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Innovative technologies for optimizing the in vitro culture of mammalian embryos

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1. Synopsis:

Since the birth of Louise Brown in 1978, assisted reproductive technologies (ARTs) have become indispensable in the treatment of infertility. Despite the advancements and success of ARTs, embryos produced in vitro (IVP) exhibit lower developmental competence compared to their in vivo-derived (IVD) counterparts [1]. A critical challenge with IVP embryos lies in the fact that post-fertilization culture conditions can significantly influence embryo quality. Key factors affecting embryo competence include the composition of the culture medium, embryo density (i.e., the number of embryos per culture medium volume), temperature, and gas balance within the incubator [2]. The main goal of in vitro embryo production is to closely mimic the in vivo environment to generate high-quality embryos capable of resulting in viable births.

In vivo, embryos develop within confined microenvironments, such as the oviduct and uterus. These natural cavities are rich in maternal factors, secreted by somatic cells of the oviductal (OF) and uterine fluid (UF), as well as embryo-derived signals. These factors, present as soluble molecules or molecular cargos enclosed within extracellular vesicles (EVs), play critical roles in promoting preimplantation embryo development and enhancing developmental competence [3-5]. Conversely, in vitro embryo culture lacks both the confined environment and the bidirectional communication between embryos and the maternal reproductive tract. As a result, individually cultured embryos often show compromised development [6]. To counteract this, embryos are commonly cultured in groups to enrich the environment with beneficial factors secreted by the embryos themselves [7-8]. However, the trend in both human and animal ART is shifting toward selecting and transferring a single euploid, competent blastocyst, aided by novel non-invasive embryo quality markers. In human clinical embryology, embryos are often cultured in small numbers per patient, making individual embryo culture an inevitable choice. Individual embryo culture systems could offer advantages over group culture, as they allow for the simultaneous analysis of spent media for metabolomic, proteomic, and non-invasive preimplantation genetic testing (niPGT), which are rapidly emerging technologies. Despite these benefits, individual embryo culture has generally shown poorer outcomes compared to group culture in the same medium volume both in human and animal ART [9].

Against this backdrop, the aim of my PhD thesis is to establish and validate an innovative method to improve the in vitro culture of individual embryos, more closely replicating the natural microenvironment of the female reproductive tract to enhance their developmental competence.

Chapter 2 explores the main factors influencing in vitro embryo culture. Specifically, it compares the characteristics and developmental outcomes of embryos produced in vivo and in vitro. It also examines the dynamic physicochemical conditions embryos experience during

their journey and development in the maternal tract, as well as the standard in vitro culture conditions. While time-lapse imaging systems for assessing embryo morphokinetics have gained widespread adoption, their utility in reliably identifying the highest quality embryos remains limited. Alternatively, omics-based approaches, which analyze the molecular composition of spent culture media and correlate it with embryonic euploidy, may soon offer a more robust solution. This highlights the need for innovative individual embryo culture systems that avoid the detrimental effects associated with isolated culture, ensuring embryo individuality while supporting the effective application of advanced methodologies [10].

Chapter 3 investigates the development of an individual embryo culture system within a confined environment using extremely reduced culture volumes, aiming to maintain developmental competence comparable to conventional group culture. The developmental competence of blastocysts produced under various culture conditions—conventional group culture (GC), semi-confined group culture (MG), and individual culture (MS)—was evaluated. Parameters such as the number of TUNEL-positive cells, lipid content, mitochondrial function, and mean cell number were analyzed in detail [11].

However, recreating a confined microenvironment for in vitro embryo development represents only one aspect of the conditions embryos experience during their transit through the maternal reproductive tract. Another critical factor is the embryo's exposure to extracellular vesicles (EVs) derived from the oviduct and uterus. To address this, **Chapter 4** examines the combined effects of a confined environment and sequential exposure to oviductal and uterine EVs, exploring their potential synergistic impact on in vitro bovine embryo culture. Developmental competence was characterized in depth under various culture conditions—group culture (GC) and microwell single culture (MS), with and without supplementation of EVs-OF and EVs-UF. Key parameters such as TUNEL-positive cells, lipid content, mitochondrial function, and mean cell number were assessed to elucidate these effects.

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2. Chapter 1:

Review

In vitro culture of mammalian embryos: is there room for improvement?

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2.1 Abstract:

Preimplantation embryo culture, pivotal in assisted reproductive technology (ART), has lagged in innovation compared to embryo selection advancements. This review examines the persisting gap between in vivo and in vitro embryo development, emphasizing the need for improved culture conditions. While in humans this gap is hardly estimated, animal models, particularly bovines, reveal clear disparities in developmental competence, cryotolerance, pregnancy and live birth rates between in vitro-produced (IVP) and in vivo-derived (IVD) embryos. Molecular analyses unveil distinct differences in morphology, metabolism, and genomic stability, underscoring the need for refining culture conditions for better ART outcomes. To this end, a deeper comprehension of oviduct physiology and embryo transport is crucial for grasping embryo–maternal interactions' mechanisms. Research on autocrine and paracrine factors, and extracellular vesicles in embryo–maternal tract interactions, elucidates vital communication networks for successful implantation and pregnancy. In vitro, confinement, and embryo density are key factors to boost embryo development. Advanced dynamic culture systems mimicking fluid mechanical stimulation in the oviduct, through vibration, tilting, and microfluidic methods, and the use of innovative softer substrates, hold promise for optimizing in vitro embryo development.

2.2 Introduction

Although several studies contributed to the design of universal or sequential culture media tailored for preimplantation mammalian embryos [1], in vitro embryo culture was essentially unchanged since the beginning of human in vitro fertilization and is still performed in relatively large media volumes under static conditions [2]. It has been shown that numerous factors and mishandlings during standard culture procedures in these systems can pose a threat to the successful development of embryos [3,4]. However, the fundamental culture conditions persist unchanged, as if the inherent biological constraints on blastocyst development from the produced zygotes had already been reached. In comparison to the few efforts devoted to the improvement of the physico-chemical embryo culture environment, a range of technologies and non-invasive markers have been increasingly developed for selecting embryos endowed with the maximal potential to implant and generate live births [5,6,7,8].

Many researchers acknowledge the significant disparities between the physico-chemical conditions of in vitro preimplantation embryo development and the dynamic environments encountered in vivo as the embryo progresses from fertilization in the Fallopian tube to implantation in the uterus. Despite this recognition, there remains uncertainty regarding the extent to which the developmental potential of in vitro cultured embryos is compromised

compared to their in vivo counterparts, particularly in humans with limited data compared to animal models. The primary objective of this paper is to review studies focusing on (1) the characteristics and performance of embryos developed in vivo vs. in vitro; (2) the dynamic physico-chemical conditions experienced by embryos during transit and development within the maternal tract; (3) the standard in vitro culture conditions; and (4) the dynamic culture systems designed in research studies and their effects on embryo development.

2.3 Natural versus In Vitro Conceptions in the Human

The efficacy of conception in vivo in humans is a highly debated and speculative topic based on relatively few old data. We still do not have definitive estimates of embryo mortality from fertilization to birth during in vivo conception in a fertile women cohort. Moreover, since the earlier marker of pregnancy is represented by a rise in hCG, we have different figures of what occurs from the second week of gestation, i.e., from implantation to birth, but still lack evidence on what has been called the black box of early pregnancy loss that covers the period from fertilization to implantation. Speculative estimates of this currently unmeasurable loss in vivo range from 10 to 70% [9,10,11,12,13].

The best estimates of the efficacy of in vivo conceptions in terms of live births per implantation are provided by three old, well-designed studies showing that about two-thirds of the in vivo conceptions detected by elevated hCG at least 1 week after ovulation result in a live birth [14,15,16]. Since these studies do not measure the pre-implantation embryo loss, embryo mortality from fertilization to birth in the human is still not known. Although speculative estimates of embryo loss before and during implantation range from 30 to 75% [17], it is currently accepted that up to 30% of conceptions do not complete implantation [18]. Therefore, it has been suggested that about 30% of the in vivo conceptions result in a live birth due to a 30% loss before implantation, a further unrecognized 30% loss after implantation but before the missed menstrual period, and a final 10% loss due to clinical miscarriage [18]. The highest estimates of live births during natural conception have been recently provided by Jarvis et al. (2016, 2017) [13,17], who re-analyzed the aggregate data from the three reliable hCG studies. Jarvis hypothesized that the criteria adopted to select fertile women cohorts did not ensure the exclusion of a proportion of sub-fertile or infertile couples. Therefore, he identified sub-cohorts of sub-fertile women to estimate the decline in pregnancy rates across successive cycles. By excluding these sub-cohorts from the initial population of women, it was determined that 40–60% of conceptions resulted in live births when considering only presumably fertile women. While human reproduction in vivo appears relatively inefficient compared to other mammals [19], studies measuring reproductive efficiency on an egg-by-egg basis suggest even lower

success in vitro [20,21,22]. However, comparing reproductive efficiency in vitro vs. in vivo requires consideration of several biases. In vivo estimates typically involve unstimulated, fertile women within the appropriate reproductive age range. Conversely, patients undergoing assisted reproduction are sub-fertile and more reproductively aged. According to Patrizio and colleagues (2007) [22], only about 5% of harvested mature oocytes led to live births. However, this analysis was based on 31 cycles involving 26 patients (with a mean age of 37 ± 3.7 years), including 10 cycles with recurrent pregnancy loss, 15 cycles with advanced maternal age, and 6 cycles for unexplained recurrent pregnancy failure. Jones et al. (2010) [23] reported a 6% efficiency of human reproduction in vitro for an age group of 25–29 years and suggested that IVF is only one-fifth as efficient as natural human reproduction. In conclusion, the differences between in vivo and in vitro conceptions in humans are still inconclusive due to the scarcity of data available in literature.

2.4 Natural versus In Vitro Conceptions in Animal Models

The success of the basic and straightforward embryo culture conditions in assisted reproductive technology (ART) is demonstrated by the birth of over 10 million babies. However, a fundamental yet unanswered question persists: to what extent do current in vitro embryo culture conditions replicate those experienced by embryos during in vivo development? In other words, is there room for improvement in the developmental rates and quality of in vitro-produced embryos to enhance clinical success rates?

While data from human ART are inconclusive, more stringent and unbiased experiments have been conducted using animal models. Studies in the mouse model showed several differences between in vitro-produced (IVP) and in vivo-derived (IVD) embryos. IVP embryos had altered ICM and TE cell numbers, lower hatching rates, increased apoptosis, lower cryotolerance, altered metabolism and oxidative stress, and differential gene expression (Table 1) [24,25,26,27,28,29,30].

Table 1. *Mouse animal model: differences between IVP and IVD embryos. ICM, inner cell mass; TE, trophectoderm; ROS, reactive oxygen species; GSH, reduced glutathione; Mmp-9, matrix metalloproteinase-9.*

Features	IVP versus IVD Embryos	References
Blastocyst cell numbers	Lower TE, lesser reduction of ICM	[24]
	Lower cell numbers	[25]
	Altered TE and/or ICM cell numbers	[26]
Blastocyst hatching	Lower hatching rate	[27]
Apoptosis	Increased TUNEL-positive cells	[28]
Cryotolerance	Lower survival of 2-cell embryos after warming	[29]
Energy metabolism	Decreased mitochondrial activity and increased glycolytic function	[25]
	Mitochondrial dysfunctions: dysregulation of 806 mitochondria-related genes validated through cytological/molecular analysis	[28]
Oxidative stress	Increased ROS; decreased GSH	[25,28]
	Increased oxidative damage of DNA, lipids, and proteins	[25]
Gene expression	Lower (~10.7-fold) expression of Mmp-9 required for successful implantation and trophoblast invasion	[27]
	Differential expression of 1000 genes involved in proliferation, apoptosis, and morphogenetic pathways	[29]
	Differential expression of 1912 genes (29 > 4-fold) involved in proliferation, apoptosis, and morphogenetic pathways	[24]
	Differential expression of genes involved in metabolism, proliferation, apoptosis, and morphogenetic pathways	[26]
	Differential expression of genes involved in cytoskeletal organization	[30]

Bovine is considered a more suitable animal model for humans in many respects. In vitro embryo culture conditions in both bovines and humans exhibit striking similarities, with many of the technologies initially developed in cattle serving as the foundation for human ART [24]. Furthermore, bovine and human embryo development display more similarities than those observed between human and murine embryonic development [25,26,27,28]. Bovines are excellent models for studying early embryo–maternal communication in humans due to their analogous cycle length, pregnancy duration, usual single offspring, and genetic imprinting patterns [29,30]. Bovine reproduction has been extensively studied for its commercial significance and the lack of ethical constraints that limit experimental studies on human embryos. A unique opportunity for comparing the competence of IVP and IVD bovine embryos arises from data collected in the cattle embryo industry. The 31st annual report of the International Embryo Technology Society (IETS) revealed that over 2 million embryos were collected or produced in cattle in 2021 [31]. Although the number of transferrable IVP embryos

exceeded that of IVD embryos for the first time in 2016 [32], accounting for the 79.7% of all transferrable cattle embryos in 2021 [31], the pregnancy and live birth rates of IVP embryos remain lower compared to their in vivo counterparts. On average, pregnancy rates after transfer of IVP or IVD bovine embryos are 40.1% and 64.1%, respectively [33], demonstrating that the competence of IVP embryos consistently lags behind that of IVD embryos [34]. In addition, IVP embryos have a reduced cryotolerance and a higher lipid accumulation compared to IVD embryos [35]. This is also exemplified by the IETS 2021 data showing that frozen IVD embryo transfer is still preferred compared to frozen IVP embryo transfer (60.5% vs. 41.3%) [31]. During development, lipids are used by the mitochondria to increase the ATP production and promote compaction and blastocyst formation [36]. However, lipids can accumulate in the embryo due to an increased uptake from the culture environment [37] or to a decreased activity of mitochondria [38]. Gad et al. (2012) [39] reported that embryos cultured in vitro at the time of embryonic genome activation are particularly affected by a down-regulation of lipid metabolism-related genes compared to embryos developed in vivo. In agreement with those findings, bovine embryos generated by IVM/IVF followed by in vivo culture in the ewe oviduct had similar cryotolerance and pregnancy rates after frozen embryo transfer than IVD embryos [40]. Havlicek et al. (2009) [41] using zygotes produced in vitro and then cultured in vivo also demonstrated that the duration of in vivo culture is crucial for cryotolerance. On the cellular and molecular level, IVP bovine embryos differ from their in vivo counterparts in many respects. Clear differences in morphology and ultrastructure [42,43,44,45], inner cell mass/trophectoderm allocation [46], intercellular connectivity [47], lipid content and profiles [48], energy metabolism [49,50,51], transcriptomic [39,52,53,54], proteomics [55], chromosome aberrations [56,57], and methylation patterns [58] underlie the higher developmental competence of IVD compared to IVP embryos. Rizos et al. (2002) [45] demonstrated that in vitro embryo culture and in vivo development produced similar blastocyst rates, but IVD blastocysts had a dramatically higher competence as shown by the increased survival after cryopreservation. Differences between IVP and IVD bovine embryos are summarized in Table 2.

Table 2. *Bovine animal model: differences between IVP and IVD embryos. ICM, inner cell mass; TE, trophectoderm*

Features	IVP vs. IVD Embryos	References
Pregnancy Rate	~ 40.1 vs. 64.1%	[33]
Blastocyst Rate	Lower blastocyst yield and quality	[45]
Hatching Rates	Lower hatching rate	[45]
Blastocyst Cell Number	Altered ICM/TE allocation	[46]
Apoptosis	Higher apoptosis rate	[35]
Cryotolerance	Reduced blastocyst cavity re-expansion after vitrification	[35]
	Increased cryodamage	[43]
	Lower survival after warming	[45]
Lipid Content	Higher lipid accumulation and down-regulation of lipid metabolism genes	[35,39,48]
Developmental Competence	Lower developmental competence	[39,52–58]
Aneuploidy	Higher chromosome aberrations	[56,57]
Ultrastructure	Differences in morphology and cell structure	[42–45]
	Reduced intercellular connectivity	[47]
Energy Metabolism	Differences in energy metabolism profile	[51]
Transcriptomic	Reduced oxidative stress response	[39]
	Down-regulation of lipid metabolism genes	[39]
	Differential expression of 793 genes, differential alternative splicing of 873 genes	[52]
	Lower abundance of peroxiredoxin 1, heat shock protein 70.1, growth and differentiation factor 9, and maternal antigen	[53]
Epigenetics	Hypomethylated genomic loci in blastocysts	[58]
	Increased Large Offspring Syndrome	[54]
Proteomic	Lower abundance of proteins related to carbohydrate metabolism and cytoplasmic cellular components	[55]
	Higher abundance of translation-related proteins after the morula stage	

The contribution of the natural environment to the embryo competence is also exemplified by the enhanced quality of IVP embryos when cultured short- or long-term in vivo in homologous or heterologous oviducts [40,41].

In conclusion, data in animal models highlight clear differences between the consequences of in vivo and in vitro environment on embryo developmental competence.

2.5 Can In Vitro Culture Increase Embryo Aneuploidy?

2.5.1 Human

Embryo aneuploidy is a major contributor to low human fecundability both in vivo and in vitro. The overall incidence of aneuploidy in newborns following natural conception is less than 1% due to the progressive elimination of aneuploid embryos through pre- and post-implantation loss. Nondisjunction of bivalents and premature separation of sister chromatids during oocyte's meiosis is responsible for embryo homogeneous aneuploidies which are maternal age-dependent both in vivo and in vitro [59,60]. However, errors during the post-zygotic mitotic divisions give rise to an impressively high incidence of age-independent mosaic embryo aneuploidies [61]. Single blastomere analysis of ART-generated embryos demonstrated that more than 75% of them can be mosaics [62,63]. Although it is still a matter of debate, the aneuploidy levels in the general vs. the ART population are hypothesized to be similar [61]. The observation of average euploidy rates ranging from 39.5 to 82.5 on a population of young oocyte donors in 42 different centers raised concerns on the possibility that controlled ovarian stimulation [64] and/or embryo culture systems can affect the euploidy of ART-generated blastocysts [65]. However, several studies did not find different aneuploidy rates in embryos of unstimulated vs. stimulated patients or in embryos from patients subjected to different doses and length of gonadotropins treatment [65,66,67,68]. The possibility that the culture system affects the embryo aneuploidy rate may indicate that the suboptimal microenvironments experienced by the embryo in vitro might induce perturbations in chromosome segregation during the post-zygotic mitosis. Several studies examined a possible correlation between culture settings and embryo aneuploidy. Culture of sibling oocytes under different extracellular pHs (6 vs. 7.5% CO2 [69]; 6 vs. 7% CO2 [70]) had no impact on blastocyst development but a significantly lower euploidy rate was found at 7 and 7.5% CO2. Different single-step embryo culture media reportedly did not affect the embryo euploidy of sibling oocytes [71]. The possible impact of embryo culture systems on euploidy rates in human ART has been recently reviewed by Swain et al. (2021) [72]. Although some data suggest that the culture system may influence the incidence of post-zygotic mitotic errors, we still do not have conclusive indications in the human.

Despite the fact that the euploidy of embryos is a fundamental prerequisite for successful implantation, the clinical significance of PGT-A is still a matter of debate. Clinical evidence

demonstrates that transfer of mosaic embryos can result in healthy live births though with lower implantation rates and increased miscarriage rates [73,74,75,76,77,78]. Although the clinical outcome of mosaic embryos depends on the type of aneuploidy involved, available data indicate that transfer of embryos with less than 50% aneuploid cells have better outcomes compared to embryos with higher levels of mosaicism [74,75,77,78,79,80]. Recent findings suggest that mosaic embryos can undergo a self-correction during development through apoptosis of aneuploid blastomeres [81]. In fact, a recent multicenter study demonstrated that the incidence of mosaicism in births from transfer of mosaic embryos is like that recorded after transfer of euploid embryos [77]. PGT-A analyzes the DNA of 5–10 biopsied trophectoderm cells and we still do not know to what extent mosaicism in trophoectoderm cells is representative of true inner cell mass or fetal mosaicism [82]. Since the live birth rate of single euploid blastocyst transfer is limited to 50–60% [83], blastocyst's euploidy is not sufficient to predict the embryo competence to implant and result in a healthy live birth. Additional markers such as blastocyst morphological grading [84] and developmental speed [85] must be considered to predict the success rates of euploid blastocyst transfer.

2.5.2 Animal Models

More rigorous research, relatively unhampered by the ethical limitations and the innumerous confounding variables present in human studies, can be conducted in animal models.

Although performed with older and technically limited procedures, fluorescent in situ hybridization and comparative genomic hybridization studies suggested a higher incidence of whole chromosome aneuploidy in IVP compared to IVD embryos in ovine, equine, and porcine models [86,87,88].

These data were confirmed and extended in recent studies using more robust procedures able to detect the extent and origin of all chromosomal errors. Tsuiko et al. (2017) [57], using the same parents, compared the genome stability of in vivo vs. in vitro conceived bovine embryos through genome-wide single-cell analysis of dissociated blastomeres. Findings showed the presence of at least one chromosomally aberrant blastomere in only 18.8% of the embryos conceived and developed in vivo, compared to 69.2% of IVF embryos and 84.6% of IVM-IVF embryos. Although this study was carried out on a limited number of cleavage-stage embryos, data clearly highlight the involvement of in vitro embryo culture and in vitro maturation on chromosome instability. Similar findings were demonstrated through single nucleotide polymorphism (SNP)-based PGT-A algorithms in the porcine model by Jochems et al. (2023) [89], who observed more errors in in vitro vs. in vivo-conceived blastocysts (79.7% vs. 13.6%). A study in the equine showed that the incidence of micronuclei which are considered indicators

of chromosomal aneuploidy was higher in IVP compared to IVD blastocysts [90]. Taken together, animal data indicate a higher genomic instability in embryos produced in vitro compared to their in vivo counterparts corroborating the need for refining current in vitro embryo culture conditions to mimic more closely the in vivo microenvironment and enhance the success and safety of ART.

2.6 Embryo Transport in the Female Reproductive Tract

During its transport in vivo along the oviduct and the uterus, the embryo undergoes several distinctive biophysical and biochemical interactions that could enhance its ability to reach the blastocyst stage and increase its competence to implant and develop to term, resulting in healthy offspring. Assisted reproduction in humans, farm animals, and studies in animal models that bypass the oviduct to achieve pregnancy have shown that embryo–maternal interactions are indeed dispensable. This may have shifted researchers' focus away from attempting to more closely mimic the natural environment in which the embryo develops in vivo.

The oviduct (Fallopian tube in humans) (Figure 1) is composed by the infundibulum (fimbria in humans), mostly lined by ciliated epithelial cells, that catches the ovulated cumulus–oocyte complex; the ampulla, site of fertilization, where ciliated cells are more represented than secretory cells; the ampullary isthmic junction that separates the lower ampulla by the isthmus; the isthmus, predominantly lined by secretory epithelial cells, and the utero-tubal junction or the intramural segment that divides the isthmus from the uterus. The mucosa is composed of primary, secondary, and tertiary folds in the ampullary segment and only primary folds in the isthmic segment. The underlying connective tissue separates the mucosa by the smooth muscle which is arranged into an inner longitudinal, a middle circular, and an outer longitudinal layer [91]. The smooth muscle is very thin in the ampulla and thick and composed of multiple layers in the isthmus [92].

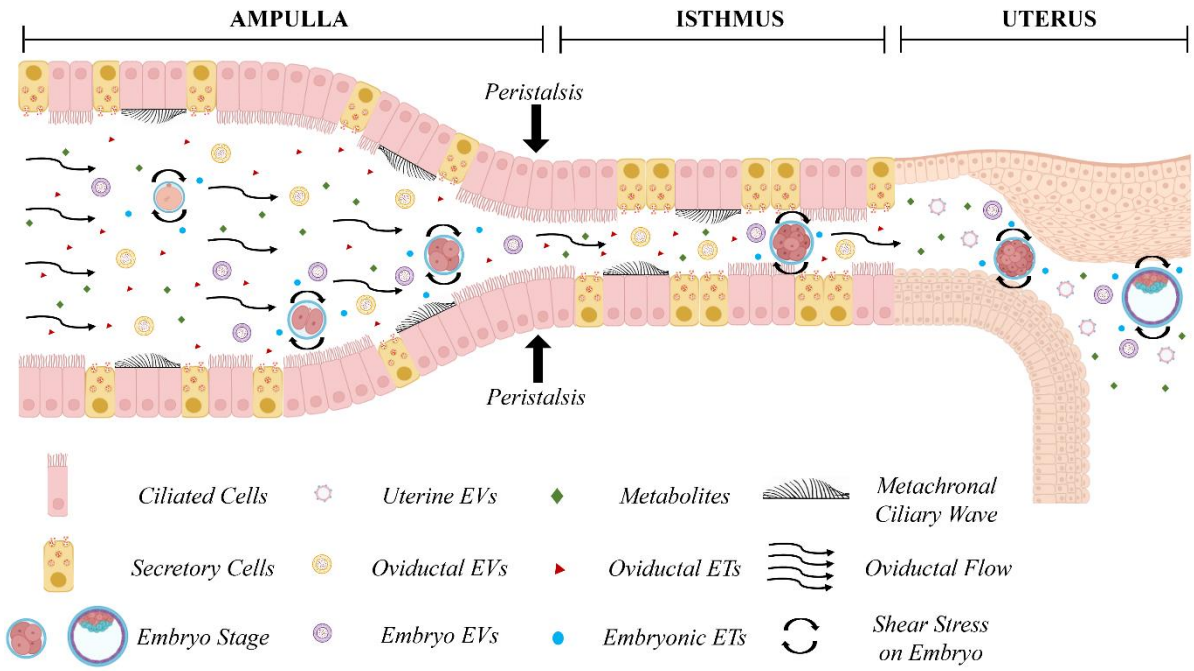

Figure 1. *In vivo embryo development in the maternal tract. Cross-talk between the embryo and the maternal tract. Oviductal, endometrial, and embryonic secretions in the form of embryotropins (ETs) and various signaling factors, enveloped or less in extracellular vesicles (EVs), are concentrated in a confined environment. Secretion of maternal fluids, metachronal ciliary waves, and peristaltic contractions exert biomechanical forces on the developing embryo during its journey through the ampulla, isthmus, and the uterus. Figure created with BioRender.com (accessed on 11 April 2024).*

Understanding the dynamics of embryo transport in the oviduct in vivo is crucial for comprehending the mechanisms underlying oviduct–embryo interactions and optimizing embryo culture in vitro. However, most available data across various species are derived from ex vivo studies, with direct in vivo imaging being rare. The duration of embryo residence in the oviduct varies among species. In humans, egg transport duration has been estimated through the recovery of embryos from the Fallopian tube or uterus following surgical sterilization at known times after the LH surge [92]. Embryos spend approximately 80 h in the oviduct [92], with transport occurring in two phases: a slow phase lasting 72 h in the ampulla, where entry into the isthmus is inhibited by a gate that relaxes under progesterone influence, followed by a rapid phase lasting 8 h in the isthmus [93]. It has been assumed that embryos reach the uterine cavity around the 8–12-cell stage, i.e., at the time of compaction, as 8-cell embryos are the latest stage recovered from the Fallopian tube, and 12-cell embryos are the earliest stage observed in the uterus. Studies in other mammals suggest that embryos develop within the oviduct approximately 4 days after ovulation [94], reaching the uterus around the 16-cell stage in bovine [95], 5.5 days in equine [96], and 4 days in mice [97]. In mice, recent in vivo imaging using optical coherence microscopy (OCM) and optical coherence

tomography (OCT) through an intravital window has provided new insights into embryo transport, enabling the determination of developmental stages and in vivo volumetric imaging and tracking of oocytes and preimplantation embryos within the native environment of the mouse oviduct [98,99]. Moore et al. (2019) [98] showed the presence of pronuclear zygotes at 0.5 day post-coitum (dpc) in the ampulla, 2-cell embryos at 1.5 dpc were found in the isthmus, and 4–8-cell embryos at 2.5 dpc were located close to the utero-tubal junction. This is consistent with older studies showing that the mouse embryo enters the uterus approximately 72 h post-coitum (0:00–2:00 h of Day 4), in the morula or early blastocyst stage [97].

The passage of the embryo within the oviduct is believed to be facilitated by three different mechanisms, the production of oviductal fluid, the beating of cilia, and the peristaltic contraction of smooth muscle [10] (Figure 1). The contribution of each factor to embryo descent is still matter of debate [100,101,102], and ciliary beating has typically been regarded as the primary propulsive force involved [101,103,104,105,106]. However, while all factors may exert a biophysical influence on the developing embryo, recent studies have underscored the primary role of muscle contraction in embryo transport. Dixon et al. (2009) [107], using video analyses and Spatio-Temporal Maps, suggested that propagating muscular contractions represent the main propulsive force driving the pendular movement of the egg along the mouse oviduct. They also noted that treatment with an L-type Ca2+ channel antagonist suppressed both muscular contractions and egg movement, while ciliary beating remained unaffected. This finding is consistent with the ability of women affected by immotile cilia syndrome to conceive, albeit with reduced efficiency [108,109], and with the fertility of mice deficient in two microRNA clusters lacking cilia but still capable of managing embryo migration from the oviduct into the uterus [110]. Wang and Larina (2020) [99], using in vivo volumetric OCT imaging and tracking of oocytes and embryos in the mouse oviduct, observed unexpected movement patterns, suggesting that egg/embryo transport is more complex than generally assumed. Cumulus–oocyte complexes rotate in groups likely propelled by cilia in the upper ampulla. Groups of fertilized oocytes in the lower ampulla undergo short-distance pulsatile oscillations while descending toward the isthmic-ampullary junction, moving backward when isthmus contracts and forward when it relaxes. At 0.5 days post-coitum (dpc), groups of preimplantation embryos remain stable in the lower ampulla, with average speeds (about 5–70 μm/s) and maximum speeds (20–230 μm/s) depending on the number of embryos present. At 1.5 dpc, embryos in the upper isthmus are relatively spread and individual embryos exhibit very rapid, bi-directional movements (40–740 μm/s) caused by propelling muscular contraction waves. In the lower isthmus, embryos nearing the utero-tubal junctions once again form groups and undergo pulsatile oscillations with reduced speeds (14.7 to 60.0 μm/s). This

study highlights that embryos experience different physical forces depending on the timing of transport along the oviduct, which could biomechanically influence embryo development.

2.7 Embryo–Maternal Tract Interactions

During the descent along the female reproductive tract, the preimplantation embryo communicates bidirectionally with oviductal cells of the ampulla, isthmus, and then with endometrial cells. The beneficial effects of culturing embryos with oviductal cells or with their conditioned media were first demonstrated by Gandolfi and Moor (1987) in the sheep [111] and by Eyestone and First (1989) in cattle [112]. Promotion of embryo development through co-culture with autologous or heterologous oviductal cells, uterine fibroblasts, cumulus cells, and cells extraneous to the maternal reproductive tract has been reported in several mammals and the human as well [113,114,115,116,117,118]. These studies prompted the use of co-culture in clinical embryology in human and domestic ruminants to more closely simulate the interaction with the maternal tract in vitro. Co-culture systems have been hypothesized to improve embryo development through (1) the removal of deleterious components of embryo metabolism and reduction in oxidative stress, and (2) active secretions of embryo-trophic factors [119]. However, co-culture has been discontinued for several reasons, potential transmission of pathogens [120], too complex methodology in the clinical context, introduction of reduced oxygen tension which ameliorates blastocyst formation [121], and uncertainty of the beneficial actions.

Along with the presence of maternal factors, the embryo itself secretes embryotropins that promote embryonic development through an autocrine action [122]. Since the oviduct is a virtual cavity, these putative embryotropins should reach a critical concentration required for their positive action on embryo development. The beneficial effects of culturing embryos in groups rather than singly in a defined culture medium volume was recognized more than 30 years ago and has been putatively attributed to the enhanced concentration of embryo-secreted autocrine and paracrine factors [123]. During in vivo conception, putative signaling factors known as embryotropins or embryokines [124,125] can be secreted by the embryo itself or by the epithelial cells lining the female reproductive tracts, but their exact nature remains elusive. Since embryo development in vitro occurs in the absence of maternally-derived signaling factors, embryotropins secreted by the embryo itself and involved in inter-embryonic communication could be responsible for the positive effects of group culture observed in most mammals studied thus far [123,126,127,128,129]. Embryos can communicate with each other or with neighboring embryos through various signaling factors, including proteins, lipids, neurotransmitters, saccharides, nucleotides, and micro RNAs [122]. Studying the proteins

released by embryos, however, is complicated by the low concentration of putative embryotropins compared to albumin and other proteins typically added in high concentrations to embryo culture media. Proteins detected in the culture media may stem from lysed cells, which are commonly found not only in arrested embryos but also in developing embryos [130]. Therefore, secretome studies should be conducted on individually cultured high-quality embryos, ensuring the absence of membrane-damaged cells [131]. Within this framework, several studies indicate that preimplantation embryos secrete various factors that may act through autocrine signaling, as they also express the corresponding receptors [122,132].

Extracellular vesicles (EVs) represent an additional recently discovered mechanism through which embryos may communicate with each other and with the female reproductive tract [133]. EVs are nanoparticles surrounded by a lipid bilayer, physiologically released from cells, which contain bioactive molecules such as lipids, proteins, DNA, mRNA, and miRNAs [134,135]. EVs comprise exosomes, microvesicles, and apoptotic bodies, which are characterized by different biogenesis mechanisms and dimensions [135]. EVs participate in cell–cell communication in various biological processes and are relevant modulators during embryo–maternal communication [136].

EVs released by preimplantation embryos, oviductal, and endometrial cells influence the developmental competence and gene expression of embryos, as well as oviductal/endometrial gene expression and functions [137]. Since the oviduct and uterus are considered virtual cavities in which embryos develop in confined fluid volumes, EVs may be present at high concentrations suitable for acting as bidirectional players in embryo–maternal tract cell–cell communication to regulate developmental competence and implantation in vivo. EVs recovered from the human Fallopian tube have been demonstrated to carry miRNAs that increase murine embryo viability in vitro [138]. Moreover, EVs isolated from media conditioned by primary human endometrial epithelial cells are efficiently internalized by human blastocysts and contain miRNAs directed toward genes involved in several processes related to embryo development [139]. Recent studies attempted to mimic in vivo conditions by sequentially culturing bovine embryos with EVs from oviductal and endometrial cells [140,141]. The addition of isthmus oviductal fluid EVs improved the development and cryotolerance of IVP blastocysts [141], whereas sequential culture with oviductal and endometrial EVs improved blastocyst cryotolerance by altering lipid content and metabolism, possibly in response to EVs' miRNA contents [140].

While these studies deepen our understanding of embryo–maternal crosstalk during in vivo development, maternal EVs are obviously absent during in vitro embryo production, and the

translation of such knowledge to in vitro embryo culture is still distant. Conversely, knowledge of the presence of embryo-derived EVs in vitro could be more readily applied to refine in vitro embryo culture in ART. Recent evidence indicates that embryo EVs may be internalized by the embryo itself, positively affecting its developmental competence. Pavani et al. (2018) [142] demonstrated that embryo EVs are taken up by zona-intact bovine embryos. Moreover, EVs extracted from media conditioned by high-density embryo group culture (25/50 μL) when added to individually cultured embryos increase blastocyst development to rates comparable to group culture, lower apoptotic cell ratios, ultimately demonstrating that EVs act as autocrine embryotropins during embryo culture. However, EVs from high-density group culture failed to increase the blastocyst hatching rates of individually cultured embryos to the same extent as reached under group culture. Generally, the developmental competence of singly cultured embryos is lower compared to group culture in terms of blastocyst cell number, hatching rates, and cryotolerance [143]. In addition, the hatching rates of IVD embryos are much higher than their IVP counterparts [45]. A recent study by the same group [1144] isolated EVs from conditioned media of individually cultured embryos that either developed to the blastocyst stage or did not. Among the miRNAs differentially expressed in conditioned media from embryos that successfully developed or less to blastocyst, the authors selected bta-miR-378a-3p for further validation. Supplementation of the embryo culture medium with miR-378a-3p mimic significantly increased blastocyst cell numbers, reduced apoptotic cell numbers, improved hatching rates, and increased the expression of genes involved in embryo development and implantation. Knowledge of EVs derived from embryos may be translated into clinical practice to establish new non-invasive biomarkers for embryo selection in ART [5]. Further research on embryo EVs carrying miRNAs or other molecular cargos is warranted to enhance embryo development and quality and for selecting high-quality embryos [145].

In this scenario, confining single embryos into submicroliter volumes may be the key to success in concentrating autocrine embryotropins to levels similar to those present in vivo or achieved during high-density group embryo culture. However, attempts to reduce the volumes of drops under oil must address (1) nutrient depletion and waste metabolite accumulation; (2) the increase in surface-to-volume ratios that enhance unwanted bidirectional solute exchanges between oil and medium; (3) increased evaporation leading to osmolarity changes [2].

2.8 In Vitro Embryo Culture: Individual versus Group Culture

It is well known that embryos benefit from being cultured together at high densities rather than individually in relatively large media volumes (Figure 2a,b).

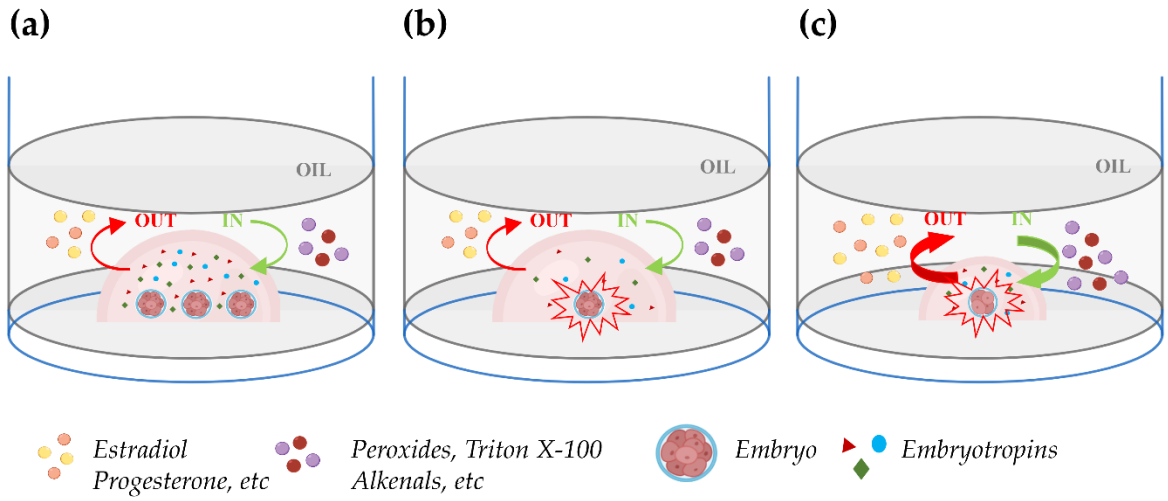

Figure 2. *Impact of embryo density during in vitro culture in drops under oil. (a) Group embryo culture in a defined volume of culture medium improves embryo development due to an enhanced concentration of embryotropins (triangles, circles, and diamonds). The green and red curved arrows, respectively, indicate unwanted in (peroxides, Triton X-100, alkenals, etc.) and out (estradiol, progesterone, etc.) apolar solute exchanges between oil and medium. (b) Individual embryo culture within the same drop volume as in the figure (a): embryo development is compromised due to the reduced concentration of embryotropins. (c) Individual embryo culture in a reduced drop volume: embryotropins are more concentrated than in (b) and the increase in the drop surface exposed to oil relative to the drop volume exacerbates the unwanted in and out exchanges between the oil and medium, impairing embryo development. Figure created with BioRender.com (accessed on 11 April 2024).*

This phenomenon, termed the "group effect", has been clearly recognized not only in mice, which are poly-ovulatory species, but also in domestic animals, which are largely mono-ovulatory. In multiple species, embryos cultured individually have been reported to exhibit slower cleavage divisions, reduced blastocyst rates and cell numbers, altered ICM-TE cell allocation, reduced hatching, increased apoptosis, altered gene expression, and reduced pregnancy rates [123,126,127,128,129,146] (Data are summarized in Table 3). The reduced blastocyst rates and cell numbers in embryos cultured individually might be explained by the low concentration of positive-effect embryo-secreted signaling molecules (Figure 2b). However, attempts to reduce the volume of drops under oil to achieve a high embryo density during individual culture generally yielded disappointing results. Lane and Gardner (1992) [147] cultured single mouse embryos in drops of 5, 10, 20, or 320 µL and reported similar blastocyst rates but higher cell numbers in embryos individually cultured in 10 and 20 µL drops. Although the lower cell numbers in 320 µL of medium could result from low concentration of embryotropins, the same finding in 5 µL drops can be due to the increased

surface exposed to oil relative to the drop volume which enhances unwanted damaging bidirectional solute exchanges between oil and medium (Figure 2c). In agreement with this hypothesis, individual culture in 5 µL in glass capillary tubes increased the blastocyst cell number to the same extent as in 10 or 20 µL drops. The beneficial effects of confinement in culture systems that avoid the harmful exchanges between oil and medium in reduced volumes microdrops was also elegantly shown by Thouas et al. (2003) [148] which cultured two mouse embryos in 1 µL glass capillaries achieving blastocyst cell numbers similar to those observed in high-density group culture with 20 zygotes in 10 µL drops under oil. Recently, Travaglione et al. (2024) showed that extreme confinement of individually cultured bovine embryos in ~70 nl microwells improved blastocyst rates compared to both conventional group culture (50 embryos/500 µL) and semi-confined group culture in microwell chambers (16 embryos/60 µL) [149]. Other studies showed that the spacing among embryos in high-density group cultures is critical. Stokes et al. (2005) [127] cultured groups of 16 IVD pig embryos in 20 µL drops at fixed distances and found maximal blastocyst and hatching blastocyst rates when the spacing was 81–160 µm. Lower rates were observed in drops with embryos in direct contact and no hatching blastocysts or blastocysts formed when the spacing was >480 and >640 µm, respectively. Similar findings were reported in the bovine model by Gopichandran and Leese (2006) [150]. Hoelker et al. (2009) [151] showed that group culture of 16 vs. 50 bovine zygotes in 500 µL of medium depresses the blastocyst rates and such effect is reverted when 16 zygotes are cultured in the same volume in a semi-confined well onto the well system.

Table 3. *In vitro embryo culture: differences between individual versus group culture. WOW, Well of the Well.*

Features	Embryo Density/Culture Conditions	Species	References
Blastocyst Rate	1/25 µL vs. 5 or 10/25 µL (49 vs. >80%)	Mouse	[123]
	1/25 µL vs. 1/50 µL (49 vs. 28%)	Mouse	[123]
	1/20 µL vs. 3–6/20 µL vs. 20/20 µL (0 vs. 6 vs. 23%)	Bovine	[126]
	16/20 µL different embryo–embryo distance (165 µm, ~20% vs. direct contact, ~8% vs. >540 µm, 0%)	Bovine	[150]
	16/500 µL vs. 50/500 µL vs. 16/500 µL WOW (21 vs. 32 vs. 31%)	Bovine	[151]
	50/500 µL vs. 16/60 µL MG vs. 1/68 nl MS (28.9 vs. 23.1 vs. 35.8%)	Bovine	[149]
	16/20 µL different embryo–embryo distance	Porcine	[127]

		(81–160 µm, ~25% vs. >640 µm, 0%) 1/30 µL vs. 3–4/30 µL (45.2 vs. 55.8%)	Human	[129]
Blastocyst Numbers	**Cell**	1 /25 µL versus 5 or 10/25 µL (34 vs. >60)	Mouse	[123]
		1/20 µL vs. 20/20 µL (77 vs. 176)	Bovine	[126]
		16/20 µL different embryo–embryo distance (decreased cell numbers at 240 µm vs. 165 µm)	Bovine	[150]
		16/20 µL different embryo–embryo distance (1–160 µm, 49.9 vs. 401–480 µm, 32)	Porcine	[127]
		1/10 or 20 µL vs. 1/40 µL (75 vs. 60)	Mouse	[147]
		2/1 µL glass capillary vs. 20/10 µL droplet (92 vs. 75)	Mouse	[148]
Blastocyst Hatching		1/25 µL vs. 5 or 10/25 µL (10.7 vs. 52%)	Mouse	[123]
		1/2 µL vs. 10/20 µL (Day 4, 49 vs. 63%)	Mouse	[146]
		2/1 µL glass capillary vs. 20/10 µL droplet (48.3 vs. 3.3%)	Mouse	[148]
Pregnancy Rate		1/30 µL vs. 3–4/30 µL (30 vs. 60%)	Human	[129]
Implantation Rate		1/20 vs. 2/20 vs. 16/20 (27 vs. 32 vs. 38%)	Mouse	[147]

As mentioned, mineral oil can be harmful especially when embryos are cultured in small drops due to the increase in the surface exposed to oil relative to the drop volume (Figure 2c). In fact, early studies on oil embryotoxicity showed that mineral oil can absorb apolar solutes from the culture medium like estradiol, progesterone, and androstenedione [152]. A recent study showed that human blastocysts express both the enzymes required to synthesize estradiol and its receptors, and supplementation of embryo culture medium with 8nM estradiol improved formation of blastocyst cavity, blastocyst hatching, and the ICM/TE ratio but only when embryo culture was not performed under oil [153]. Mineral oil can also exert detrimental effects on embryo culture releasing a number of embryotoxic solutes into the culture medium like peroxides, alkenals, aldehydes, Triton X-100, and zinc [154,155,156,157,158]. In summary, high embryo density is the key of success to optimize embryo development but individual culture in drops of reduced volumes under oil is not the right way to increase the embryo density.

Recently, semiconfined individual embryo culture in microwell chambers based on the concept of the well onto the well originally developed by Vajta (2000) [159] has been largely adopted in human ART to follow each embryo individually through time-lapse morphokinetics. Culture of embryos in multiple microwells under one drop of shared medium should allow to concentrate putative, beneficial embryo-secreted factors in each microwell while the shared drop of medium should avoid the depletion of nutrients and the accumulation of waste metabolites. However, such devices preclude the possibility to non-invasively assess the competence of individual embryos through analysis of the spent media. Research in this field in the human and animal models clearly indicates the huge potential of non-invasive assessments on spent media to identify the developmental competence and the genetic status of individual embryos to transfer in a future ART setting [6,7,8].

2.9 Advanced Culture Systems to Mimic Fluid Mechanical Stimulation on In Vitro Embryo Culture

Recent studies have underscored the critical role of mechanical forces experienced by embryos within the oviduct and their implications for the optimization of in vitro embryo culture [160,161,162,163]. As aforementioned, in the dynamic environment of the oviduct, the embryo encounters a multitude of biomechanical cues and dynamic movements crucial for its transport and development. These include mechanical forces exerted by the oviduct wall, shear stress resulting from oviductal and subsequently intrauterine fluid pressure, as well as constriction from the peristaltic contractions of smooth muscle of the oviduct, and the metachronal ciliary wave of the oviductal cells resulting in a peristaltic-ciliary flow, which propel the embryo towards the uterus [92,104,164,165]. Understanding and replicating these biomechanical microenvironments in vitro have become areas of intense research focus, aiming to ultimately improve ART outcomes. Findings of the effects of advanced culture systems on embryo development in different species are summarized in Table 4.

Table 4. *Advanced culture systems designed to replicate the biomechanical microenvironment of the maternal reproductive tract. ROS, reactive oxygen species; TE, trophectoderm.*

Advanced Culture Systems	Microenvironmental Feature Mimicked	Key Findings	References
Microchannel System	Continuous media perfusion flow rates 0.1–0.5 µL/h	Impaired mouse embryo development	[166]
	No media perfusion	Improved mouse embryo cleavage and blastocyst rates	[167]
Rotating Wall Vessels	Shear stress (1.2 dynes/cm²)	Impaired mouse embryo development Up-regulation of stress signaling pathways	[168]

Micro-funnel with Pulsatile Movement	Peristaltic contractions and metachronal ciliary wave	Improved mouse blastocyst hatching, mean cell numbers, and pregnancy rates	[169]
Micro-modulated Syringe Pump	Compressive strain and duration of peristaltic contractions	Improved 8-cell stage bovine embryo development	[161]
Micro-vibration Systems	Metachronal ciliary wave (5 s/30–60 min or 10 s/60 min)	Vibration during in vitro maturation improves porcine blastocyst rates	[170]
	Metachronal ciliary wave (44 Hz, 5 s/60 min duration)	Improved day 3 embryo quality, blastocyst, and pregnancy rates in human	[171]
	Metachronal ciliary wave (42 Hz, 5 s/60 min duration)	Improved 8-cell embryo rate, hatched blastocyst rate, and blastocyst cell numbers in mouse Improved human blastocyst rates, implantation, and pregnancy rates	[172]
	Metachronal ciliary wave (5 s/60 min at 20, 40, or 80 Hz)	Increased bovine blastocyst rates at 40 Hz Reduced blastocysts cell number at 80 Hz	[173]
	Metachronal ciliary wave (20–42 Hz for 5 s/60 min)	Reduced mouse blastocyst rates	[174]
	Metachronal ciliary wave (45 Hz for 5 s/60 min)	Improved bovine blastocyst cryotolerance, epigenetic, and transcriptional status	[175]
	Metachronal ciliary wave (42 Hz for 5 min/60 min)	No improvements of human blastocyst and aneuploidy rates	[176]
Tilting Embryo Culture System (TECS)	Metachronal ciliary wave	Improved blastocyst rates and cell numbers in both mouse and human embryos	[162,177]
TECS/Microchannels	Metachronal ciliary wave associated with straight microchannels (200 μm) with physical constrictions (150 or 160 μm)	Improved bovine embryo cleavage	[178]
Microfluidic Devices with Electrowetting (EWOD)	Oviductal embryo transport (electrical pulses—60~68.5 VRMS at 500 Hz)	Improved mouse blastocyst hatching Healthy live births	[179]
	Oviductal embryo transport (electrical pulses >1 kV/cm for 30 ms)	No improvements of porcine cleavage and blastocyst rates Increased ROS levels	[180]
Microfluidic Systems (Static or dynamic)	Softness of oviductal wall	Improved mouse blastocyst and hatching rates, and TE cell numbers	[181]
		Improves culture of bovine and ovine zona-free nuclear-transfer embryos	[182]
	Softness of uterine wall	Enables early post-implantation mouse embryo development	[183]

	Enables post-hatching culture of bovine embryos	[184,185]

Detailed investigations, particularly in the mouse model [186], revealed that to accurately mimic physio-mechanical conditions, sequential and distinct mechanical (i.e., shear stress, compressive strains, pressure) impulses may be necessary. However, early attempts to introduce a dynamic setup with continuous media perfusion at relatively high flow rates (0.1–0.5 µL/h) to culture embryos within microchannels (250 µm deep and 1 mm wide) yielded impaired embryo development, characterized by decreased proportions of morulae and blastocysts, and high abnormal eight-cell embryos compared to static microdevices [166]. Although the precise mechanism underlying this negative effect remains unclear, it could depend on physico-chemical factors including high shear stress on the embryo and/or the continual removal of growth-promoting autocrine factors [123,187]. Previous studies have proved that mouse embryos cultured in oil-suspended microdrops into rotating wall vessels (1.2 dynes/cm2 shear stress) exhibited an elevated susceptibility to shear stress beyond physiological levels in E2.5 embryos (compacted eight-cell/early morula stage) compared to E3.5 embryos (early blastocyst stage) and an up-regulation of stress-signaling pathway constituents. Furthermore, an increased lethality of embryos deprived of zona pellucida was observed after unphysiological shear-stress induction, demonstrating that excessive shear stress impairs embryo development compared with static embryo culture [168]. In this context, defining the magnitude of shear stress acting on human embryos during tubal transport poses a significant challenge, given the variability in reported velocities (average velocities 0.1 µm/s and maximum velocities around 8.6 µm/s) and uncertainties in extrapolating findings from animal models or womens' oviductal tissue samples [188,189,190,191]. Establishing a safe range and duration of shear stress demands the development of precise, reliable, and controllable engineering systems. For instance, a low shear stress value <1.2 dyn/cm2 is often considered safe [188]. Incorporating a gentle surrounding fluid flow into embryo culture systems is highly complex [192,193]. In this direction, Heo et al. (2010) contributed to the integration of oviductal contractions and ciliary currents developing a micro-funnel with a physiologically-based pulsatile movement. Such a dynamic system improved mouse embryo development in terms of blastocysts hatching (Microfunnel-pulsatile 71 vs. 23% Microfunnel-control and 31% Microdrop-control), mean cell number (Microfunnel-pulsatile 109 + 5 vs. 60 + 3 Microfunnel-control, and 67 + 3 Microdrop-control) and pregnancy rates [169]. Other researchers emulated the in vivo compressive strain and duration of peristaltic constrictions through a micro-modulated syringe pump connected to a membrane-based microfluidic system

highlighting the importance of the selection of the correct stimulus amplitude (150 Pa) and duration (2 s) in long-term bovine embryo culture [161]. Other studies have investigated the positive impact of physical stimulation, such as micro-vibration, to simulate the human Fallopian tube metachronal ciliary wave ranging from 5–20 Hz in vivo [194] on embryo development. Mizobe et al. (2010) observed a positive impact of micro-vibration on blastocyst rates when applied during in vitro maturation of pig oocytes [170]. In humans [171], mice [172], and bovines [173], increased blastocyst rates and reduced blastocysts mean cell numbers were observed with 40 Hz and 80 Hz frequencies, respectively. A detrimental effect of micro-vibration (20–42 Hz for 5 s/60 min) was reported on mouse embryos when applied during the early stage of embryo development in comparison with the control static group (47% vs. 56%, respectively) [174]. A recent investigation has suggested that micro-vibration (45 Hz for 5 s/60 min) induces an increased cryotolerance of bovine embryos along with improvements in epigenetic and transcriptional status in blastocysts [175]. A paired randomized controlled trial demonstrated that dynamic culture of human embryos with micro-vibration (42 Hz frequency for 5 min/60 min) did not improve formation of transferrable blastocyst (58.3% vs. 57.1%), aneuploidy rates (20.0% vs. 33.3%), or the blastocyst development rate (67.1% vs. 63.1%) vs. static groups, contrasting previous findings [176]. In striving to replicate mechanical stimuli akin to ciliary beating that move the embryo during transport within the female reproductive tract, a tilting embryo culture system (TECS) has been implemented. This approach demonstrated that estimated shear forces of ~0.7 dyn/mm2 improve blastocyst rates (mouse, TECS vs. control: embryo density 5/50 µL, 59 vs. 46%; embryo density 10/500 µL, 42% vs. 27%), cell numbers (TECS vs. control, mouse: 77 ± 4 vs. 66 ± 4; human: 43 ± 3 vs. 34 ± 3) in both mouse and human embryos [162,177]. An additional study explored the impact of the combined mechanical stimulations including a tilting culture system and straight microchannels (200 µm) with physical constrictions (150 or 160 µm) on bovine early embryo development, demonstrating an enhanced embryo cleavage (constricted vs. straight channel: 56.7 ± 13.7 vs. 23.9 ± 11.0%) with no differences in the blastocyst rates [178]. In the attempt to recapitulate the movements of embryos in the oviduct, a digitalized microfluidic device powered by electrowetting on a dielectric (EWOD), at a relatively low energy, was used to manipulate microculture droplets, demonstrating an enhancement of blastocyst hatching (dynamic vs. static: 50 vs. 21.2%) and the ability to produce normal live births in the mouse model [179]. However, concerns persist regarding the potential impact of the electric field on preimplantation embryos. While some studies suggest that certain levels of electrical stimulation (1.36 kV/cm for 40–60 ms) can enable fertilization in human oocytes [195], others indicate an increase in reactive oxygen species levels, for example in porcine embryos,

exposed to electric pulses >1 kV/cm for 30 ms [180]. Another aspect to consider when trying to emulate in vitro the in vivo environment is the compression exerted by the oviductal wall on the embryo. The isthmus lumen diameter is relatively small, with certain sections being comparable to the diameter of a human embryo. Additionally, the uterine epithelium exhibits softer mechanical properties (102–103 Pa) compared to the conventional polystyrene petri dish (1 GPa) with a difference of nearly six orders of magnitude [181,196,197]. Advanced culture systems based on substitutive substrates have been developed in this direction by using static and/or dynamic microfluidic approaches as an alternative to conventional polystyrene petri dishes. Materials with different stiffnesses including polyacrylamide gels [183], agarose gels [182,184], alginate gels [185], PDMS [198] and collagen gels [181] were tested for their suitability for embryo culture, highlighting the positive impact of a low elastic modulus of the culture surface on the early preimplantation embryo developmental rate and allowing the in vitro post-implantation development. Accordingly, studies reported that the 3D type I collagen gels (1 kPa stiffness) culture system showed higher rates of blastocyst (64 ± 9.1 vs. 50 ± 18%), and hatching blastocyst stages (54 ± 25 vs. 21 ± 16%), as well as increased trophectodermal cell numbers (TE, 65 ± 13 vs. 49 ± 12 cells), compared to those cultured in a conventional polystyrene dish [181]. Furthermore, volume of media and oil may also influence forces exerted upon the embryo during various movement schemes, and factors such as friction or impact against platform edges may also be important variables. The use of microfluidic innovative systems has been proposed for customizing in vitro experiments for the manipulation of small volumes in the order of nanoliters to culture single embryos, recapitulating fluid volumes in the oviduct [199]. Microfluidic approaches that reduce volumes compared to the standard microdrop techniques have been demonstrated to have similar behavior in terms of blastocyst attachment (33.22 ± 5.6 vs. 45.64 ± 8.4%) [199] or an enhanced embryo development [167]. Future studies introducing fluid flow to such systems in which autocrine and paracrine factors are concentrated in reduced volumes could lead to higher pregnancy and implantation rates in ART. All these considerations lead us to understand that an optimal embryo culture system should incorporate the capacity to mimic, at a microscale, wide-ranging consistencies, mechanical forces, and manipulation of minute volumes in the nanoliter range, all essential aspects for accurately recapitulating embryo development in vitro and gaining widespread embryo culture implementation [163,167].

2.10 Conclusions

Current embryo culture systems in ART fail to accurately replicate the biophysical factors and signaling cross-talk that embryos experience in vivo. The consequences of such discrepancies on the developmental competence of in vitro-produced embryos compared to their in vivo

counterparts are challenging to discern in human ART. However, research in animal models, particularly in bovine species, more clearly demonstrates the lower developmental competence and higher genomic instability of IVP embryos compared to IVD embryos.

Three main factors of the in vivo environment are currently not replicated in in vitro embryo culture systems: (1) the biophysical conditions experienced by embryos in the maternal tract, such as oviductal fluid flow, metachronal ciliary waves, peristalsis, and the low elastic modulus of biological surfaces in contact with the embryo; (2) oviductal and endometrial soluble embryotropins or other molecular cargos enclosed in extracellular vesicles that have positive effects on embryo development; (3) confinement in sub-microliter volumes that enhances the concentration of embryo-secreted factors beneficial to the embryo.

In animal models, in vitro embryo development is generally improved under high embryo density conditions. However, in human ART, the relatively low number of embryos and the need to monitor the development of individual embryos preclude the possibility of in vitro culture at high embryo densities. Semi-confined culture in well-of-the-well devices has been widely adopted in human ART to allow the collection of morphokinetic data of individual embryos. Nevertheless, such culture systems do not permit the collection of individual embryo spent culture media for non-invasive analysis of embryo developmental competence and euploidy.

Although several research studies indicate that dynamic culture systems can improve embryo developmental competence, we are still far from faithfully recapitulating the high and poorly understood complexity of biophysical factors acting during in vivo embryo development. Nevertheless, the increasing interest of research groups in this field holds promise for establishing a more physiological, confined, and dynamic individual embryo culture system allowing the collection and analysis of non-invasive markers of embryo developmental competence.

2.11 References

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3. Chapter 2:

Article

Individually cultured bovine zygotes successfully develop to the blastocyst stage in an extremely confined environment

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3.1 Abstract:

The possibility of detecting the developmental competence of individually cultured embryos through analysis of spent media is a major current trend in an ART setting. However, individual embryo culture is detrimental compared with high-density group culture due to the reduced concentration of putative embryotropins. The main aim of this study was to identify an individual culture system that is not detrimental over high-density group culture in the bovine model. Blastocyst rates and competence were investigated in a conventional (GC) group, semi-confined group (MG), and individual culture (MS) in a commercial microwell device. Main findings showed that: (1) individual embryos can be continuously cultured for 7 days in ~70 nL microwells (MS) without detrimental effects compared with the GC and MG; (2) MS and MG blastocysts had a reduced number of TUNEL-positive cells compared to GC blastocysts; (3) though blastocyst mean cell numbers, mitochondrial activity, and lipid content were not different among the three culture conditions, MS blastocysts had a higher frequency of small-sized lipid droplets and a reduced mean droplet diameter compared with GC and MG blastocysts. Overall, findings open the way to optimize the development and competence of single embryos in an ART setting.

3.2 Introduction:

Despite the continuous development of new procedures for selecting euploid and competent embryos to transfer in human assisted reproductive technologies (ART), only a few studies have been focused on biophysical aspects of current in vitro preimplantation embryo culture procedures, which has remained essentially unchanged over the years as we had already reached the maximum levels of efficiency [1-3]. However, studies in animal models and domestic species clearly indicate that in vitro-produced (IVP) embryos have a lower developmental competence than their in vivo-derived (IVD) counterparts, in terms of blastocyst rates, euploidy, cryotolerance, gene expression, and pregnancy rates [4-11]. In vivo, embryos develop within confined microenvironments—the oviduct (or fallopian tube in humans), and the uterus—virtual cavities where highly concentrated maternal and embryo-derived factors, in the form of proteins and/or other molecular cargos enclosed within extracellular vesicles, promote preimplantation embryo development and affect embryo developmental competence [12-16]. Although the bidirectional crosstalk between the embryo and the maternal reproductive tract is obviously absent during in vitro embryo culture, evidence indicates that the potential benefits of autocrine embryo secretions, the so-called embryotropins, could depend on the specific culture system adopted [17]. In vitro embryo development is ameliorated when multiple embryos are cultured in a certain volume of culture

medium compared to a single embryo cultured in the same volume. Such an improvement, known as a "group effect", has been suggested to arise from the increased concentrations of embryotropins during group embryo culture [12,13]. According to these concepts, different studies were addressed to improve the in vitro embryo culture conditions, reducing the volume of the culture medium drops under mineral oil [18,19]. However, reduced drop volumes exerted detrimental impacts on bovine embryo development [20], likely due to the increased surface exposed to oil relative to the drop volume, resulting in a higher in and out exchange of apolar solutes between the oil and medium [21-27]. Vajta et al. (2021) introduced a semi-confined group culture procedure, termed "Well of the Well" (WOW), whereby multiple embryos individually reside within microwells while sharing a common drop of medium with the other embryos (reviewed in [1]). This approach should maintain the benefits of group culture through the establishment of a semi-confined microenvironment in which positive-effects autocrine secretions are enriched, while the common drop of medium prevents the depletion of nutrients and the accumulation of waste metabolites [1,28,29]. Different microwell devices have been largely adopted in human ART to allow for the collection of morpho-kinetic parameters of individual embryos, though the actual advantage of embryo morphokinetics for the selection of high-quality embryos remains unclear [30,31]. The current trend in human ART is to select and transfer a single euploid and competent blastocyst through the application of novel non-invasive embryo quality markers [32]. "Omics analysis", i.e., the determination of metabolome, transcriptome, secretome, genome, etc., of single embryos, studying the spent culture media of individual embryo cultures to evaluate the euploidy and developmental competence of embryos to transfer could soon become a reality [33]. Under this scenario, the development of individual embryo culture systems without detrimental effects compared to group culture becomes mandatory to ensure an actual embryo's individuality and allow for the application of these methods. As obvious ethical reasons prevent direct experimentation on humans, in the present study, the bovine was selected as an animal model for its closer similarity to human embryo development compared to the mouse model [34,35]. In this perspective, we established an individual embryo culture that maintains quality and viability comparable to conventional group culture, mimicking the reduced volume experienced by embryos in the oviductal tract.

3.3 Materials and Methods

3.3.1 Oocyte Retrieval and In Vitro Maturation

Bovine ovaries were obtained from Di Tella S.R.L. slaughterhouse (San Marcellino, Caserta, Italy; CEE accreditation number 1403/M) and transported to the laboratory within 3 h at 33 °C. Ovaries were washed with pre-warmed physiological saline supplemented with 100 IU Penicillin and 100 µg/mL streptomycin, and cumulus oocyte complexes (COCs) were collected into 15 mL conical-bottom tubes (Falcon; Milan, Italy) containing 70 µL of heparin 10 mg/mL through aspiration from antral follicles (2–8 mm diameter) using an 18-gauge needle. COCs with a compact, at least three-layered non-atretic cumulus, and an oocyte with homogeneous cytoplasm were selected, washed in wash medium (Stroebechmedia, Copenhagen, Denmark), and matured for 22–24 h in groups of 50 in four-well dishes (Nunc—Termo Fischer Scientific, Roskilde, Denmark) containing 500 µL of IVM medium (Stroebechmedia, Copenhagen, Denmark) at 38.5 °C, 6% CO2 in air, 95% humidity.

3.3.2 Semen Preparation, In Vitro Fertilization and Embryo Culture

Frozen bovine semen from three bulls (0.5 mL straws—approximately 15–20 × 106 spermatozoa per straw—motility after thawing >70%) was obtained from Intermizoo S.p.a (Padova, Italy). Straws were thawed in a water bath at 37 °C for 1 min, and semen was rinsed in 10 mL of semen wash medium (Stroebechmedia, Copenhagen, Denmark) by centrifugation at 170× g for 10 min. The sperm pellet was resuspended in 700 µL of IVF medium, sperm concentration was determined with a Burker chamber, and sperm motility was evaluated by a Sperm Class Analyzer (SCA, Microptic S.L., Barcelona, Spain) using a Makler chamber placed on a microscope stage at 38 °C. Sperm motility was analyzed at a Nikon TE 2000 (Nikon, Tokio, Japan) inverted microscope connected to a Basler Vision Technology A312 FC camera (Basler, Ahrensburg, Germany) with a positive-phase contrast 10× objective. At least 200 cells and four/five fields were acquired and analyzed for each sperm suspension. Progressive motility and kinetics were evaluated by SCA in terms of curvilinear velocity (VCL), straight-line velocity (VSL), and average-path velocity (VAP). The following software settings were used: 25 frames/s, 10 frames/object, 10 µm/s velocity limit for slow sperm, 25 µm/s velocity limit for average sperm, 50 µm/s velocity limit for rapid sperm, 50% minimal linearity, and 70% straightness for progressive fast sperm. For in vitro fertilization, 45–50 matured COCs for each well were suspended in 250 µL IVF Medium (Stroebechmedia, Copenhagen, Denmark), inseminated with 250 µL of sperm suspension (2 × 106/mL—final sperm concentration), and co-incubated for 19–22 h at 38.5 °C, 6% CO2 in air, 95% humidity. At 19–22 h post insemination (p.i.), the presumptive zygotes (n = 1073) were denuded by

vortexing (2 mL volume, max velocity for 3 min) and then cultured under the different culture conditions, as reported below, in IVC medium (Stroebechmedia, Copenhagen, Denmark) for 7 days at 38.5 °C, 6% CO2, 6% O2 and 88% N2, 95% humidity. At day 8 p.i., blastocyst rates and stages were assessed, and blastocysts were treated for determination of cell numbers, TUNEL-positive cells, mitochondrial activity, and lipid content, as detailed below.

3.3.3 Culture Devices and Loading of Embryos or Presumptive Zygotes

Each experiment was performed using commercial polystyrene microwell chambers (GERI® chambers—GENEA BIOMEDX, Sidney, Australia) and NUNC™ four-well dishes. The GERI® chamber consists of 4 wells, one of which includes 16 contiguous microwells that allow for the spatial confinement of single embryos (Figure 1a). Each microwell has an inverted truncated cone shape, with a base diameter of 430 µm, an upper diameter of 500 µm, and a height of 400 µm. Therefore, each microwell has a volume of 68 nL and a surface (exposed to oil)/volume ratio of 2.85 (Figure 1b). GERI® chambers were loaded following two different procedures: (i) microwell group culture in a semi-confined environment (MG), in which 16 embryos (or presumptive zygotes) were loaded into microwells in a shared 60 µL medium drop (embryo density 1/3.75 µL) and then covered by 3 mL of mineral oil (Strobechmedia, Copenhagen, Denmark) (Figure 1c); (ii) microwell single culture in a confined environment (MS), in which 16 embryos (or presumptive zygotes) were loaded as in MG and then the shared 60 µL drop of medium was removed through two repeated 60 µL aspirations with the pipette tip vertically positioned at the center of microwell array (embryo density 1/68 nL) (Figure 1d). In all experiments, conventional group embryo culture (GC), consisting of groups of 50 presumptive zygotes/500 µL per well (embryo density 1/10 µL), served as control.

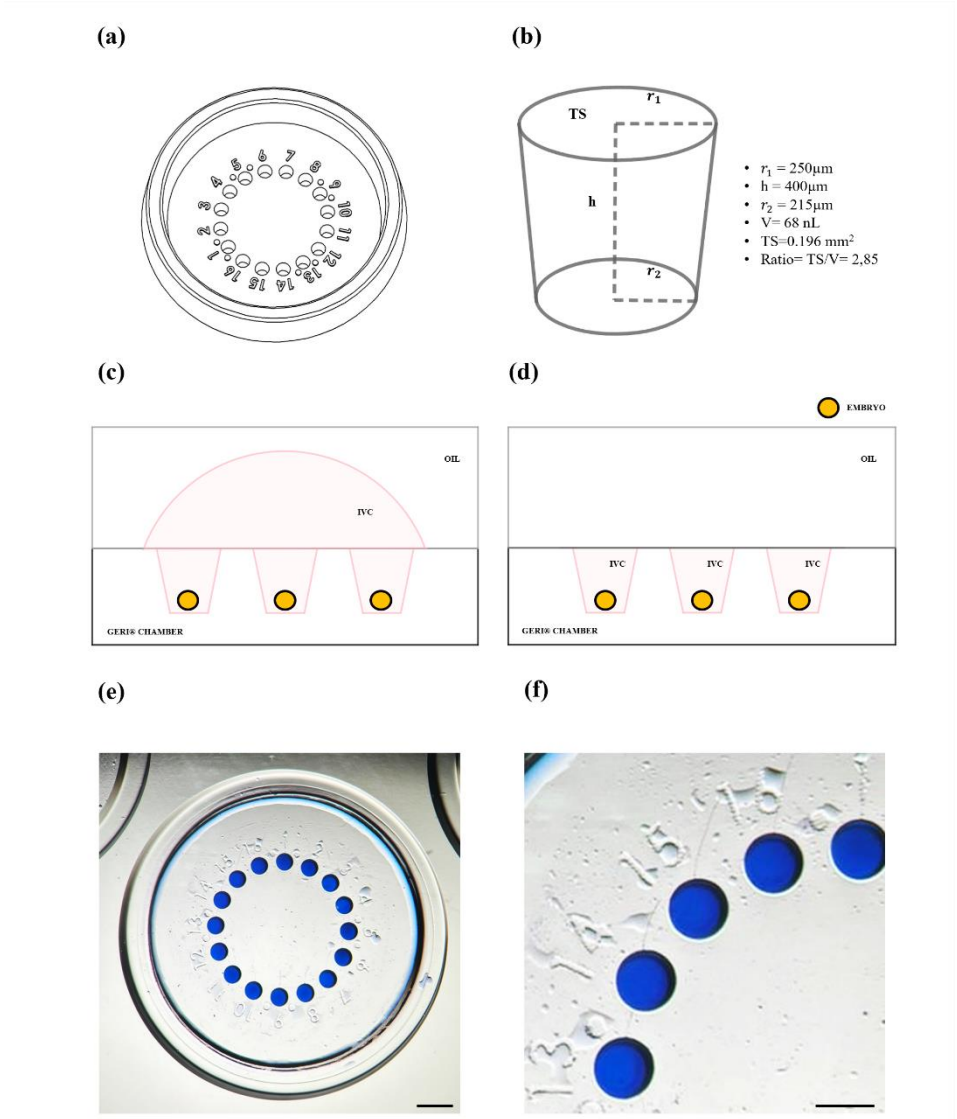

Figure 1. *Drawings of the WOW-based GERI® chamber (a–d) and representative images of MS dye loading experiment to verify medium communication between adjacent microwells (e,f). (a) Schematic drawing of GERI® chamber main well. (b) Dimensions, volume and surface/volume ratio of an individual microwell. TS = top surface; ratio = ratio of surface exposed to oil (TS) and microwell volume (V). (c,d) Drawings of MG (c) and MS (d) culture conditions. IVC = embryo culture medium. (e,f) Representative images of dye-loaded MS after 7 days incubation (e) showing lack of communication (f) between adjacent microwells. Bar = 1 mm (e); bar = 0.5 mm (f).*

Preliminary dye loading experiments were performed to demonstrate the lack of medium communication between adjacent microwells during individual culture under the MS condition. To this end, in a first series of experiments, after MS loading, a few nL of toluidine blue 1% was gently injected into microwells through a Cook® Flexipet® Pipette (Cook, Milan, Italy). Micrographs of dye-loaded MS were acquired before and after incubation for 7 days. At the end of culture, diffusion of the dye from a dye-loaded microwell to the adjacent non-dye-

loaded microwell was seldom present. However, this dye loading procedure did not faithfully reflect the MS embryo loading procedure as an additional volume of stain was injected into the microwells. For this reason, in a second series of experiments, toluidine blue was directly dissolved in IVC medium, microwell chambers were loaded according to the MS procedure (Figure 1e), and micrographs were acquired before and after 7 days incubation (Figure 1f) to verify the possible presence of residual films of dye putting adjacent microwells in communication. Findings showed the lack of residual films of culture medium putting adjacent microwells in communication, demonstrating that embryos are individually cultured under this procedure.

3.3.4 Experimental Design

Experiment 1: The first series of experiments (n = 5; total oocytes 673) was addressed to investigate the blastocyst rates and quality obtained after 3 days of culture in conventional group culture (GC) followed by culture for a further 5 days in semi-confined (MG) or confined (MS) environment. To this end, embryos cultured 3 days p.i. in GC were left undisturbed or randomly allocated in MG and MS and cultured until day 8 p.i. in 6% CO2, 6% O2 and 88% N2, with 95% humidity at 38.5 °C. Blastocyst rates and stages were recorded at day 8 p.i. Blastocyst cell numbers and percentage of TUNEL-positive cells were determined as described below.

Experiment 2: Results of experiment 1 indicated that day 3 p.i. embryos transferred to MG and MS were able to develop until the blastocyst stage but with reduced rates compared to conventional GC. Therefore, in experiment 2, presumptive zygotes at day 1 p.i. (n = 6; presumptive zygotes 776) were loaded after removal of cumulus cells in GC, MG, and MS, cultured until day 8 p.i. and analyzed as described in experiment 1.

Experiment 3: Day 7 blastocysts produced in GC, MG, and MS (n = 3; 60 blastocysts) as described in experiment 2, were treated for determination of mitochondrial activity and lipid content as indicators of developmental competence, as detailed below (Figure 2).

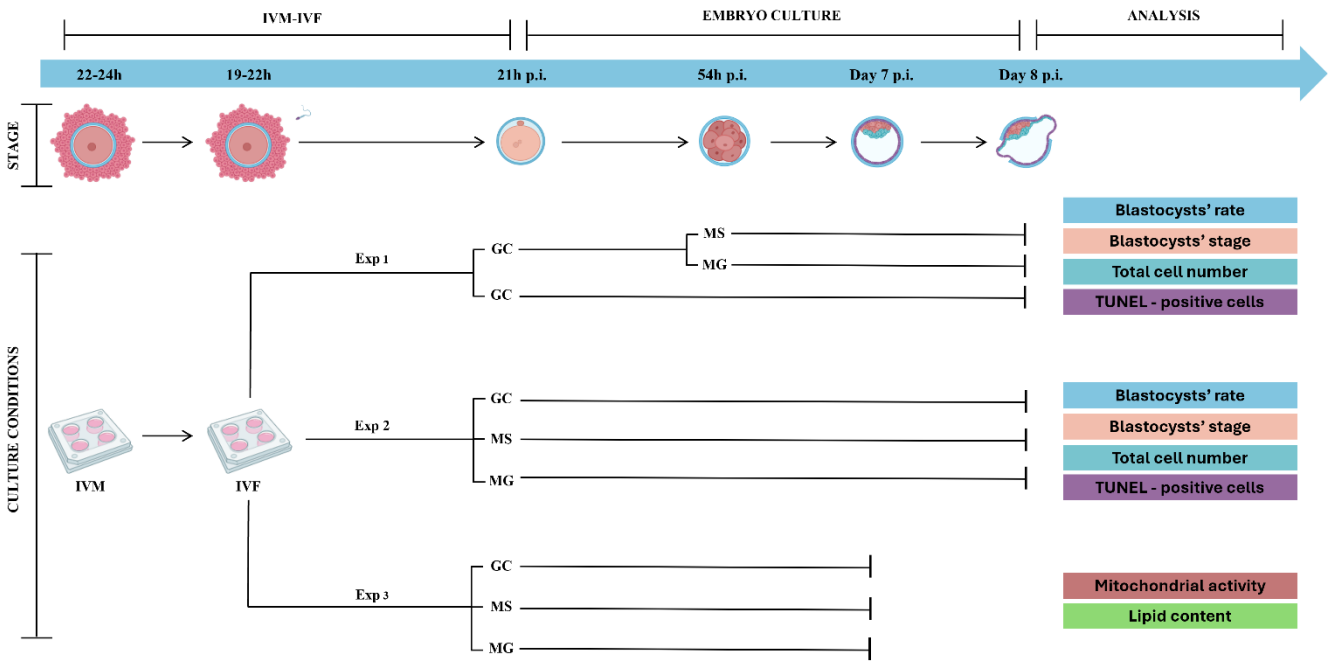

Figure 2. *Graphic experimental design. GC = group culture; MG = microwell group culture; MS=microwell single culture; p.i. = post insemination. Figure created with BioRender.com (accessed on 11 April 2024).*

3.3.5 Embryo Development and Quality Assessment

For all culture conditions, cumulative blastocyst yields were recorded at day 8 p.i. under a Nikon SMZ18 (Nikon, Florence, Italy). Blastocysts rates were assessed and expressed as percentages of blastocysts on cleaved embryos. Blastocyst developmental stages were expressed as percentages of early-expanded blastocysts and hatching/hatched blastocysts on total blastocysts.

3.3.6 TUNEL Assay

The TUNEL assay was used to assess DNA fragmentation in blastocysts (n = 167). The terminal free 3′-OH ends of DNA were labelled with dUTP conjugated to red fluorescent dye tetramethylrhodamine (In Situ Cell Death Detection Kit, TMR red, Merck, Milan, Italy) using terminal deoxynucleotidyl transferase. At day 8 p.i., blastocysts from GC, MG, and MS cultures were fixed in 4% paraformaldehyde (PFA, Sigma Aldrich, Milan, Italy) in PBS overnight at 4 °C. After three 5 min washings in PBS with 0.1% polyvinylpyrrolidone (Sigma Aldrich, Milan, Italy) (PBS-PVP), blastocysts were permeabilized in 0.1% Triton X-100 (Sigma Aldrich, Milan, Italy), 0.1% sodium citrate (Sigma Aldrich, Milan, Italy) for 30 min at 4 °C, washed three times for 5 min in PBS-PVP, and then incubated for 1 h in the TUNEL reaction mixture at 37 °C in the dark according to the manufacturer's instructions. Blastocysts were then washed in PBS-PVP, labelled with 10 µg/mL Hoechst 33342 (Sigma Aldrich, Milan,

Italy) for 7 min at room temperature (r.t.), washed again, mounted on a microscope slide in 10 µL of PBS-PVP, covered with 24 × 24 mm coverslips, and sealed. Images were acquired at a Nikon Eclipse TE2000-U connected to a Nikon DS-5Mc video camera with a 40×, 1.3 N.A. objective through NIS-element BR 4.6 software (Nikon, Florence, Italy). Hoechst-stained nuclei were visualized through a UV filter (λex 350 nm, λem 461 nm), and TUNEL-positive nuclei through a TRITC filter (λex 544 nm, λem 570 nm; Nikon, Florence, Italy). Negative controls were prepared omitting terminal deoxynucleotidyl transferase in the reaction mixture, while positive controls were prepared through pretreatment with 1 mg/mL DNase I for 10 min at r.t. Blastocyst total cell and TUNEL-positive cell numbers were quantified using the 'total cell counter' of Image J 1.54f software (NIH, New York, NY, USA).

3.3.7 Blastocyst Mitochondrial Activity and Lipid Content

Blastocyst staining: Day 7 blastocysts (n = 60; 20 for each culture condition) were assessed for mitochondrial activity and lipid content. Blastocysts from each culture condition were washed twice in IVC medium, incubated for 30 min at 38.5 °C in 400 nM MitoTracker DeepRed (Molecular Probes, Eugene, OR, USA) in IVC medium, washed 3 × 5 min in PBS-PVP, and then fixed in 4% PFA 30 min at r.t. Fixed blastocysts were permeabilized in 0.1% saponin in PBS for 30 min and stained 1 h at r.t. in 20 µg/mL Bodipy (493/503, Thermofisher Scientific, Parma, Italy) in PBS-PVP. After 3 × 5 min washings in PBS-PVP, the blastocysts were stained for 30 min in 10 µg/mL Hoechst 33342 in PBS-PVP, washed 3 × 5 min in PBS-PVP, mounted on microscope slides in 50% glycerol-PBS, covered with round coverglass with spacers to retain the blastocyst three-dimensional shape, and sealed. Blastocysts were imaged with a 60× oil immersion objective at a FV3000 OLYMPUS laser-scanning confocal microscope (Olympus Italia, Segrate, Italy) equipped with a 488 nm argon laser (emission 500–540 nm) for visualization of lipid droplets, a 640 nm laser (emission 650–750 nm) for visualization of mitochondria.

Assessment of mitochondrial activity: total fluorescence signal intensity was quantified as follows: 8–12 3 µm serial optical sections and a maximal projection were acquired for each blastocyst. Image analysis was performed using ImageJ software (NIH; ImageJ version v1.54i software). Following selection through the freehand selection tool, each blastocyst maximal projection was measured to determine its area and its integrated density (IntDen), corresponding to pixel intensity. An area outside the blastocyst was measured for obtaining the background fluorescence. Fluorescence intensity in each blastocyst was determined as follows: relative fluorescence = IntDen − (area of selected blastocyst × mean background fluorescence). Fluorescence intensities were expressed in arbitrary units (a.u.).

Assessment of lipid content: Blastocysts lipid quantification was performed by analyzing the total area of lipids in each blastocyst. Three 1024 × 1024 images were captured for each blastocyst: one equatorial and two in the middle of the resulting halves. Images were analyzed using the 'nucleus counter' tool, set to quantify droplet areas with the ImageJ software. Lipid quantity was corrected by the total embryo area to account for varying blastocyst sizes [36]. In addition, an analysis for lipid droplets (LDs) in terms of size frequency was conducted on the same blastocysts previously examined. A series of 8-bit z-stack images were captured for each embryo at 3 μm intervals, starting from the top slice (containing the first LDs capture) to the final slice. The acquired optical sections were analyzed through the Image J 1.54f software (NIH, USA). LDs parameters were measured through the following steps: background subtraction (rolling ball radius = 10), threshold adjustment (55–255), binary image conversion, watershed transformation (to remove the potential LDs clusters), and analyzing particles tool (excluding particles with the mean area <0.07 μm2 to avoid counting background noise). Additionally, an Excel file was created to categorize LDs size frequency into five classes: 0.07–0.3, 0.3–5, 5–15, 15–30, and >30 μm2. For all analyzed parameters, the value of each slice was calculated separately, and the mean value per slice was determined.

3.3.8 Statistical Analysis

Each experiment was conducted at least in biological triplicate, and the results were presented as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism Software (version 8.02 for Windows, GraphPad Software, Boston, MA, USA). For the analysis of mitochondrial activity and LDs parameters, one-way analysis of variance (ANOVA) was employed, followed by Tukey's pairwise comparison tests. Blastocyst rates, stages, total cell numbers, and TUNEL-positive cells were expressed as cumulative percentages and analyzed using Fisher's exact test for pairwise comparisons when an overall significance was observed. $p < 0.05$ was considered statistically significant.

3.4 Results

Experiment 1: In the first series of experiments, the cleavage rate at day 3 p.i. was 88.8%. Blastocyst's rate at day 8 p.i. (Figure 3a) in GC was markedly higher than that in MG and MS (29.9 vs. 19.6 and 16.7%; $p < 0.001$). No differences were observed among the three culture conditions in terms of blastocyst's stages (Figure 3b). Blastocyst cell numbers and percentages of TUNEL-positive cells were assessed as markers of developmental competence. The mean blastocyst's cell number was not significantly different among the three culture conditions (GC, 137.8 ± 71.7; MG, 137.3 ± 67.2; MS, 129.5 ± 59.9) (Figure 3c). Conversely, the percentage of TUNEL-positive cells cultured in GC was significantly higher compared to that of both MG and MS blastocysts (9.7 vs. 6 and 6.1%; $p < 0.01$) (Figure 3d). Figure 3 shows representative images of blastocysts cultured in GC (Figure 3e), MG (Figure 3f), and MS (Figure 3g) stained with Hoechst 33342 to visualize cell nuclei (blue) and TUNEL to visualize DNA-fragmented cells (red).

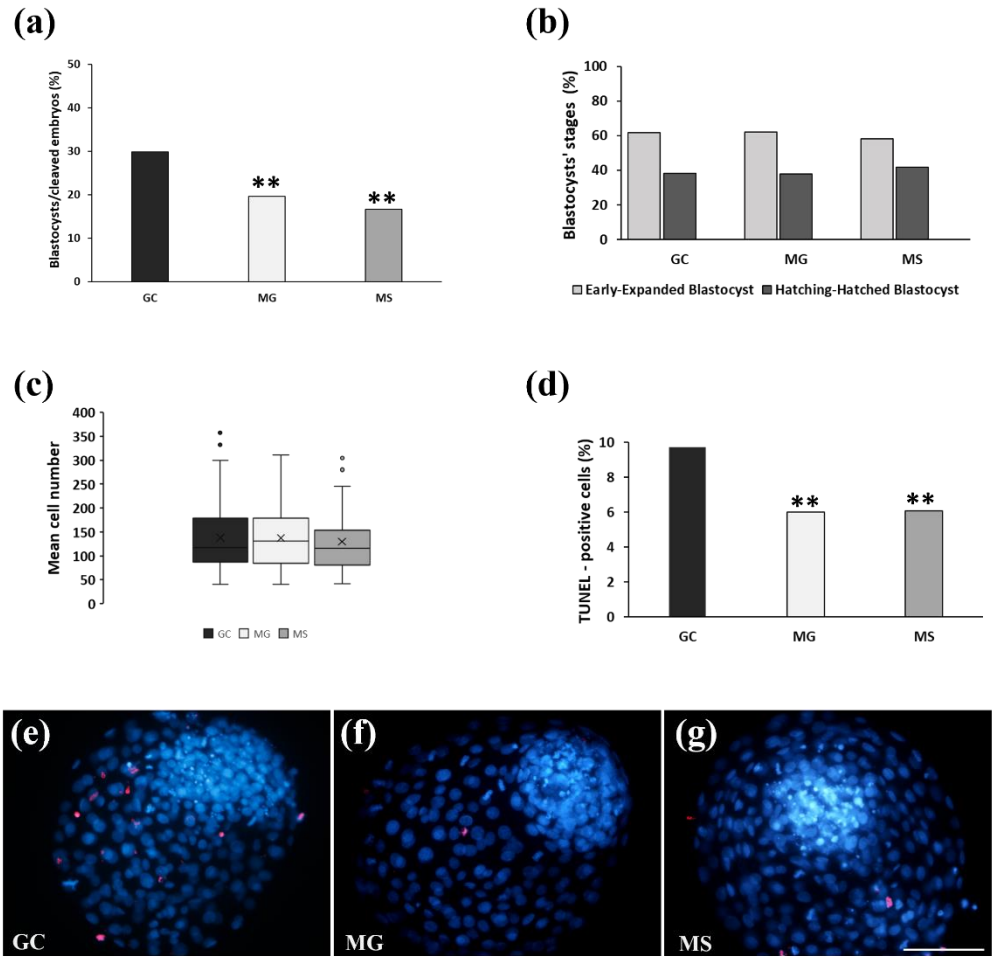

Figure 3. *Experiment 1: Uninterrupted culture in GC compared with culture in GC until day 3 p.i. followed by MG and MS culture until day 8 p.i. (a) Blastocyst's rates. (b) Blastocyst's stages. (c) Blastocyst's mean cell numbers. (d) Percentages of TUNEL-positive cells. (e–g) Representative images of cell numbers (blue) and TUNEL-positive cells (red) in blastocysts cultured in GC, MG and MS. Bar = 100 µm. **, $p < 0.01$ vs. GC.*

Experiment 2: The findings of Experiment 1 showed a reduced blastocyst's rate in embryos cultured in MG and MS from day 3 to day 8 p.i. compared with those uninterruptedly cultured in GC. In Experiment 2, presumptive zygotes were uninterruptedly cultured under the three conditions from day 1 to day 8 p.i. The following cleavage rates were detected at day 3 p.i. under the three embryo culture conditions: GC, 86.6%; MG, 83.8%; MS, 84.9%. Interestingly, blastocysts rate in MS (35.8%) was similar to GC (28.9%) and significantly higher than MG (23.1%; $p < 0.05$) (Figure 4a). No significant differences in blastocyst's stages (Figure 4b) and mean cell numbers (Figure 4c) were detected among the three culture conditions (GC, 137.8 ± 71.7; MG, 137 ± 75.3; MS, 137.1 ± 60.3). Conversely, the percentages of TUNEL-positive cells in blastocysts cultured in MG and MS were significantly lower than those in GC (7.9 vs. 7.4 and 9.7%; $p < 0.01$) (Figure 4d). Figure 4 shows representative images of blastocysts cultured in GC (Figure 4e), MG (Figure 4f), and MS (Figure 4g), stained with Hoechst 33342 to visualize cell nuclei (blue) and TUNEL to visualize DNA-fragmented cells (red).

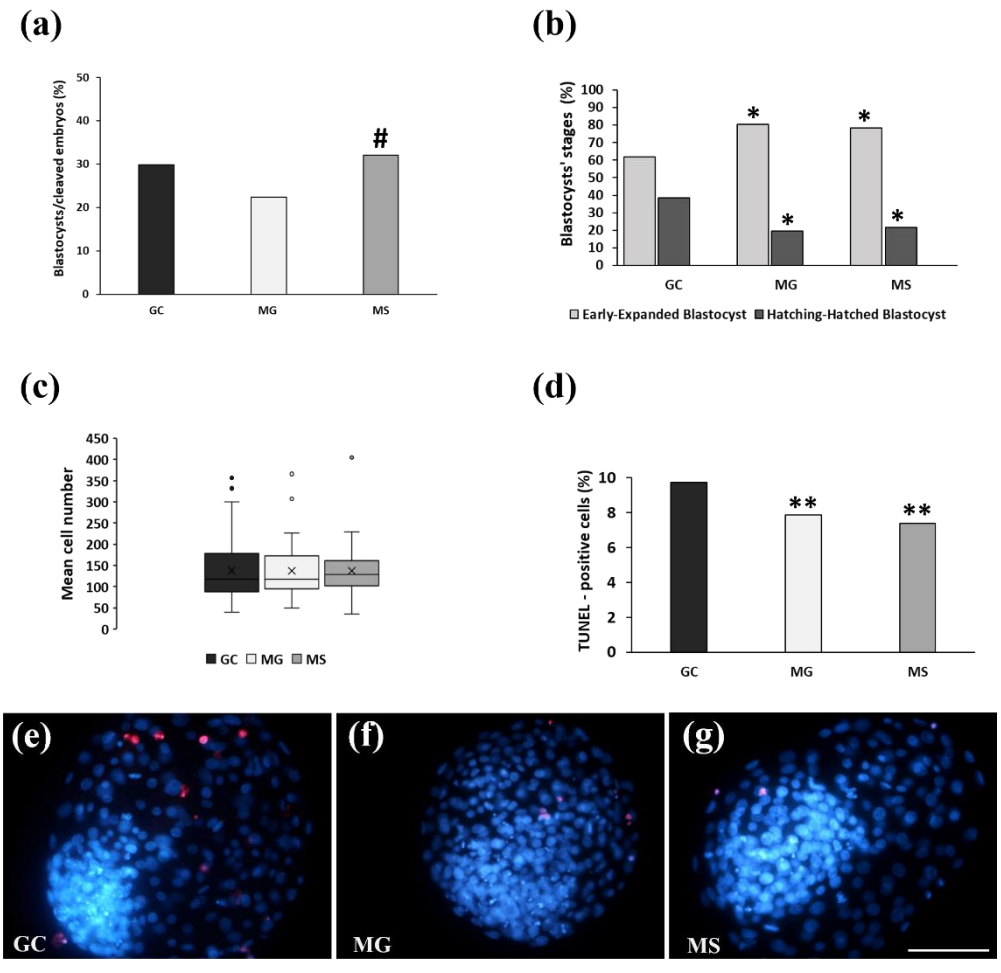

Figure 4. *Experiment 2: Culture of presumptive zygotes from day 1 to day 8 p.i. in GC, MG and MS.* ***(a)*** *Blastocyst's rates.* ***(b)*** *Blastocyst's stages.* ***(c)*** *Blastocyst's mean cell numbers.* ***(d)*** *Percentages of TUNEL-positive cells.* ***(e–g)*** *Representative images of cell numbers (blue) and TUNEL-positive cells*

*(red) in blastocysts cultured in GC, MG and MS. Bar = 100 µm. #, p < 0.05 vs. MG; *, p < 0.05 vs. GC; **, p < 0.01 vs. GC.*

Experiment 3: Presumptive zygotes were cultured under the same conditions described in Experiment 2. The following cleavage rates were detected at day 3 p.i. under the three embryo culture conditions: GC, 81.7%; MG, 87%; MS, 87%. Mitochondrial activity and lipid analysis (LDs area; LDs size frequency) of day 7 blastocysts were evaluated as additional markers of developmental competence. Although not statistically significant, the mitochondrial activity was higher in MG and MS compared to GC blastocysts (Figure 5a). Figure 5 shows representative images of blastocysts cultured in GC (Figure 5b), MG (Figure 5c), and MS (Figure 5d), labeled in vivo with MitoTracker DeepRed to visualize mitochondrial activity. LDs size frequency quantification showed a significantly higher number of classes in a range 0.07–0.3 and 0.3–5 µm2 and a lower mean diameter (1.8 ± 0.48 µm) in MS compared to MG (2.53 ± 0.10 µm) and GC (2.28 ± 0.43 µm) conditions (Figure 6a,b). Blastocyst's lipid contents were comparable among the three culture conditions (GC, 0.29 ± 0.15; MG, 0.38 ± 0.17; MS, 0.37 ± 0.25 µm2) (Figure 6c). Furthermore, the total number of LDs per blastocyst was slightly higher in MS (949 ± 50.18) compared to GC and MG (757.81 ± 200.15; 720.43 ± 200.37) (Figure 6d) conditions, although not statistically significant. Figure 6 depicts representative 3D reconstruction images of blastocysts cultured in GC (Figure 6e), MG (Figure 6f), and MS (Figure 6g), stained with Hoechst 33342 to visualize cell nuclei (blue) and BODIPY to visualize LDs (green). It was notable that in MS, a greater abundance of smaller size LDs occurred compared to GC and MG conditions (Figure 6e–g).

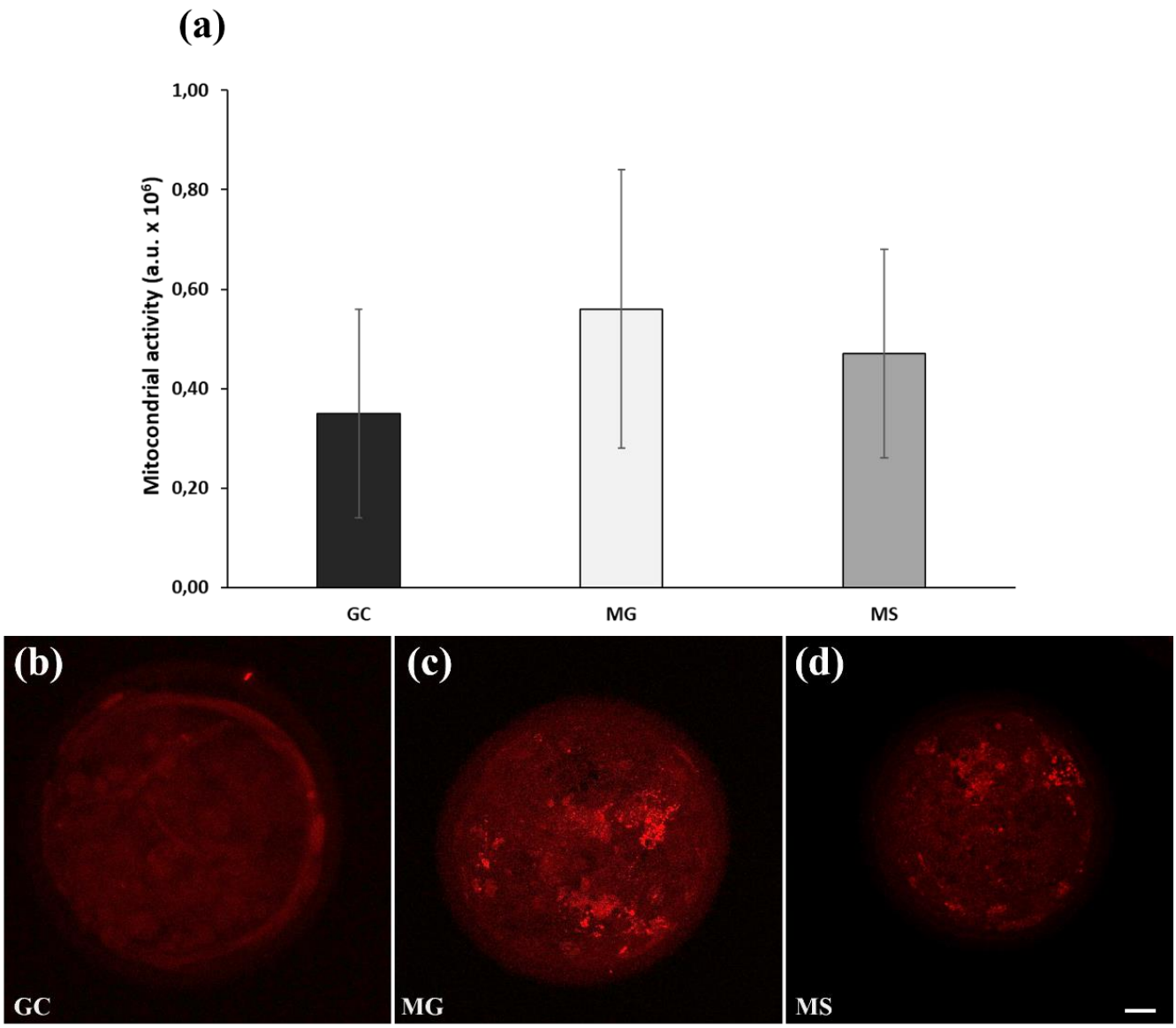

Figure 5. *Experiment 3: Culture of presumptive zygotes from day 1 to day 7 p.i. in GC, MG and MS. (a) Mitochondrial activity expressed in arbitrary units (a.u.). (b–d) Representative images of mitochondrial activity in blastocysts cultured in GC, MG and MS and labeled in vivo with MitoTracker DeepRed. Bar = 20 µm.*

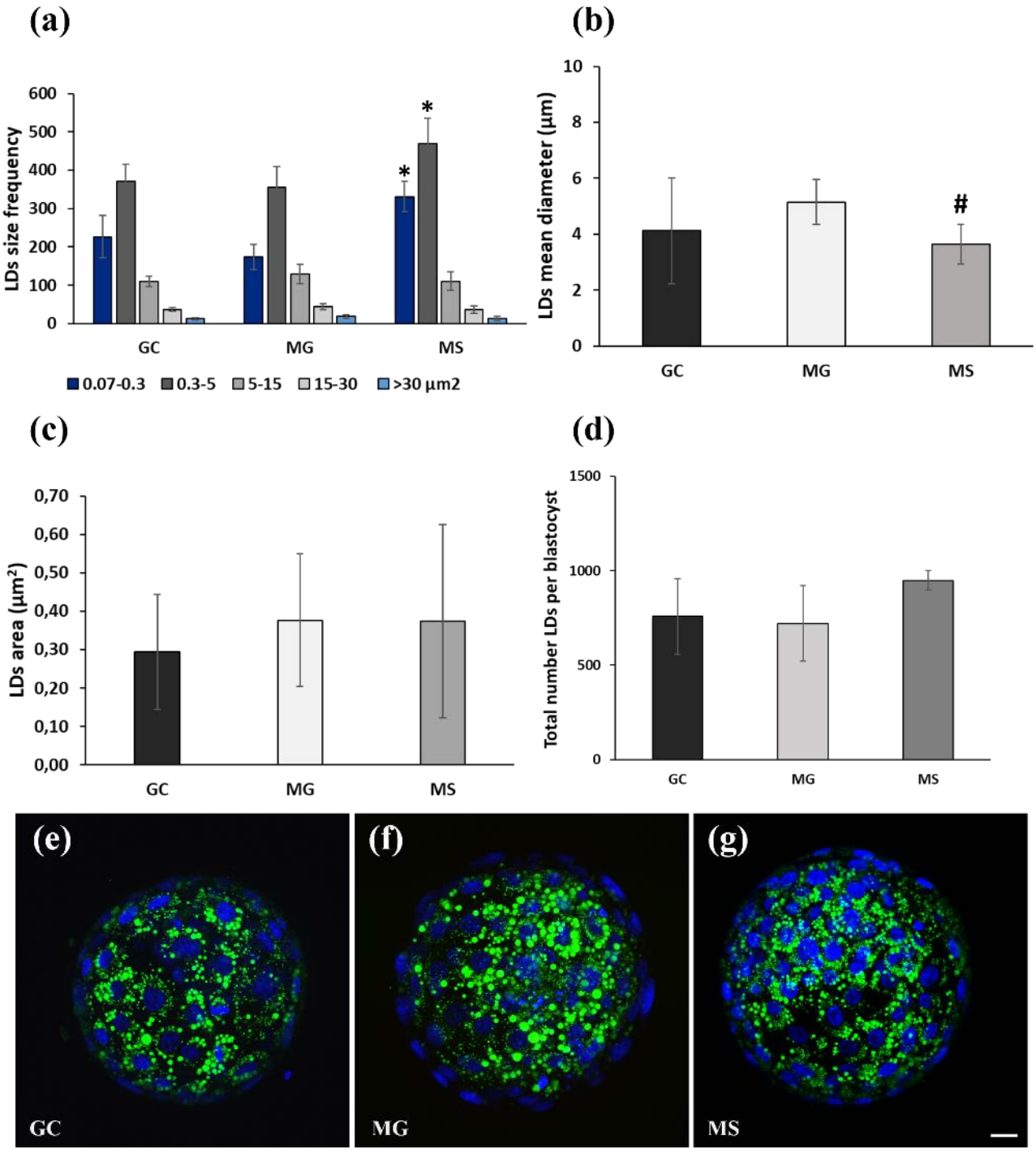

Figure 6. *Experiment 3: Culture of presumptive zygotes from day 1 to day 7 p.i. in GC, MG and MS. (a) LDs size frequency expressed in µm2. (b) Mean diameter of LDs. (c) Quantification of the total area of lipid droplets (µm2). (d) Total number of LDs per blastocyst. (e–g) Representative images of cell numbers (blue) and Bodipy–stained LDs (green) in blastocysts cultured in GC, MG and MS. *, $p < 0.05$ vs. GC and MG; #, $p < 0.05$ vs. MG. Bar = 20 µm.*

3.5 Discussion

The possibility of selecting competent blastocysts to transfer through omics assessment on spent culture media is a current trend in assisted reproduction both in human and domestic animal species [37,38,39]. However, such a procedure depends on the possibility of efficiently culturing individual embryos. Individual embryo culture negatively affects the ability to reach the blastocyst stage and blastocyst developmental competence compared with high-density group embryo cultures [17,40,41,42,43,44,45]. Thus far, the "group effect" has been clearly demonstrated in a number of poli- and mono-ovulatory species, including humans, (though controversial data were reported on the latter species) [12,13,44,46,47,48]. The lack of efficiency of individual versus group cultures depends on a number of factors, the dilution of embryo-secreted positive effects embryotropins being the most unfavorable condition [18,19]. Attempts to increase the concentration of such positive factors through a reduction in the volume of single embryo culture drops under oil must cope with the enhanced unwanted bidirectional apolar solute exchanges between oil and medium due to the increase in the drop surface relative to its volume and the possible deprivation of nutrients and accumulation of embryo-damaging waste metabolites [20,21,24,49,50]. Under this context, the most striking finding of the present study is the ability of bovine zygotes to be individually and continuously cultured for 7 days until the blastocyst stage in only ~70 nL of IVC medium without any detrimental effect in terms of mean cells numbers, mitochondrial activity and lipid content compared to standard group culture. To our knowledge, this is the first proof that such an extremely confined, static individual culture has an efficiency similar to high-density standard group culture. The minimal medium volume required to continuously culture a bovine embryo from day 1 to day 8 p.i. is thought to be a compromise among nutrient deprivation, waste metabolites accumulation, and putative embryotropins concentrations. In the case of culture in drops under oil, a further constraint of minimal required volume for individual embryo cultures is represented by the enhanced drop surface exposed to oil relative to the drop volume associated with its reduction. Such a constraint likely derives from the enhanced in and out unwanted solute exchanges between oil and medium when drop volume is progressively reduced. Oil has been shown to sequester from the culture medium apolar solutes such as steroid hormones and release toxic solutes like alkenals, aldehydes, triton X-100, etc., damaging embryo development [21,22,23,24,25,26,27]. This concept seems to be in contrast with the present findings, as the calculated surface/volume ratio during single microwell culture is 2.85, i.e., approximately equal to the ratio of 2.5 μL drops (approximating the drop geometry to a hemisphere) in which bovine zygotes reportedly do not successfully develop to the blastocyst stage. Carolan et al. (1996) cultured bovine embryos individually in droplets of

1, 2, 5, 10, or 20 µL overlaid with mineral oil and showed that embryo development was seriously compromised in droplet sizes of less than 10 µL. Moreover, individual culture in 10 and 20 µL droplets produced blastocysts with lower cell numbers and hatching rates compared with group cultures [20]. Similar findings were reported by O' Doherty et al. (1997), showing detrimental effects on blastocyst rates when embryos were individually cultured in 10 µL drops compared to high-density group culture [51]. Conversely, our main findings demonstrate that in individual cultures, approximately 70 nL is enough to allow for blastocyst development. Compared with individual cultures in 10 µL drops, the putative embryotropins concentration achieved in ~70 nL of culture medium should be more than 140-fold higher. It can be speculated that such a higher concentration of positive-effect embryo secreted factors counterbalances the well-known detrimental effects of oil overlay at the same surface/volume ratio. This is in agreement with studies demonstrating the feasibility of extremely confined culture, using the microcapillary "Glass Oviduct" system (GO) in which oil is only in contact the two ends of the capillary, achieving an extremely low surface/volume ratio. GO systems support single bovine embryo development until the blastocyst stage for 7 days of uninterrupted culture in 1 µL [52,53]. In the present study, individual culture (MS) was compared with standard group culture (GC) at a density of 1 embryo/10 µL and semi-confined group culture (MG) in a microwell chamber at 1 embryo/3.75 µL. Blastocyst rates in MS were not different from those achieved in GC and were significantly higher than those achieved in MG. The mean cell numbers were not different among the three culture conditions, whereas the proportion of apoptotic cells in MS and MG was reduced compared to GC. Similarly, Sugimura et al. (2010) showed a reduced apoptosis in microwell semi-confined group culture compared to standard group culture at a density of 1 embryo/5 µL, while blastocyst morphological quality and inner cell mass and trophectoderm cell numbers were similar under the two culture conditions [54]. In the first experiment of our study, embryos were initially cultured in GC and then transferred to MG and MS at day 3 p.i. Although the mean cell numbers in blastocysts were consistent across all embryo culture conditions, those developed in MG and MS exhibited significantly fewer apoptotic cells compared to those in GC. However, both microwell culture conditions showed a significant decline in blastocyst formation rates compared to GC culture. This could be explained by the interrupted culture in MG and MS compared to a continuous culture in GC. Transfer in the microwell devices at day 3 involved a change in medium leading to the depletion of putative embryotrophic factors accumulated during the first 3 days of development and an additional manipulation at a time close to compaction, which reportedly represents a critical stage of embryo development [55,56,57,58]. Blastocyst mitochondrial activity and lipid content were assessed as additional

markers of developmental competence [36,59]. No significant differences were detected among the three culture conditions in terms of mitochondrial activity, lipid content (per blastocyst area), and the number of lipid droplets per blastocyst. However, a higher prevalence of smaller-sized LDs was revealed in the MS condition. Lipids provide an energy reserve during the initial stages of embryo development before the activation of the embryonic genome and play a key role in plasma membrane biosynthesis [59]. However, excessive lipid droplet formation and increased droplets size, due to perturbed lipid metabolism and mitochondrial function, impair embryo developmental competence and cryotolerance [59,60,61,62]. During morphogenesis, lipid droplets accumulate and grow in size until the morula stage and decrease at the blastocyst stage due to an increased lipid demand [63,64]. The enhanced lipid accumulation in embryos is a consequence of the stress experienced during the in vitro culture procedures [59]. Although no differences in terms of lipid area and LDs number per blastocyst among the three culture conditions were found, it can be hypothesized that the reduced size of lipid droplets in MS blastocysts might be attributed to a higher metabolic activity (and possibly competence) of individually cultured embryos. Consistent with this hypothesis, lipid droplets >6 μm in diameter have been associated with the presence of immature mitochondria and reduced developmental competence [64,65]. Although the blastocyst mean droplets diameter was <6 μm across the three culture conditions, a significantly lower diameter was present in MS compared with MG blastocysts. Further studies are needed to clarify whether individually cultured blastocysts have an enhanced lipid metabolism and cryotolerance compared with embryos cultured in groups.

3.6 Conclusions

One of main goals in ART is the establishment of an effective individual culture to select euploid high-quality embryos for transfer. The demonstration that individual bovine zygotes can be successfully cultured until the blastocyst stage in ~70 nL of medium would open the way to successfully reach this goal through the application of time-lapse technology coupled to omics analysis on spent media [37,66,67]. Although currently such a nanoliter volume is poorly compatible with the analytical techniques for the detection of single embryo cell free DNA, metabolites, secreted proteins, microRNA, etc., the exponential progresses in this field could allow for the manipulation and analysis of pico- to nanoliter volumes of spent media in the near future. The finding that a bovine embryo can be effectively cultured in approximately 70 nL of medium in a microwell geometry where excessive detrimental solute exchanges between the oil and medium are prevented underscores the importance of extreme confinement in optimizing individual embryo culture. This paves the way for establishing confined

individual cultures in larger microwells, which are more conducive to the collection and analysis of spent media.

3.7 References

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5. Final remark

The findings and methodologies presented in this thesis underscore the potential of optimizing in vitro culture systems to improve the developmental competence of mammalian embryos. By recreating elements of the in vivo microenvironment—such as confined conditions and exposure to extracellular vesicles—the results highlight promising avenues for bridging the gap between in vivo and in vitro embryo development. Despite notable advancements, current in vitro culture systems remain unable to replicate the intricate interplay of biophysical and biochemical factors found in the female reproductive tract. This limitation is reflected in the reduced developmental competence and genomic stability of in vitro-produced embryos compared to their in vivo-derived counterparts. The experimental results from this work demonstrate the efficacy of confined culture systems in enhancing individual embryo development while preserving the potential for advanced analytical techniques, such as non-invasive preimplantation genetic testing. Furthermore, the sequential addition of extracellular vesicles from oviductal and uterine origins reveals a synergistic effect on improving embryonic quality, mimicking natural embryonic-maternal communication. This research provides a foundational step toward refining in vitro embryo culture practices in both human and animal-assisted reproduction technologies. However, further studies are needed to elucidate the optimal combination of biophysical parameters and molecular signaling factors that support embryo development. The integration of these insights into clinical practice holds the potential to significantly improve ART outcomes, thereby addressing some of the persistent challenges in the field of reproductive biology.