day 5 good quality and day 5 bad quality embryo media. However, at day 8, the mean concentration of EVs isolated from day 8 bad quality embryo media (7.17×10^8/ml) was 1.26-fold higher compared to that of day 8 good quality embryo media (5.68×10^8/ml, $p < 0.05$) (Fig. 5A and C). Furthermore, a gradual drop in the NP concentrations in the control media (8.65×10^8/ml, 6.47×10^8/ml, and 5.37×10^8/ml at day 2, day 5 and day 8 respectively) was observed as the incubation duration prolonged.

The size profiles of EVs isolated from embryo conditioned media and NPs isolated from control media were further analyzed to determine if there were differences between groups in specific size ranges. Particles in size ranges of 61–90 nm, 91–120 nm, and 121–150 nm were compared as this is the size range in which the NTA results can be expected to be most accurate. Furthermore, this size range corresponds to the expected size range of exosomes. At day 2, statistically significant differences were noted between good quality embryo media and control media concerning 61–90 nm and 91–120 nm sized particles (Fig. 6B and C and between good quality embryo media and bad quality embryo media with regards to 91-120 nm sized particles (Fig. 6C). Moreover, at day 8, differences were noted between good and bad quality embryo media in terms of 61–90 nm and 91–120 nm sized particles (supplementary materials, S2). However, on day 5, no differences were observed between any of the groups based on any of the size ranges analyzed (supplementary materials, S3).

Interestingly, significant differences in the mean diameters of the EVs isolated from the media conditioned by individually cultured bovine embryos and NPs isolated from controls were observed at day 2 and day 8 (Fig. 7),). At day 2, the mean (±CI) diameter of EVs isolated from good quality and bad quality embryo conditioned media were 166 ± 9 nm and 159 ± 8.8 nm, respectively and were higher compared to the mean concentration of NPs isolated from the control media which was 150 ± 9 nm ($p < 0.05$). In

Table 2
Morphological assessment of embryo development.

Timepoint of media collection	Day 2	Day 5	Day 8
Presumptive zygotes (n)	35	35	35
Not cleaved by day 2 (%)	26.66 ± 2.05	23.80 ± 3.10	24.76 ± 3.11
Cleaved by day 2, but subsequently degenerated (%)	26.66 ± 4.11	31.42 ± 3.56	31.42 ± 4.85
Developed to morula by day 5, but subsequently degenerated (%)	21.90 ± 1.55	17.14 ± 1.34	20.95 ± 3.39
Developed to blastocysts by day 8 (%)	24.76 ± 2.05	27.61 ± 2.80	22.85 ± 4.85

Data are represented as counts (n) or mean ± SEM of percentage. At each time point, conditioned media were collected from 105 samples (n = 35, triplicates for each time point of media collection). Embryo quality was assessed based on the morphological parameters.

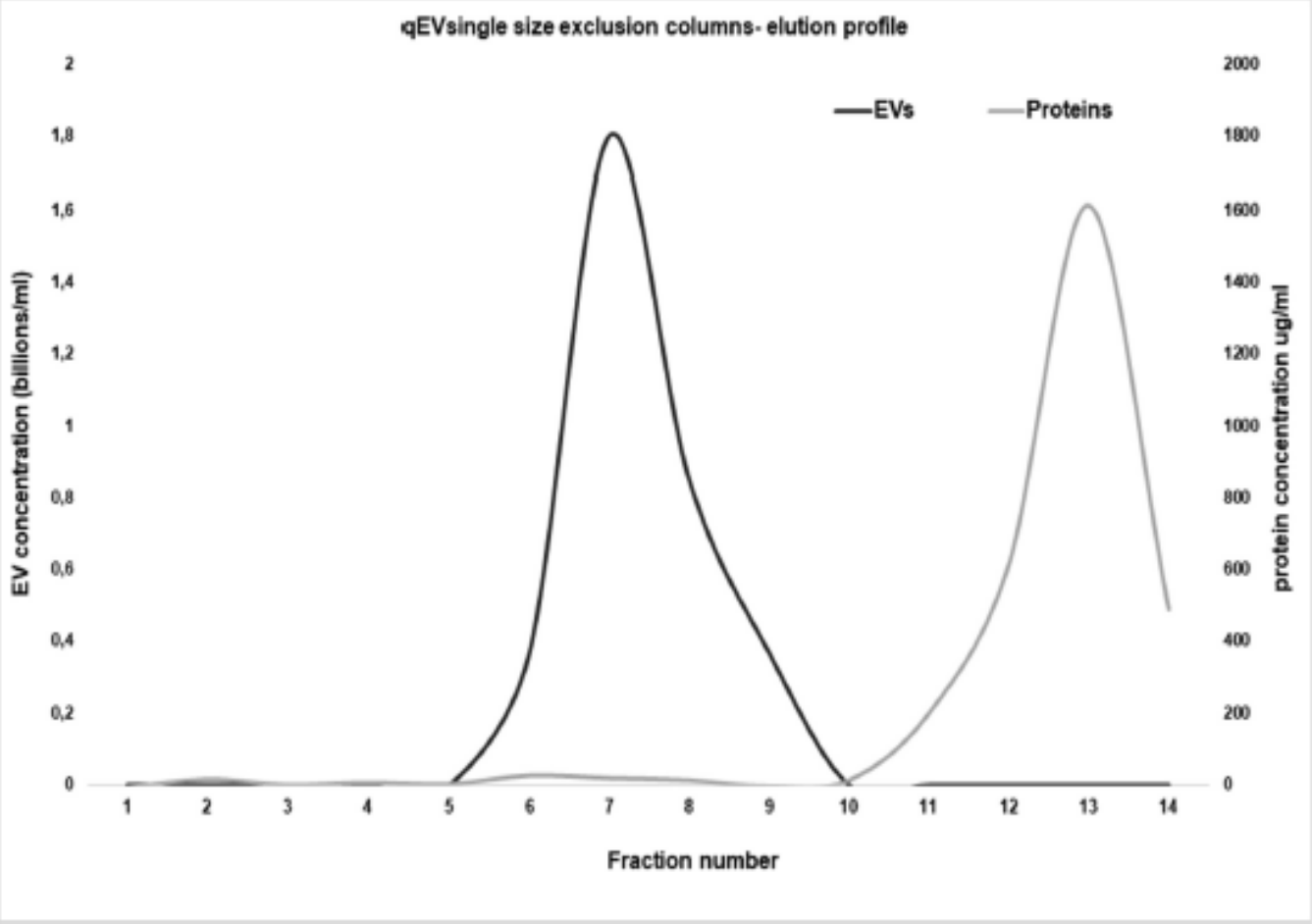

Fig. 1. Elution profile of qEVsingle size exclusion chromatography columns. The elution profile of the column was tested using RPMI-1640 supplemented with 10% FBS. Fractions 7–8 were enriched with EVs, while fractions 6 and 9 had detectable quantities of EVs. Proteins were only detectable after the 10th fraction and peaked at fraction 13. Each fraction is 200 μl.

contrast, on day 5, there were no differences in the mean diameters of EVs isolated from the good quality and bad quality embryo media. However, at day 8, the mean (±CI) diameter of EVs isolated from good quality embryo media (163 ± 7 nm) was higher compared to that of bad quality embryo media (154 ± 11 nm) ($p < 0.05$). Moreover, a gradual increase in the mean diameters of the NPs isolated from control media, as the incubation duration prolonged, was observed.

4. Discussion

Isolation of EVs from culture media conditioned by single bovine embryos is challenging due to the low sample volume and the limitations of the isolation and detection technologies. In this study, primarily, we attempted to isolate and characterize the EVs in the media conditioned by single bovine embryos along the entire preimplantation development i.e. 2 cell, morula and blastocyst stages. However, Melisho et al.(2017) isolated EVs from individually cultured bovine blastocysts, which were released between day 7–9 of *in vitro* development [21]. Moreover, those embryos had been cultured in groups till day 7 to select the blastocysts to culture them individually from day seven onwards. Moreover, recently, the same group further investigated the release of EVs from individually cultured bovine embryos during blastulation [26].

According to the guidelines of the International Society for Extracellular Vesicles (ISEV), depletion of EVs from culture media is expected to be carried out when such media are used for cell culture in EV-based research [27]. It has been previously shown that EV-depletion has an impact on the development of embryos in terms of quality [25]. As a part of this study, we carried out *in vitro* production of the embryos, both individually and in groups, in EV-depleted media to assess their development. Impaired blastocyst formation rate in EV-depleted embryo culture media (3%) compared to individual embryo culture in regular IVC media (39.3%) indicated that EV-depleted IVC media is lacking some vital elements, needed for embryos to develop to blastocysts. Due to the process of EV depletion, the media may have lost other vital factors, along with EVs, if there is any, from the media that are crucial for embryo growth. In contrast, when the embryos were cultured in groups within EV-depleted conditions, the blastocyst rate was not affected. This indicates that in the group culture systems, despite the culture media is EV-depleted, the embryo-derived EVs and other paracrine molecules would help to promote the development of embryos. Indeed, improved embryonic development had been demonstrated when *in vitro* produced (IVP) embryos were cultured in groups in many mammalian species [35,36]. Therefore, EV-depleted IVC media were not used in the subsequent individual embryo culture experiments due to: (i) the poor individual embryonic development under EV-depleted conditions; (ii) claims of the previous studies that BSA used to supplement culture media do not carry EVs [25]. Therefore, IVC media supplemented with regular BSA was used for the subsequent single embryo culture experiments.

For isolation of EVs from media, qEVsingle/70 nm SEC columns were used. These columns were designed for isolating EVs from small sample volumes of up to 150 μl and have an optimal recovery

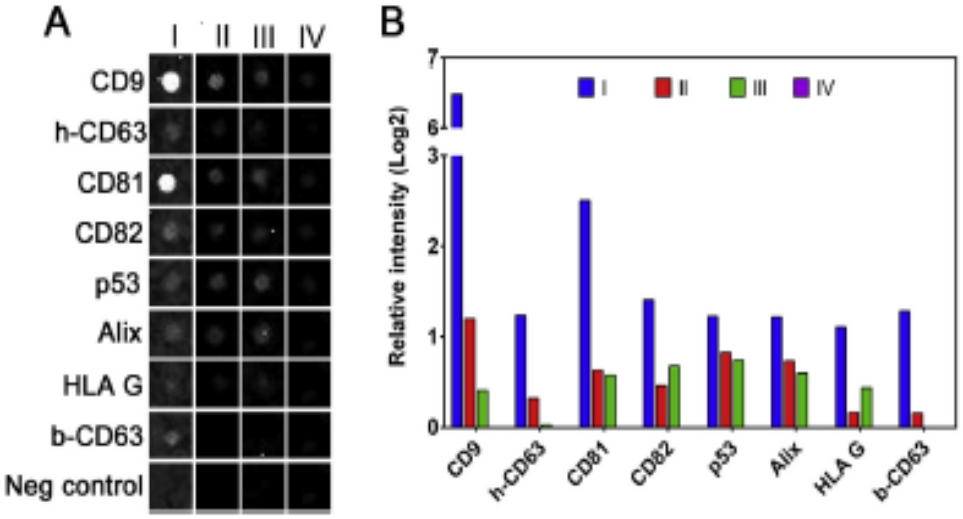

Fig. 2. Phenotyping of the EV population in bovine embryo conditioned media. EVs in the embryo conditioned media and controls were profiled using an EV Array printed with different capturing antibodies. For embryo conditioned media, culture media collected from 40 individually cultured embryos that developed to morula by day 5 were pooled (2 ml). For control media, culture media incubated for five days without embryos were pooled (2 ml). From each group, 100 μl were aliquoted and used as media before EV isolation (sample II and IV of figure 8). From the remaining media samples, EV isolation was carried out, concentrated to 100 μl each (sample I and III of figure 8). (**A**) Microarray spots obtained by fluorescent detection with anti-bovine CD9 antibody. Positive signals were only detected from the visualized spots. (**B**) Histogram of the log 2 transformed fluorescence intensities relative to the negative control spot. **Sample I:** EVs isolated from media conditioned by individually cultured bovine embryos for five days, **Sample II:** Culture media conditioned by individually cultured bovine embryos (before SEC), **Sample III:** NPs isolated from culture media incubated for 5 days without embryos, **Sample IV:** Culture media incubated for 5 days without embryos (before SEC).

range of 70–1000 nm. They can separate nanoparticulate materials within this size range from proteins and other particles in the media based on size differences. The benchmarking of the qEV-single SEC columns enabled to identify the fractions containing EVs while separating them from the proteins and other smaller molecules in the samples. We were able to isolate and detect nanoparticles in embryo conditioned media and the control media samples in all types of samples incubated up to days 2, 5 and 8.

The characterization of EVs isolated from embryo conditioned media was carried out using EV Array, NTA, TEM, and SEM. EV Array technology was used to identify the EVs using antibodies against EV-, surface- or surface-associated markers. The major advantage of this technology over other available methods to detect EV proteins is that it requires only a very limited quantity of samples [31]. The test was strongly positivity for CD9 and CD81 and less strongly positive for CD63 together with CD82, p53, Alix and HLA G. This confirmed the secretion of EVs to the culture media by individually cultured bovine embryos as early as day 5 of *in vitro* culture. However, in terms of NPs isolated from day 5 control media, only very weak signals were observed for some of the markers. It indicates that despite control media have higher levels of NPs, as measured by NTA, they do not respond to EV Array antibodies. Considering the high sensitivity of the EV Array, it is possible to conclude that there are no EVs in the control media. This finding corroborates with the findings of Pavani et al. (2018) who demonstrated that BSA lyophilized powder used for supplementing

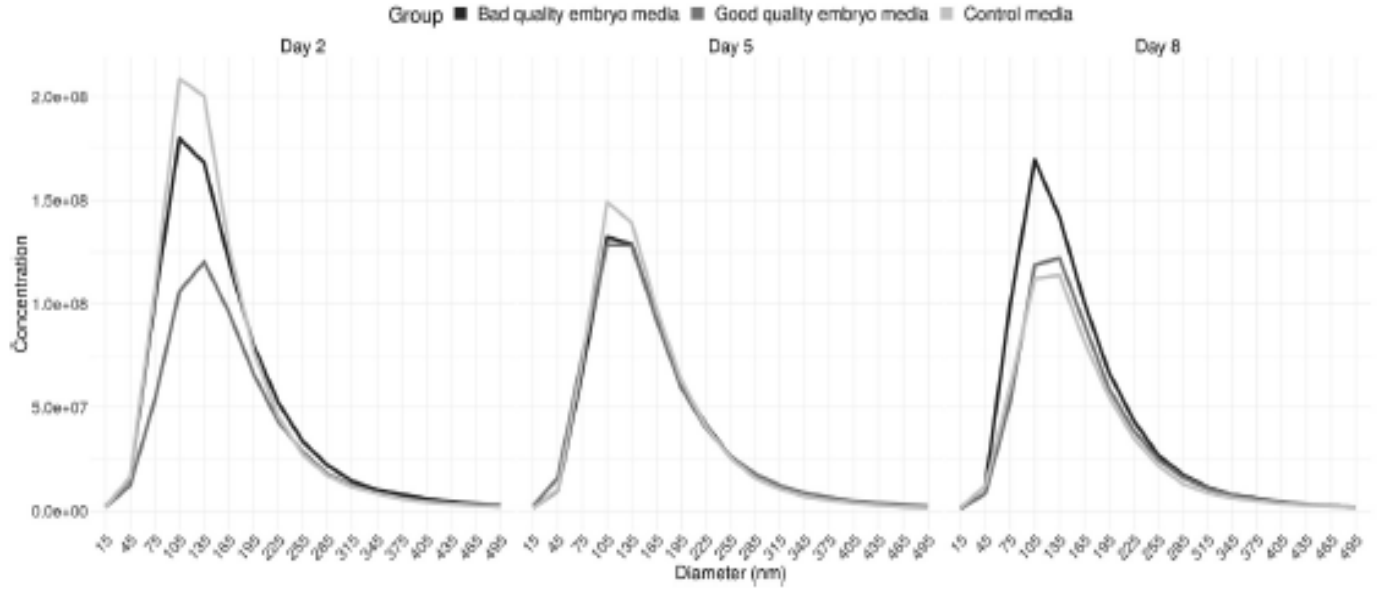

Fig. 3. Nanoparticle Tracking Analysis based characterization of average size profiles of EVs isolated from day 2, day 5, and day 8 embryo conditioned media and NPs isolated from control media samples. "Day 2 good quality embryo media" (n = 23); "Day 2 bad quality embryo media" (n = 22); "Day 2 control" (n = 15); "Day 5 good quality embryo media" (n = 25); "Day 5 bad quality embryo media" (n = 19); "Day 5 control" (n = 15); "Day 8 good quality embryo media" (n = 24); "Day 8 bad quality embryo media" (n = 20); "Day 8 control" (n = 15). Concentrations are expressed as particles/ml.

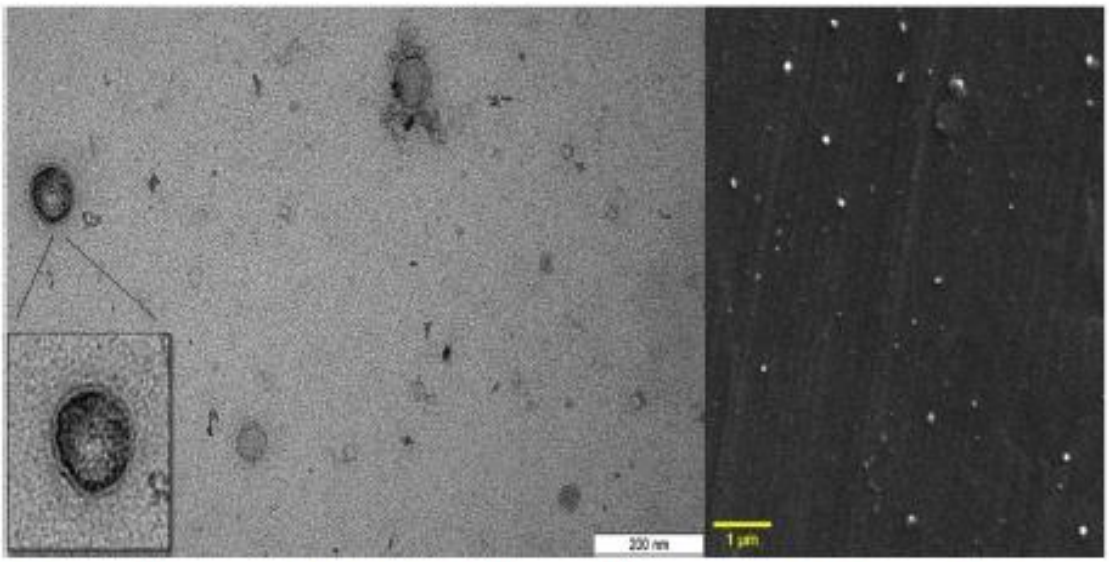

Fig. 4. Electron microscopy-based characterization of EVs. (**A**) TEM imaging of EVs isolated from a pool of individually cultured bovine embryo (n = 60) conditioned media developed to morula by day 5. The majority of EVs were in a size range of 50–150 nm. The magnified part of the image zoomed in to an individual EV exhibiting cup-like shape typical to EVs, that is associated with sample processing. The Scale bar is 200 nm. (**B**) SEM imaging of EVs isolated from a pool of individually cultured bovine embryo (n = 60) conditioned media developed to morula by day 5. White dots in the dark background indicate EVs which are heterogeneous in terms of their size. Scale bar is 1 µm.

IVC media did not contain EVs [25]. To characterize the BSA derived NPs, they carried out western blot assays and immunogold staining

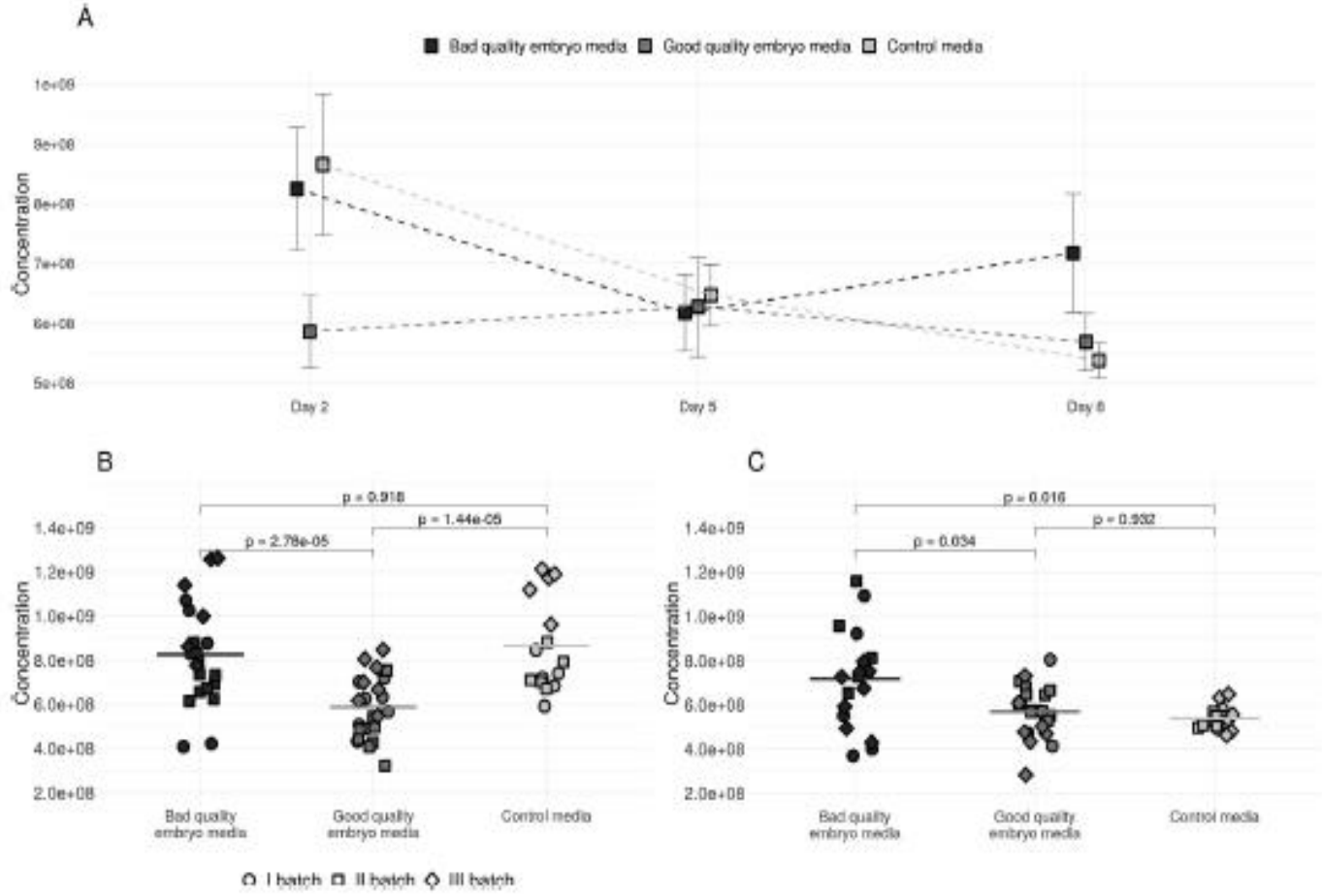

Fig. 5. Changes in the concentrations of EVs (per ml) isolated from culture media conditioned by individually cultured bovine embryos and the NPs (per ml) isolated from control media. (**A**) Mean concentrations of EVs isolated from media conditioned by individually cultured bovine embryos at day 2, 5, and 8 post-fertilization and the NPs isolated from control media. (**B–C**) Comparison of the concentrations of EVs isolated from media conditioned by individually cultured bovine embryos at day 2 and day 8, respectively. "Day 2 good quality embryo media" (n = 23); "Day 2 bad quality embryo media" (n = 22); "Day 2 control" (n = 15); "Day 5 good quality embryo media" (n = 25); "Day 5 bad quality embryo media" (n = 19); "Day 5 control" (n = 15); "Day 8 good quality embryo media" (n = 24); "Day 8 bad quality embryo media" (n = 20); "Day 8 control" (n = 15). Different shapes in **B** and **C** indicate the measurements of the samples from 3 replicates and each such shape represents the mean of the three measurements of each biological sample. Differences were considered to be statistically significant if $p < 0.05$. Error bars are 95% confidence intervals.

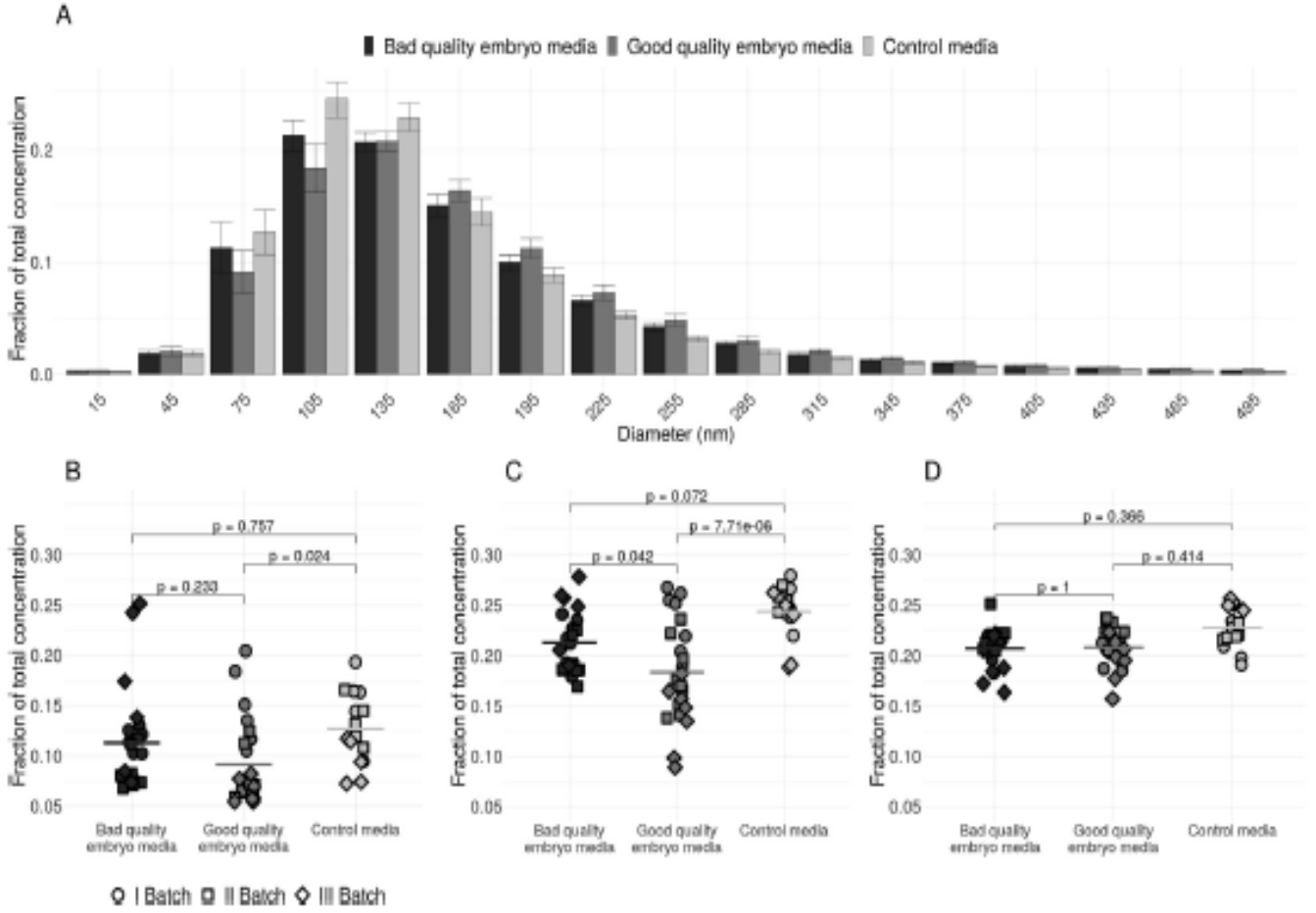

Fig. 6. Comparison of the concentrations of EVs (per ml) isolated from day 2 embryo conditioned media and the NPs (per ml) isolated from day 2 controls, based on size ranges. (A) The overall distribution of the particles isolated from day 2 good quality embryo media (n = 23), Day 2 bad quality embryo media (n = 22) and day 2 controls (n = 15). Data are presented as fractions of the total concentration. (B, C, D) Comparison of the concentrations of EVs/NPs isolated from day 2 embryo conditioned media and controls, based on size ranges: B; 61–90 nm, C; 91–120 nm, D; 121–150 nm. Different shapes in B, C, and D, indicate the measurement of the samples from three replicates and each such shape represents the mean of the three measurements of each biological sample. Differences between groups were considered to be statistically significant if $p < 0.05$. Error bars are 95% confidence intervals.

of these particles and concluded that BSA derived NPs were unlikely to include EVs [25]. Stolk et al. (2015) isolated EV-like NPs from BSA ('sham' BSA EVs) and tested them in comparison to the mesenchymal stem cell (MSC)-derived EVs [37]. They reported that such 'sham' BSA EVs were different from the MSC-derived EVs in terms of surface protein markers. Furthermore, the EV Array used in our study demonstrated the enrichment of EVs in samples subsequent to purification with qEVsingle columns and their concentration with Amicon® Ultra-2 10 K centrifugal filters.

NTA based characterization of the EVs isolated from day 2, 5 and, 8 embryo conditioned media showed that most of the particles are distributed within the 30–300 nm size range. Similarly, the control media also had NPs most abundantly in the same size range indicating the non-specificity of qEVsingle SEC columns for EVs. With regards to the sample measurement using NTA, in the scatter mode, it tracks nanoparticles irrespective of whether they are EVs or not. Therefore, NTA-ZetaView® can not specifically identify NPs as EVs. TEM imaging showed EVs in size range of 50–150 nm. However, EVs were not abundant in these images, which is possibly due to the low concentration of EVs in embryo conditioned media or loss during processing. The SEM also visualized nearly round-shaped EVs isolated from embryo conditioned media.

Biological analyses are evolving towards single-cell technologies, such as single-cell genomics, that provide a clearer understanding of complex biological processes at a single-cell level. Preimplantation embryos, such as morula, are composed of a limited number of pluripotent cells. Therefore, studying single embryos warrants single cell-based technologies for better results. The ISEV has proposed Minimal Information for Studies of Extracellular Vesicles ("MISEV") in 2014 and updated in 2018 [27]. As most of the EV studies are based on EVs deriving from bulk tissues and cell cultures with higher cell quantities, achieving such requirements is not much of an issue for those studies. In contrast, when dealing with single cells or a limited number of cells such as preimplantation embryos, this is a very challenging task due to the scarcity of the materials and the limitations of the EV-isolation and detection technologies.

Considering the results of SEM, TEM, and the EV Array analysis, we conclude that the NPs present in the culture media conditioned by embryos in the current investigation is indeed EVs. Hence, it is justified to use the term EVs for NPs purified from bovine embryo conditioned culture media. Such proof of evidence was not present for the NPs isolated from the control media; hence they were considered as NPs.

It was observed that the mean EV concentration of the culture media conditioned by day 2 good quality embryos were lower compared to day 2 bad quality embryos, considering the concentration of NPs in day 2 controls as the baseline/background. At day 2, the embryos of both these groups were morphologically similar though their subsequent development was different. Therefore, the concentration of EVs isolated from day 2 conditioned media foreshadowed the prospective development of the embryos, as those

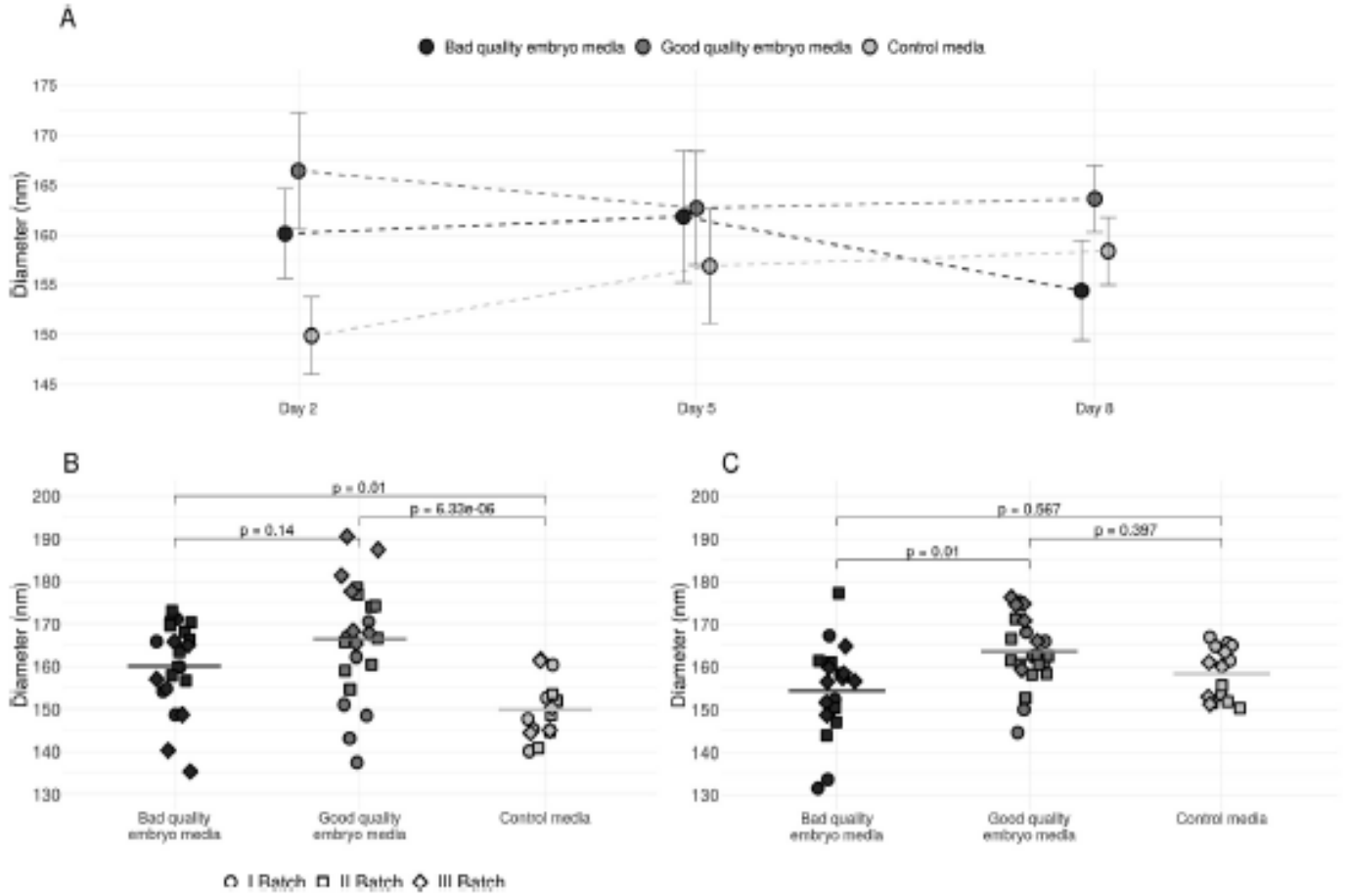

Fig. 7. Mean diameters of EVs isolated from culture media conditioned by individually cultured bovine embryos and the NPs isolated from control media. **(A)** Mean diameters of EVs isolated from media conditioned by individually cultured bovine embryos at day 2, 5, and 8 post-fertilization and NPs isolated from controls. "Day 2 good quality embryo media" (n = 23); "Day 2 bad quality embryo media" (n = 22); "Day 2 control" (n = 15); "Day 5 good quality embryo media" (n = 25); "Day 5 bad quality embryo media" (n = 19); "Day 5 control" (n = 15); "Day 8 good quality embryo media" (n = 24); "Day 8 bad quality embryo media" (n = 20); "Day 8 control" (n = 15). **(B and C)** Statistical comparison of the mean diameters of EVs isolated from media conditioned by individually cultured bovine embryos at day 2 and day 8, respectively. Different shapes in B and C indicate the measurement of the samples from 3 replicates and each such shape represents the mean of the three measurements of each biological sample. Differences between groups were considered to be statistically significant if $p < 0.05$. Error bars are 95% confidence intervals.

embryos producing fewer EVs were more likely to develop to blastocysts. Moreover, it is possible that while developing embryos release EVs to the culture media, they uptake other NPs existing in the culture media. Similarly, they may reuptake the released EVs. Therefore, we propose the hypothesis of a possible dynamic exchange of NPs and EVs between the embryo and its surrounding microenvironment, resulting in the net decrease of concentration of particles in the embryo conditioned media.

The media conditioned by embryos that degenerated after reaching the morula stage (Day 8 bad quality embryo media) had a higher mean EV concentration compared to the embryos that developed to blastocysts by day 8 (Day 8 good quality embryo media). It is possible that when the embryos are degenerating, they release more EVs to the media. This observation corroborates with a recent study by Melisho et al. (2019), who reported that non-viable early blastocysts derived EVs were higher in concentration compared to viable early blastocyst derived EVs [26]. In contrast, the current study has investigated three morphological levels of preimplantation embryo development i.e. 2 cell stage, morula and blastocyst. A previous study by the same researchers showed that the concentration of vesicles released by IVF blastocysts with arrested development is higher compared to competent PA blastocysts [21]. Contrary to day 2 and day 8, mean concentrations of EVs isolated from day 5 good quality and bad quality embryo media were not different. Subsequent analysis of particle concentrations based on size ranges demonstrated differences in the concentrations of EVs between good quality and bad quality embryo media in day 2 and day 8 samples. This observation further proves the physical differences in EVs isolated from good quality and bad quality embryo media.

In general, the sizes of most of the EVs isolated from embryo conditioned media ranged between 30 and 300 nm. This size range corroborates with previous studies which reported bovine embryo-derived EVs are in the similar size range [21,25]. The average diameters of EVs isolated from day 2 good quality and bad quality embryo conditioned media were higher compared to the NPs in day 2 control media. Moreover, the difference of the average diameters of EVs isolated from day 8 good quality embryo media and day 8 bad quality embryo media were statistically significant, with bigger size demonstrated for good quality embryo-derived EVs in our study. However, the study by Melisho et al. (2017) did not observe a difference in EV sizes released by competent and non-competent blastocysts [21]. This could be due to the differences in the EV isolation methods and NTA methods used in the 2 studies.

The limiting factor for the exchange of EVs between the embryo and its environment (including the culture media), compared to the somatic cells, is the presence of *zona pellucida* (ZP). The ZP is an outer covering of mammalian oocytes and early embryos consisting of highly modified glycoproteins [38]. Its porosity and permeability have been thoroughly studied [39,40]. The ultrastructure of the ZP

of *in vivo* and *in vitro* created zygotes are different, and *in vitro* zygotes were found to have higher pore density [41]. An ultrastructural study of the ZP in bovine embryos showed that the average number (per 5000 μm^2) and average diameter of the outer pores in ZP varied between different early embryonic development stages with mean outer pore diameters of zygotes and morula being ca 223 nm and 155 nm, respectively [42]. The average diameters of the EVs isolated from all the 3 stages of embryos were of similar sizes and supported the possibility of permeation by most of the NPs, including EVs, across the ZP. However, the permeability of molecules across the ZP is determined not only by their size but also by their biochemical and physicochemical properties. Turner and Horobin (2004) demonstrated this in mouse embryos using colored probes and could predict the passage of lipid and lipid-containing molecules [40]. However, this model could not predict the permeability of ZP to proteins and nucleic acids. Therefore, pore sizes and the permeability of the ZP would decide the size and type of NPs, including EVs, that would pass in or out of the embryos across the ZP.

The NTA-based quantification of the EVs in the conditioned media, in combination with morphological grading of embryos, can be used to develop a scoring system that would grade the embryos for uterine transfer. Such kind of combined analysis would enhance embryo grading process and would make it more trustworthy compared to simple morphology-based embryo grading, and thus, would assist embryologists in selecting the best embryos for transfer. In a recent study by Melisho et al. (2019), a novel model has been constructed to identify viable embryos using a combination of EV characteristics and blastocyst morphokinetics [26]. This kind of combined non-invasive embryo scoring system would enhance the outcome of IVF treatment in the future. In that regard, the presence of regular embryo culture media, rather than EV-depleted media, would be more supportive as it provides an optimum environment for the embryo culture. Moreover, studying the surface characteristics of these particles, such as surface proteome, zeta potential, and the molecular cargo, such as nucleic acids and proteins, would provide more information regarding the usability of EVs as a possible biomarker of embryo developmental capacity.

In conclusion, the depletion of EVs from the culture media had a negative impact on the individual embryo culture, as was evidenced by a drop in the blastocyst formation rate in EV-depleted media. Individually cultured preimplantation bovine embryos secrete EVs to the culture media. The concentrations of nanoparticles in the embryo conditioned media are modulated based on the developmental stage of the embryo and embryo quality, and thus, may indicate embryo's further potential for development. Further advancement of the current technologies is needed to enable profiling EVs deriving from single cells such as zygotes and 2 cell staged embryos. This would provide more useful information about the early preimplantation embryo quality and lead to identifying novel biomarkers that would be decisive in selecting embryos for transferring to the uterus.

Author contributions

M.N., K.D, and A.F and U.J developed the concept; K.D., M.N., and A.F. designed the experiments; M.N. developed and optimized individual embryo culture systems and produced embryos *in vitro*, Y.R. produced embryos for TEM and EV Array-based characterization; K.D. and A.L. experimented for the optimum EV purification system for single embryos; K.D. carried out EV purification from embryo conditioned media and NTA analysis, and sample preparation for EV characterization by EV Array, TEM and SEM; K.G. and G.B. contributed for EV purification and NTA analysis; M.M.J. and R.B. performed EV Array and provided written materials in methods and results; A.A. performed TEM and provided written materials in methods and results; S.B. performed SEM and provided written materials in methods and results; F.L. and K.D. carried out data analysis, K.D. wrote the initial draft of the manuscript with conceptual contributions from A.F., M.N., K.G., J.V. and G.B.; and all the authors participated in discussing the initial draft and agreed to the final manuscript; A.F., U.J., and A.S. supervised.

Declaration of competing interest

The authors have no conflicts of interest.

CRediT authorship contribution statement

Keerthie Dissanayake: Conceptualization, Methodology, Validation, Investigation, Data curation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Monika Nõmm:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Freddy Lättekivi:** Formal analysis, Data curation, Writing - original draft, Visualization, Writing - review & editing. **Yosra Ressaissi:** Investigation, Writing - review & editing. **Kasun Godakumara:** Investigation, Writing - review & editing. **Arina Lavrits:** Validation, Writing - review & editing. **Getnet Midekessa:** Investigation, Writing - review & editing. **Janeli Viil:** Validation, Writing - review & editing. **Rikke Bæk:** Investigation, Writing - review & editing. **Malene Møller Jørgensen:** Investigation, Writing - review & editing. **Sourav Bhattacharjee:** Investigation, Writing - review & editing. **Aneta Andronowska:** Investigation, Writing - review & editing. **Andres Salumets:** Writing - review & editing, Supervision, Resources, Funding acquisition. **Ülle Jaakma:** Methodology, Project administration, Resources, Writing - review & editing, Supervision, Funding acquisition. **Alireza Fazeli:** Conceptualization, Methodology, Project administration, Writing - original draft, Resources, Writing - review & editing, Supervision, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2020.03.008.

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In vitro embryo production requires an enriched microenvironment with various vital cell-secreted factors. *In vitro* cultured single bovine embryos have demonstrated lower blastocyst rate compared to grouped cultured embryos. We assumed that extracellular vesicles (EVs) within an embryo culture system may affect normal *in vitro* development. This study aimed to assess the supplementation effects of bovine embryo-derived EVs on the development of individually cultured bovine embryos. Bovine oocytes were *in vitro* maturated (IVM) for 24 h and then *in vitro* fertilized (IVF). In preliminary experiments, we established that group cultured embryos in EV depleted Bovine Serum Albumin (BSA) media successfully completed their development; while single cultured embryos were only able to reach the morula stage and then degenerated. Hence, we tested EVs supplementation effects in droplets of EV depleted BSA media covered by mineral oil. EVs used for supplementation were produced from single embryos cultured for 8 days in droplets of BSA culture media under mineral oil. Conditioned medium was collected on day 5. EVs were purified, using Izon columns, from embryos which reached the blastocyst stage and embryos which cleaved on day 2 then degenerated. Non-EV supplemented single embryos cultured in BSA media were considered as control. Purified EVs were characterized by nanoparticle tracking analysis and transmission electron microscope (TEM). A total of 8.8 $\times 10^6$ particles/ml, which we assumed to be the approximate amount of EVs that a single embryo may release during *in vitro* culture, was supplemented to each droplet on day 4 post-fertilization. Cleavage rates were 70 and 80% for the supplemented groups and 86% for the control. Morula rates were 40%, 47%, and 47% respectively. No blastocyst was observed within the supplemented groups while the control group counted 33% of blastocysts. Our study suggests that BSA EVs support single cultured embryos to complete their development and that a single embryo needs a significant amount of EVs to reach the blastocyst stage. More researches are needed to understand the role of culture media EVs in supporting single embryo development.