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REVIEW

Preimplantation embryo metabolism and culture systems: experience from domestic animals and clinical implications

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Abstract Despite advantages of in vitro embryo production in many species, widespread use of this technology is limited by generally lower developmental competence of in vitro derived embryos compared to in vivo counterparts. Regardless, in vivo or in vitro gametes and embryos face and must adjust to multiple microenvironments especially at preimplantation stages. Moreover, the embryo has to be able to further adapt to environmental cues in utero to result in the birth of live and healthy offspring. Enormous strides have been made in understanding and meeting stage-specific requirements of preimplantation embryos, but interpretation of the data is made difficult due to the complexity of the wide array of culture systems and the remarkable plasticity of developing embryos that seem able to develop under a variety of conditions. Nevertheless, a primary objective remains meeting, as closely as possible, the preimplantation embryo requirements as provided in vivo. In general, oocytes and embryos develop more satisfactorily when cultured in groups. However, optimization of individual culture of oocytes and embryos is an important goal and area of intensive current research for both animal and human clinical application. Successful culture of individual embryos is of primary importance in order to avoid ovarian superstimulation and the associated physiological and psychological disadvantages for patients. This review emphasizes stage specific shifts in embryo metabolism and requirements and research to optimize in vitro embryo culture conditions and supplementation, with a view to optimizing embryo culture in general, and culture of single embryos in particular.

Keywords In vitro embryo production · In vitro maturation · Oocyte maturation · In vitro fertilization · Intracytoplasmic sperm injection · In vitro embryo culture

Capsule Successful in vitro production of embryos may be enhanced by utilizing contemporary knowledge of oocyte and embryo metabolism to closely match in vitro environments with gamete and zygote requirements.

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Introduction

The ultimate goal of assisted reproductive technologies (ART) is the birth of healthy offspring. Despite rapid progress in the field of ART for both humans and domestic animals, challenges remain. One example is that defined media still do not reflect the natural microenvironments of the female reproductive tract where gametes interact and embryos develop. Therefore, further optimization of culture conditions would provide a better environment especially for single oocytes, thereby circumventing problems associated with ovarian super stimulation and transfer of multiple embryos.

Defining optimal factors and substrates for the entire in vitro embryo production process has been an area of intensive research leading to understanding those most critical for early preimplantation embryonic development. However, the dynamic and complex nature of in vivo conditions makes it difficult to emulate such microenvironments in vitro with defined media. Complicating our assessment of culture methods is the plasticity of gametes and preimplantation embryos, with acceptable development reported for a wide variety of culture conditions. Nonetheless, there are opportunities to define better culture systems that more closely match the physical and chemical interactions between gametes and embryos with the diverse microenvironments presented by the

female reproductive tract. Improvements in culture systems should be focused on embryo metabolism at every stage of in vitro embryo production (IVP). In vivo, the embryo is exposed to a dynamic environment as its metabolic needs evolve from early to later preimplantation stages. Consumption of sodium pyruvate and other carboxylic acids is relatively greater at precompaction stages, coincident with the relatively more oxygenated environment of the oviduct. Once in the uterus, usually at compacted morula or early blastocyst stages, the oxygen tension is decreased and glucose metabolism increases to accommodate higher energy demands. The metabolic pathways and shifts in requirements of preimplantation mammalian embryos are depicted in Fig. 1. The switch from low to high glucose consumption occurs in most species [1, 2] and places a high priority on meeting the changing metabolic needs of embryos in vitro. More optimal IVP systems would result in fewer "developmental block" issues and facilitate production of more high quality embryos for successful fresh transfers or cryopreservation.

Here we review current understanding of optimal conditions for meeting the metabolic requirements during in vitro embryo culture, pointing out existing obstacles and promising avenues of research for improvement. The links between oocyte quality and embryo quality and useful assessment techniques are explored. In addition, current research in animal models on oocyte maturation (IVM) and in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) are summarized for implications to human clinical situations. Our intention is to provide a comparative review of current knowledge of bovine and other mammalian IVP models as a reference for enhancement of embryo production in domestic animals and potential application to human ART.

In vitro maturation

In vitro maturation in human ART is mostly used as a last resort, especially when patients do not respond well to superstimulation protocols; the resulting low maturation rates or overall poor developmental competence may potentially be inherent to the intrinsic quality of the oocyte and not due to the IVM procedure per se. Consequently, the poor IVM results have raised concerns and thus have limited the use of this procedure in human medicine [3, 4]. In addition, the ICSI technique further limits the application of IVM for human oocytes since the first steps prior to and during fertilization are bypassed by the ICSI procedure. However, IVM may be suitable for patients with polycystic ovarian syndrome who are at higher risk of developing ovarian hyperstimulation syndrome when they undergo follicle superstimulation treatments [3].

There has been considerable progress in refining protocols for in vitro maturation of oocytes of livestock species with potential application to human IVM [3, 5]. Controlling the delay of meiotic progress by means of adenylate cyclase activators, cyclic adenosine monophosphate (cAMP) analogues, or phosphodiesterase inhibitors is very critical during IVM. In addition, optimal conditions for cumulus cells surrounding the oocyte need to be considered as there is a complex, yet well orchestrated, bi-directional communication between these two cell types. Oocyte-secreted factors (OSF [5]) are involved in cumulus cell pathways that are directly related to the oocyte's welfare i.e. regulation of apoptosis, metabolism, proliferation, expansion and differentiation. Recently, natriuretic peptide type C (NPPC) and its receptor (NPR2) were reported as essential for the maintenance of meiotic arrest in mouse oocytes [6]. The proposed mechanism in vitro is through production of cyclic guanosine monophosphate (cGMP) by exogenous NPPC binding to its receptor on cumulus cells. Consequently, cGMP is transferred, through gap junctions, to the oocyte where it plays a role in the inhibition of the phosphodiesterase 3A (PDE3A), thereby maintaining meiotic arrest by protecting cAMP levels from the degrading effects of active PDE3A. In vitro, exogenous estrogen seems to be required for the maintenance of NPR2 [6]. Collectively, cAMP and cGMP modulators along with OSF may result in improved protocols for in vitro maturation with potential application for ART.

During maturation, migration of mitochondria and lipid droplets to the center of the oocyte and location of cortical granules beneath the oolemma are good indicators of oocyte competence [7]. The novel maternal effect gene, peptidylarginine deiminase 6 (PADI6), which appears to be restricted in the mouse to the oocyte and early embryo, is required for the formation of cytoplasmic lattices and directly related to the formation of microtubules [8]. The latter are responsible for the microorganelle redistribution during maturation and, thus, it is worthwhile to characterize this pathway to optimize IVM approaches. Since PADI6 is a Ca^{2+} sensitive enzyme, the role of Ca^{2+} oscillations is relevant in PADI6 function during maturation/fertilization [9].

The potential for providing an *assisted metabolism* to oocytes that have been exposed to a sub-optimal in vivo environment is a promising area for research. An altered follicular environment may result in changes in glucose metabolism [10] that profoundly affect viability of cumulus oocyte complexes during maturation. In cases of hyperglycemia, enzymes related to glycolysis such as hexokinase may become saturated and glucose may be repartitioned to other pathways such as the hexosamine biosynthesis (HBP [fuel sensing]) or Polyol pathways. Consequently, imbalances to the redox system may occur as well as undesirable O-linked glycosylation of serines and threonines normally responsive to kinases for activating other necessary pathways in meiotic progression [10]. Nitration of these same amino acids might also be more common in an ammoniagenic environment [11] resulting from diets (e.g.

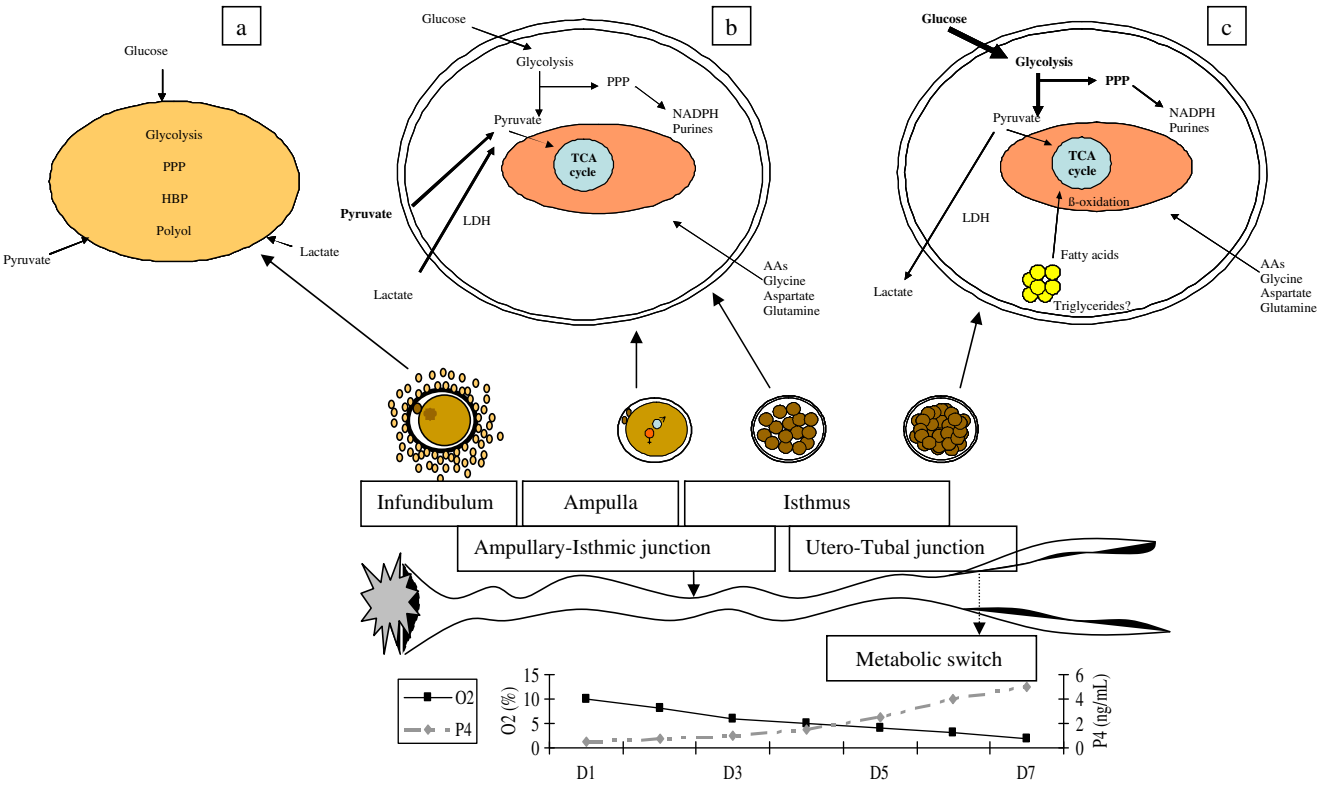

Fig. 1 Summary of embryo metabolic events during preimplantation. **a** Within the cumulus oocyte complex, the oocyte emphasizes glycolysis and pentose phosphate (PPP) pathways. Glycolysis provides an important source of energy via hexokinase activity. PPP provides reducing equivalents (NADPH) and production of pentoses for the synthesis of purines. The hexosamine biosynthesis pathway (HBP) is important for extracellular matrix expansion of the cumulus cells and o-linked glycosylation of important amino acids residues involved in the cell cycle progression. Polyol pathway provides sorbitol and fructose as other sources of energy and it contributes to maintenance of redox balance. **b** After fertilization, the zygote depends on tricarboxylic acids such as pyruvate and lactate as the primary energy sources. **c** Bovine morula compaction is an important event that occurs at day five after fertilization in the bovine embryo and marks the beginning for higher glucose consumption. Thus, in preparation for a hypoxic environment in the uterus and coincident with the increase in plasma progesterone (P4), the preimplantation embryo at morula stage switches its metabolism from low to high glucose utilization via glycolysis and PPP pathways

ruminants) or media high in amino acids, thus, oocyte and embryonic developmental competence would be compromised.

Cumulus-oocyte complexes cultured in media supplemented with serum usually perform better than those cultured in serum-free media supplemented with synthetic macromolecules. However, in some situations, serum from patients (e.g. patients with recurrent pregnancy loss or contaminated commercial batches) may contain undesirable components that could potentially be detrimental for IVM. Evidence of rescuing factors, such as paraoxonase 1 (PON1 [in oocytes]) and preimplantation factor (PIF [in embryos]), suggests that oocytes may be improved by an optimal redox environment and thus increase the chances of fertilization or subsequent embryo development [12, 13]. Further research on these various factors is needed to better characterize under which conditions they might be beneficial as supplements for IVM. In addition, metabolic pathways in vitro are regulated by substrate availability in media and gas phase conditions [3, 14]. Exploring the latter options may result in optimization of IVM for a specific case when standard IVM conditions do not work due to a metabolic enzyme alteration.

In vitro fertilization/intracytoplasmic sperm injection

The technique of intracytoplasmic sperm injection (ICSI) is nearly unique as a means to achieve fertilization in the face of severe male infertility-related conditions [15]. Although ICSI is the most widely used technique for micro-assisted fertilization in humans, it remains inefficient for most other species due to issues related to initiation of the normal cascade of events associated with activation of the arrested MII oocyte.

Over the last few years, several studies for improvements of ICSI in livestock have been conducted, especially where IVF systems are currently inefficient (e.g. equine IVF). As reviewed by García Roselló et al. [16], the future of ICSI, in laboratory and livestock species, will most likely deal with problems not associated with male infertility. Nevertheless, this will provide an excellent opportunity for refinement of

protocols to optimize the technique. Malcuit et al., working with cattle, indicated that some of the signaling mechanisms that lead to the activation of the phosphoinositide pathway and generation of Ca^{2+} oscillations during natural fertilization (vide infra) were not replicated by ICSI [17]. The problem appeared to stem from the inability to induce Ca^{2+} oscillations and that activation by an exogenous agent was required to initiate embryo development. Several methods for exogenous activation after ICSI have been reported such as applying calcium ionophores (e.g. ionomycin) in combination with 6-DMAP [18], ethanol [19], and roscovitine plus cytochalasin B [20]. Further, in human males with complete globozoospermia, ICSI along with assisted oocyte activation using calcium ionophores has resulted in pregnancies and childbirths [21, 22]. To date, global non-human blastocyst rate from ICSI remains at approximately 20 %.

Early fertilization events have been widely characterized in several species. Release of a spermatic phospholipase C_ζ (PLC_ζ) is needed for the hydrolysis of oocyte-cytoplasmic phosphatidylinositol-4, 5-bisphosphate (PIP_2) with formation of diacyl-glycerol phosphate (DAG) and inositol 1,4,5-trisphosphate (IP_3). Subsequently these products stimulate resumption of meiosis by the arrested MII oocyte and extrusion of the second polar body followed by formation of female pronuclear envelope and pronucleus. This series of events depends on Ca^{2+} oscillations originated by sperm PLC_ζ that take place within a few hours after the spermatozoon enters the ooplasm. These oscillations are essential and result in downstream production of molecules necessary for MII oocyte activation (reviewed in [9, 17, 23, 24]). However, alternative mechanisms involved during oocyte activation seem to show a certain degree of redundancy. One example is the sperm-borne protein with conserved AA sequence throughout species, post-acrosomal WW domain binding protein (PAWP), that induces MII oocyte activation in a Ca^{2+} oscillation-related manner [25]. Further, it has been shown that not only Ca^{2+} oscillations are needed for the progression of the cell cycle, but also zinc "sparks", a conserved mammalian mechanism, are required early after fertilization/activation [26]. Collective integration of MII oocyte activation processes may need to be addressed.

Sperm selection based on physiological processes with potential practical applications have been reported [27] where single layer centrifugation (SLC) and hyaluronan binding assays (HBA) are becoming the method of choice. Laser technology might be used to further improve ICSI, especially for livestock species as a model for potential human application. Recently, a real time system was developed using laser technology and fluorescent imaging to measure individual sperm performance efficiently [28]. This system offers an advantage for sperm selection since it allows measurement of mitochondrial membrane potential. Thus, studies of sperm energetics could be performed to characterize individual sperm not only from valuable high genetic merit animals and endangered species, but also from human patients being referred for IVF services. A possibility of improving ICSI results, at least in livestock species, may be to use this technology after the swim-up method to further optimize sperm selection from the pool of elite quality sperm (M.W. Berns, personal communication). Therefore, development of better approaches is needed to guarantee that the fertilizing spermatozoon expresses optimum performance i.e. capable of undergoing acrosome responsiveness, hyperactivation, and activation of the arrested MII oocyte. Along these lines, alternative options for assisted oocyte activation after ICSI may need to be addressed such as the use of PLC_ζ or PAWP to trigger the necessary, more natural, Ca^{2+} oscillations or zinc sparks. Cloning research in livestock species may also be directly benefited by such studies.

Embryo culture systems

The existence of diverse embryo culture media and methods has made definition of the optimal components of embryo culture media very challenging. In vivo, the embryo is exposed to a dynamic environment as its metabolic needs evolve from early to later preimplantation stages. However, despite greater understanding of the biology of the embryo and its interaction with the oviduct and uterus, it has been very difficult to emulate the different stage-regulated embryo metabolic pathways in vitro. Soon after fertilization the embryo benefits from a well oxygenated environment as in the oviduct and it shows substrate preference for tricarboxylic acids such as pyruvate and lactate. However, as the embryo develops to morula there is a metabolic switch that needs to be activated to supply an alternative source of energy in response to a more hypoxic environment in the uterus (refer to Fig. 1).

Emerging new technologies such as microfluidics, time-lapse cinematography and metabolomics may provide further advances for producing good quality embryos in vitro before transfer to improve the likelihood of successful pregnancies and also to reduce the number of embryos transferred per cycle.

Preimplantation embryo metabolism and in vitro environment

The pioneering work by Yanagimachi and Chang [29] reported the first IVF using golden hamsters and led to other important milestones in the creation of mammalian embryo culture media. Important progress on in vitro embryo culture was made by Tervit and coworkers in developing a synthetic oviduct fluid (SOF) medium based on biochemistry and physiology studies of the ovine oviduct [30, 31]. Studies by Ménézo et al. [32] provided insight on human in vitro embryo development. From the 70's onwards, findings of Bavister and

coworkers using murine models resulted in major advances toward meeting embryo metabolic requirements [33]. Critical work by Biggers and coworkers in the 80s and 90s, using several mammalian species, created a sequential simplex optimization media (SOM), and later improvements (KSOM) allowed the 2-cell block to be overcome at last [34–36]. Studies by Gardner and collaborators during this time led to important findings on embryo metabolism and creation of Gardner's media [37]. Further work on livestock species resulted in critical improvements or development of new media such as Ménézo's B2 [38], CR1aa [39] and SOFaa [40].

The extensive research activity carried on from about 1970 to 2000 was the basis of today's commercial media formulations for human and other mammalian species, but many of the advances in culture systems have been the result of empirical adjustments. As the embryo migrates from the oviduct to the uterus, a metabolic switch from low glucose to high glucose consumption occurs in most species [41–43]. Interestingly, equine embryos seem to show a different pattern in substrate preferences throughout preimplantation development, preferring hyperglycemic (>15 mM) and hypoxic conditions (5 % CO_2, 5 % O_2 [44]). In any species, media formulation should account for this switch in metabolic pathways to most efficiently produce ATP depending on embryo-stage preference. Various chemical modulators and nutrients with established metabolic effects for optimizing embryo development are listed in Table 1 and their metabolic effects are described in this section.

Oxidative metabolism is the main source for ATP especially at early (precompaction) embryonic stages, sodium pyruvate being the preferred tricarboxylic acid substrate. One of the earlier problems faced by researchers was the effect of glucose in media at precompaction stages and the attendant undesirable early onset of glycolysis resulting in embryo growth retardation or embryo development block (reviewed by Thompson [41]). However, supplementation of culture media with ethylenediaminetetraacetic acid (EDTA) overcame this issue with its inherent "Crabtree-effect" allowing embryos to continue further cell cycles [1, 45]. After the problem of preventing early onset of glycolysis was solved by the addition of EDTA, attention turned to enhancement of glucose metabolism at postcompaction stages. Compounds with the opposite effect of EDTA not only enhance glucose consumption, but improve embryonic development and competence. Oxidative phosphorylation uncouplers such as 2, 4 dinitrophenol (DNP) partially inhibit oxidative phosphorylation while enhancing glycolysis [1]. Another metabolic regulator, phenazine ethosulfate (PES), is an electron acceptor for reduced nicotinamide adenine dinucleotide phosphate (NADPH) thereby enhancing the pentose phosphate pathway [46]. In addition, PES inhibits fatty acid synthesis [47]. Since DNP and PES both favor the metabolism of glucose, but via two different metabolic pathways, it would be of interest to evaluate the effects on the redox status of combining both chemicals under different glucose supplementation levels. Since these metabolic regulators may play a role in the fatty acid metabolism, the idea of combining their use with polyunsaturated fatty acid (PUFA) is also attractive.

Assisted metabolism may be necessary to aid in the regulation of glucose uptake in postcompaction in vitro produced embryos. This approach would permit improved efficiency of ATP production by competent embryos while also rescuing embryos that would otherwise fail. Along these lines, Lopes et al. [48] reported bovine embryo competence in relation to expression of glucose transporter 1 (GLUT-1) and glucose-6-phosphate dehydrogenase (G6PD) mRNAs. They demonstrated that metabolically active blastocysts use more oxygen and glucose than less competent embryos, indicating greater capability for glycolysis [49]. Further, Leese et al. reported a functional metabolic "quietness" of viable preimplantation embryos, in contrast to impaired embryos which demonstrated increased energy consumption [50]. Thus, there is a differential threshold between embryos with a certain degree of impairment showing higher (altered) metabolic rates and "quiet" embryos with an optimal degree of metabolic activity that could be measured at both pre and post compaction stages. Relevance of glucose transporters becomes critical, as embryo compaction occurs and glucose metabolism is enhanced, coincident with expression of insulin and IGF-I receptors. These receptors act in concert with glucose transporters, especially GLT-8, an insulin sensitive GLUT (reviewed [51]). According to a recent report, male and female bovine embryos have different requirements for glucose. Male embryos showed higher developmental rates compared to female embryos due, at least in part, to a higher metabolic rate of glucose [52]. This finding could significantly impact media formulation especially for the dairy or beef industry where the markets for female or male embryos are more popular, respectively.

Oxygen tension plays an important role in the expression of hypoxia inducible factors required for the up regulation of genes involved in the process of enhanced glucose metabolism and overall embryonic developmental competence postcompaction [42]. Today's embryo culture systems rely on a controlled oxygen tension of 5–10 % because there is extensive evidence for detrimental effects of higher oxygen tension in culture without supplementation of antioxidants or co-culture with somatic cells. Even the slightest increase in oxygen tension above the aforementioned range resulted in embryos with higher rates of apoptosis (among other developmental variables) than those cultured under controlled hypoxic conditions (5 % O_2; [53]).

Amino acid (AA) metabolism is gaining research interest in human and bovine species. Current sequential IVC systems provide the usual non-essential AAs during precompaction stages and a full array of essential and non-essential AAs at peri and postcompaction stages (reviewed by Gardner [54]

Table 1 Past and current focus on embryo metabolism in livestock species

Metabolic area	Factor studied	Reference
-Modulation of glucose consumption at precompaction stage	EDTA	Reviewed in Thompson et al. [1]
-Enhancement of glucose consumption at pericompaction stages	DNP	Thompson et al. [1]
	PES	De La Torre-Sanchez et al. [46]
	HIF	Harvey [42]
-Utilization of internal lipid stores	L-Carnitine	Sturmey et al. [70]
		Sutton-McDowall et al. [71]
-Progesterone-related nutrient metabolism	AA + Glucose	Clemente et al. [68]
		Hugentobler et al. [121]
		Garcia-Herreros et al. [52]
-Sex-dependent nutrient requirements	AA	Sturmey et al. [60]
	Glucose	Garcia-Herreros et al. [52]

Abbreviations: *EDTA* ethylenediaminetetraacetic acid, *DNP* 2, 4—Dinitro phenol, *PES* Phenazine ethosulfate, *HIF* Hypoxia inducible factors, *AA* amino acids

and Feugang et al. [43]). Although providing an array of AA supports preimplantation embryo development, there has not been extensive research on the AA requirements at specific embryo stages. Besides the knowledge that alanine and glycine are the two major AAs required in higher concentrations for IVC, methionine is emerging as also being critical due to its interaction with folate [55]. The interaction of folate/methionine metabolism seems especially important because methionine is present in supraphysiological concentrations in contemporary culture media formulations, whereas, folate is not present in most IVC media formulations. The implications are that the embryo may deplete folate stores from the oocyte during advanced embryonic cellular cycles resulting in potential interruptions in the aforementioned metabolic cycle. This adverse situation may be exacerbated with an excess of methionine available for intracellular trans-methylation and possible epigenetic alterations [55]. However DNA methylation of blastocyst nuclei was unaffected by methionine concentration at several concentrations in vitro [56]. In addition, these authors determined that methionine requirement during bovine preimplantation development is between 14 and 21 μmol/l and similar to the concentrations found in the bovine reproductive tract. The interaction of methionine and folate needs to be considered further.

Embryos of ruminants, as well as other mammals have requirements for both essential and non-essential AAs. Besides methionine, the essential AA lysine participates in metabolic pathways (e.g. donating carbon skeletons) and more importantly can be an epigenetic target site in histone tails to modulate chromatin structure [57]. Another essential AA is histidine, which seems to be related to interferon tau expression (IFNτ; embryonic antiluteolytic cytokine required and secreted in ruminants for maternal recognition of pregnancy; [58, 59]). In addition to increased requirements for essential AAs as embryo development progresses, AA preference by gender has been reported recently. In vitro produced female embryos showed preference for arginine, glutamate and methionine whereas male embryos showed preference for phenylalanine, tyrosine and valine [60]. For these reasons AA embryo requirements in vitro need to be established for improved development and conception rates per species.

According to metabolic and nutritional needs, the mammalian embryo shows a marked difference in requirements at early and late preimplantation stages. For this reason, sequential media have proved successful [7, 41, 43, 54, 61]. Different sequential media formulations have been reported to achieve acceptable rates of embryo development in vitro; however, there is usually limited information available on success rate following embryo transfer. Sequential culture strategies consider at least two phases covering pre-and-postcompaction stages, but, there remains much controversy regarding sequential vs. one-step culture with both similar and contradictory results [62].

The success of an IVP laboratory may stem not only from improvements of the IVC per se, but from the entire IVP system [54]. The latter includes: incubation conditions, gas phase, culture media, oil overlay, tissue culture ware/contact supplies, and embryo density and the volume of medium. In addition, embryologist or technician skills have to be considered as part of the system. One important area that deserves more attention is pH regulation in livestock embryo culture media. Emulation of physiological pH at the different preimplantation embryo stages may be critical for optimizing single and overall embryo culture systems. Therefore, the use of new pH zwitterionic buffering systems that would maintain a desired pH with minimal variations, during culture or handling, could result in more suitable environments for improved embryo development [63].

Defined media and supplements improving embryo production

Over the past 20 years a marked evolution in IVP systems has occurred from using surrogate xeno-oviducts and embryo co-

culture conditions with somatic cells toward the optimum goal of producing embryos in defined serum-free media [7, 61, 64, 65]. The use of serum was the basis for the development of culture systems that did not require co-culture conditions. Although supplementation of IVC media with serum or albumin is widely acknowledged to support improved embryo development relative to serum-free conditions, these supplements may also include detrimental factors that potentially impair embryo development. When using serum-containing media, a problem is encountered in culturing embryos destined to undergo cryopreservation procedures. Excessive accumulation of triglycerides may occur that results in detrimental effects on post-thaw performance. However, a recent study on mitochondrial ultrastructure assessed by transmission electron microscopy indicated no differences between embryos treated with serum vs. serum-free groups suggesting that serum may not be a major detrimental factor at least at the mitochondrial level before bovine embryo genome activation (EGA; [66]). Nonetheless, for these and other reasons, such as minimizing the risk of disease transmission and evaluating the effect of specific factors during IVC on embryo performance, there is continuing demand for reliable and scientifically well-defined cell and tissue culture methods [67]. However, during the process of defining systems we must consider their adaptation to the physiological requirements of the embryo during culture [41]. Table 2 summarizes consensus results in the literature for blastocyst rates among the common livestock animals utilizing varied culture media formulations.

Optimal defined culture media for embryo production appear to require a combination of hormones, cytokines and antioxidants in order to replace serum or albumin with other macromolecules. Recently, more competent embryos have been reported when growth factors or cytokines are added either singly or in combination to the embryo culture media throughout the preimplantation stages. The major roles of these factors are in regulation of apoptosis, cell differentiation and proliferation. Table 3 summarizes the effects of various growth factors and hormones for improving embryo development in defined media.

An important hormone for potential relevance in vitro is progesterone (P4) because of its requisite role in the maintenance of pregnancy. Results from trials studying the effects of P4 on preimplantation embryo development have been controversial. The hormone itself is lipophilic and in culture systems that usually rely on overlay of oil to avoid droplet evaporation, the P4 may be depleted from the media by the time embryos are transferred into the droplet. Preimplantation bovine embryos express P4 receptors but there is no effect of in vitro P4 supplementation on elongated embryos post hatching [68]. Considering earlier stages, embryos supplemented with a low dose of P4 (1 ng/ml) metabolized more glucose at postcompaction stages [69]. Although P4 supplementation during culture did not improve embryo characteristics, these authors concluded that subtle changes in overall glucose partitioning and metabolism could have long term effects. In a more recent report, Ferguson et al. (2012) reported that the addition of P4 to the embryo culture stimulated the rate of embryo development and improved hatching rates. More research on effects of P4 in vitro seem warranted since the rise in circulating P4 levels that occurs after estrus and insemination is coincident with the embryo entering the uterus and the timing and switch from low to higher glycolytic rates.

A promising area for the improvement of single embryo culture systems is the utilization of embryonic lipid stores as an alternate source of energy especially for species such as bovine, equine, and porcine where implantation occurs later than in human and murine species [70, 71]. Intriguingly, in invertebrate species lipid droplets seem to regulate the function of proteins related to embryo genome activation (EGA) by maintaining them in an inactive state. Thus, lipid stores may function not only as a source of energy reserve, but also as a potential reservoir for signaling factors that play a role in cellular fate, in a spatiotemporal way, for mammalian early embryo development [72].

Oxidative stress and antioxidants

Much research on IVP has focused on the damaging effects of an oxidative environment and the inherent creation of reactive oxygen and nitrogen species (RONS) that may impair embryo development. The reduced glutathione (GSH) to oxidized glutathione (GSSG) ratio is the usual indicator of choice for measuring the redox status. Based on ratios of GSH/GSSG, the cytoplasm is a strong reducing environment whereas the endoplasmic reticulum (ER) is significantly less reducing [73]. Consequently, excessive supplementation of reducing agents in media to offset oxidative damage has resulted in controversial outcomes as slight redox imbalances are detrimental for embryo development.

In eukaryotic cells there are two major systems responsible for maintaining a reduced state inside the cell. Glutathione and thioredoxin (Trx) systems along with their respective reductases provide protection to proteins and enzymes during catalytic reactions in an interactive way [73, 74] in order to maintain the reducing environment of the cell, to detoxify RONS [75], and to modulate various cellular activities such as gene expression, cell proliferation, survival and cell death [76]. Disulfide bonds provide another mechanism of cellular protection and are essential for the folding and stability of proteins at the level of the ER. Under normal conditions, the most commonly used antioxidants such as β-mercaptoethanol (β-ME), GSH and taurine provide a buffer against oxidizing agents. However, an excess of antioxidants may compete for oxidizing equivalents in the ER and, thereby, promote an unfolded protein response due to an excessive accumulation of misfolded proteins [73]. Consequently, an excess of

Table 2 Consensus average embryo blastocyst rates among large animal domestic species cultured with different media formulations

Media type	Species	In vitro procedure	Blastocyst rate (%)	Reference
KSOM	Porcine	IVF	32.1	Hashem et al. [122]
	Bovine	IVF	37.1	Felmer et al. [123]
SOFaa	Porcine	Cloning	38.0[a]	Lagutina et al. [124]
	Ovine	IVF	24.1	Garcia-Garcia et al. [125]
	Ovine	IVF	28.7	Wan et al. [126]
	Bovine	IVF	34.9	Sanches et al. [127]
CR1aa	Equine	ICSI	25.5[b]	Matsukawa et al. [128]
	Ovine	IVF	25.5	Wan et al. [126]
	Bovine	IVF	36.1	Somfai et al. [129]
DMEM/F-12	Equine	ICSI	42.0[c]	Hinrichs [130]
NCSU-23	Porcine	IVF	30.5	Hashem et al. [122]
	Porcine	Cloning	28.5[a]	Lagutina et al. [124]
	Porcine	IVF	32.8	Kim et al. [131]
PZM	Porcine	IVF	27.4[d]	Hu et al. [132].
	Porcine	IVF	27.5[e]	Yoshioka et al. [133]
IVD101	Bovine	IVF	37.1	Hoshi [134]
	Bovine	IVF	21.7	Somfai et al. [129]
Gardner (G1/G2)	Ovine	IVF	21.5	Garcia-Garcia et al. [125]
	Bovine	IVF	27.8	Sanches et al. [127]
IVF VET Solutions	Bovine	IVF	69.0[f]	Albuz et al. [135]

[a] Overall average blastocyst rate from 3 different donor cell lines

[b] Blastocyst rates originated from expanded oocytes

[c] Highest blastocyst rate reported for equine ICSI-produced embryos

[d] Blastocyst rate achieved by supplementing Vitamin C (20 μg/ml)

[e] Blastocyst rate reported at day 5 post IVF

[f] High blastocyst rate achieved by a novel approach, stimulated physiological oocyte maturation (SPOM), during IVM

reductants would disrupt not only multiple ER functions, but also signaling pathways that rely on low physiological concentrations of RONS as well as redox-sensitive transcription factors viz. *reducing stress* [73]. Alternatively, an excess of RONS produced without sufficient antioxidant protection may lead to disequilibrium of the redox balance viz. *oxidative stress* characterized by RONS targeting and damaging DNA, RNA, protein and lipids [74].

Many attempts to minimize oxidative stress have been made during the past two decades. Culturing embryos under hypoxic conditions (i.e. 5–10 % O_2) in combination with antioxidants partially compensated for the antioxidant contributions of co-culture cells. Studies using antioxidants under high and low oxygen tension conditions have resulted in controversial findings. Choe et al. (2010), using the swine model, studied the effect of the combination of GSH, β-ME and cysteine on embryo development. Treatment groups had a greater number of developing embryos than the control [77], however, higher concentrations of these antioxidants might have increased embryo yield because high O_2 culture conditions (20 % O_2 and 5 % CO_2) were used. In contrast, guaiazulene (a component of various chamomile species with antioxidant properties) had no positive effect on embryo development under low oxygen tension (5 % O_2) [78].

Melatonin has been reported to be a potent antioxidant. This hormone is a scavenger of free radicals and recent findings on melatonin either alone or in combination with taurine have been reported in buffalo and bovine species [79, 80]. Overall, there were positive effects in the treatment groups compared to the controls for both studies, however, they used 20 % O_2 and 5 % CO_2 conditions. Thus, the use of antioxidants may yield greater benefits under high O_2 tension.

Recent attention has been given to phytochemicals containing antioxidant properties [81]. For example, Jang et al. [82] and Lee et al. [83] tested the effects on bovine embryos of astaxanthin (carotenoids group) and 3, 4-dihydroxiflavone (phenolics group), respectively. Based on their culture conditions, they demonstrated that these phytochemicals had positive effects on cell proliferation, differentiation, and viability i.e. reduction of ROS, lipid peroxidation and apoptotic cell numbers.

Due to the increased interest in the redox systems and their interactions, more studies that not only measure the effectiveness of single factor supplementation, but also consider potential interactions of a cocktail of factors, antioxidant and hormones are needed, especially for single embryo culture. Expression of EGF receptor may participate in the regulation of glutathione peroxidase-1 (GPX-1) along with GSH to maintain cellular homeostasis [11]. Since both GPX-1 and Trxr are selenoenzymes, research on the interaction of both enzymes in light of the unfolded protein response status is of interest, especially when the GSH system belongs to the primary redox buffer that is competing with the Trx system for reducing equivalents. In addition, there seems to be an

Table 3 Supplementation with embryotrophic factors towards improving (more) defined media

Factor	Actions	Reference
Colony-stimulating factor 2 (CSF2)	Blocked apoptosis in bovine embryos through regulatory actions on apoptosis genes. CSF2- embryos tended to secrete more IFNτ.	Loureiro et al. [136, 137]
Epidermal growth factor (EGF)	Besides its role on proliferation, migration and differentiation, EGF is involved in reduction of apoptosis during preimplantation and placentation. Higher cryotolerance has been reported in bovine embryos treated with EGF at IVM.	Gordon [7]; Hambruch et al. [138]; Dhali et al. [139]
Fibroblast growth factor 2 (FGF2)	FGF2, but more importantly FGF2 receptor activity were necessary for optimal development	Fields et al. [140]
Preimplantation factor (PIF)	Involved in modulation of local immunity, promoting decidual proadhesion molecules and enhancement of trophoblast invasion. In addition, PIF was found to be expressed in the trophoblast layer during the first two trimesters of human pregnancy.	Stamatkin et al. [13, 141]; Weiss et al. [142]; Barnea and Sharma [143]
Insulin, transferrin and sodium selenite (ITS)	Insulin exerted its actions on glucose transport and AA uptake along with antioxidant protection from transferrin and selenium.	Gordon [7]
GH (somatotropin) and IGF-I	In combination stimulated embryonic development. Although the GH effects have been reported as not mediated by IGF-I in the bovine IVM model, both hormones appear to have critical roles in the modulation of apoptosis and metabolism. Embryonic development is stimulated by the combination.	Moreira et al. [144]; Bevers and Izadyar [145]; Kölle et al. [146, 147]; Pozzobon et al. [148]; Kuzmina et al. [149]; Velazquez et al. [150, 151]; Makarevich et al. [152]
IGF-I, IGF-II, FGF2, transforming growth factor beta1 ($TGF_{\beta 1}$), leukemia inhibitory factor (LIF) and CSF2, singly and in combination.	Results from the combination were similar to culture under serum conditions, but not greater.	Neira et al. [153]
Activin A	In bovine species, fourth cell cycle (lag phase) is longer for in vitro derived embryos than in vivo counterparts (~50 h vs. ~24 h). Use of activin A shortened the lag phase and was also beneficial at later (5–8 days post insemination [dpi]) stages, however, more rapid rates of development were associated with higher rates of apoptosis.	Gordon [7]; Trigal et al. [154]
Thyroid hormone (TH)	Important during early pregnancy but the use in vitro is minimal. On the bovine embryo TH is involved in lowering apoptotic rates as well as improved hatching rates post thawing for cryopreserved embryos.	Ashkar et al. [155]

effect of preimplantation factor (PIF) on the regulation of Trx and GSH systems. This 15-AA polypeptide is a conserved sequence throughout mammalian species and it is produced by the viable embryo. Thus, integrating PIF on redox studies could provide more information on the regulation of the balance of such important systems.

The use of alternative macromolecules

Recent evidence has shown that embryos cultured in vitro remain inferior to those developed in vivo [84]. In addition, when embryos are cultured individually this inferior quality is more marked. However, culture of single embryos under defined conditions is highly desirable in human clinical situations, but presents challenges, e.g. single embryos (or COCs) in culture lack the benefits of embryotrophic factors from group culture and serum or albumin in the media. Variation from batch to batch of serum or albumin (i.e. contamination) is of concern and could potentially impair embryo development. Consequently, in an attempt to standardize the IVP production by making more defined culture media, many synthetic macromolecules have been used to replace animal-derived products. These include polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), ficoll and knockout serum replacement

(KOSR [defined cocktail of factors]), but the results of using these synthetic macromolecules have generally been disappointing. More promising results have been reported for recombinant human serum albumin (rHSA) in combination with hyaluronan (HA) that are the most abundant macromolecules in the female reproductive tract [85, 86]. Table 4 summarizes different macromolecules and/or approaches to culture embryos.

Assessing embryo quality

Better non-invasive markers and improved ways to determine oocyte quality are needed, since morphological assessment is still the method of choice to select "good" quality oocytes. In addition to TLC, there are other non-invasive approaches for assessing the quality of embryos either for fresh transfer or cryopreservation. For example, assay of preimplantation factor (PIF) in media [13] may serve as a universal embryo viability marker since it is secreted by viable embryos of several species including mice, cattle and humans. This approach may represent a reliable tool to facilitate embryo selection for transfer into the recipient patient. Another non-invasive technique using the nanorespirometer to measure oxygen consumption as a marker of embryo viability has been reported [48, 49, 87]. Based on the same principle, the development of an embryoscope that combines time-lapse cinematography and an oxygen microsensor has been used to measure oxygen consumption rates and to determine embryo developmental competence [87].

Using amino acid profiling as another non-invasive approach, Sturmey et al. compared the developmental potential of bovine in vivo and in vitro derived embryos [60]. They were able to detect differences between the two embryo sources, through increased AA media depletion by the in vitro group and also a sex-specific preference for amino acid depletion (Table 1). In humans, metabolomic profiling using near infrared spectroscopy is another effective non-invasive tool to predict embryo development [88]. Non-invasive means of evaluating embryo quality and potential can be used to optimize decisions for embryo transfers (single vs. multiple) or cryopreservation procedures (by embryonic developmental stage) for maximum success [88–91].

Although non-invasive approaches are improving, invasive ones have been extremely helpful in finding candidate genes to predict embryonic survival. It may be possible to establish transcriptomic landmarks specific for embryonic stage as critical markers for embryo quality [92]. Bovine transcriptome analysis has revealed that part of the decreased reproductive rate in pure breed livestock species might be related to a high degree of inbreeding [65]. Other transcriptomic differences have been reported for IVF blastocysts vs. degenerative embryos [93], in vivo vs. in vitro derived embryos [94], and blastocysts that did or did not result in pregnancy after transfer [95]. In addition, within these analyses, the relative expression

Table 4 The use and benefits of alternative macromolecules in bovine embryo culture systems

Macromolecule	Culture	Main findings	Reference
rHSA + HA	Group	Inclusion of both macromolecules plus citrate resulted in high rates of bovine blastocyst development.	Lane et al. [85].
BSA + HA	Group	The addition of BSA was necessary throughout the entire culture period and resulted in higher blastocyst rates irrespective of HA treatment. However, supplementation of HA at day 4 post insemination increased mitochondrial DNA (mtDNA) copy numbers at the blastocyst stage.	Palasz et al. [86].
KOSR	Group	KOSR at 20 % O_2 yielded the highest pre-implantation embryo rates.	Mingoti et al. [156]
human embryonic stem cell derived bioactive material (hES-BM)	Group	Results from staining methods and measures of gene expression of pluripotency, embryogenesis and growth markers were significantly higher than in the control group treated with fetal bovine serum. The authors concluded that hES-BM could be used as a new supplement for bovine preimplantation embryo development.	Kim et al. [157]
Agarose-embedded helper embryos	Group (co-culture)	Enhanced developmental competence and embryo quality markers e.g. involved in implantation and normal calf delivery (PGHS2, TXN, PLAU).	Senatore et al. [158]; Deb et al. [159]
Somatic cell feeders	Group	Cryotolerance was positively influenced by the presence of somatic cells and was reflected in survival and hatching rates as well as differential cell counts.	Shirazi et al. [160]
Heterologous cumulus cells	Single (co-culture)	Gene expression for qualitative markers (TP53, BAX, SHC1, SHC and IGF2R) was reportedly similar for groups of embryos co-cultured singly or in groups with or without heterologous cumulus cells.	Goovaerts et al. [161]

of 18 genes was similar for in vivo and in vitro derived bovine embryos and whether resulting in delivery or not [95].

Alternative culture approaches

Under large-scale embryo production for livestock, group size in culture may not represent issues since the number of embryos per session typically allows groups of 10–25 embryos per droplet (usually 20–100 μL). By contrast, assisted reproduction clinics offer individualized services that represent a challenge in terms of group size culture conditions. Thus, reliable single (or small-group) COCs/embryo culture systems that can be replicated are necessary.

A prospective study with human oocytes submitted to either IVF or ICSI, Ebner et al. (2010) tested a new type of culture dish and measured the effects of individual culture, individual culture with contact to neighbors, and group culture (n=9–15) of zygotes [96]. The results showed that group culture was best in terms of compaction and blastulation when compared to individual culture. Although there was only a trend for more births under group-culture treatment, the authors recommended group culture and also suggested a reduced volume of culture droplet or increased embryo density. Other studies investigating the effects of small-group or single embryo culture and continuous vs. sequential media have been encouraging. In mice, the effect of embryo density and microdrop volume on embryo development was tested and overall, no differences were found in terms of cleavage or blastocyst formation [97]. However, embryos cultured singly had fewer ICM and TE cells than those cultured in groups. Nonetheless, groups as small as 2 embryos (in a microdrop from 0.5 to 2.0 μL) gave similar results to group culture in a 10 μL droplet. The effect of polyester mesh sections on the culture of COCs and embryos has been tested in an effort to reduce the labor inherent to the manual preparation for well of the well ("WOW") dishes. Results have indicated that oocyte maturation and embryo development may be carried out in polyester meshes (~170 μm opening) with similar developmental rates as those obtained by WOW or control group-culture [98, 99].

A human IVF study investigated the effects of continuous uninterrupted single medium culture without replacement of fresh media vs. sequential media [100]. No significant differences were detected between the two approaches in terms of embryo quality for d-3 transfers, however, for transfers at d-5 the continuous approach resulted in a greater number of suitable embryos than sequential media. A subsequent study confirmed that although embryo quality was similar following uninterrupted culture vs. sequential media, the embryo utilization rate (embryos available for vitrification or fresh transfers over the total number of embryos) was higher for continuous culture [101]. Interestingly a study using the bovine model reported that preimplantation embryonic development was better in a two-step sequential culture method than in the uninterrupted single media conditions as relative quantitative expression of gene markers related to embryo development showed higher values [102]. A study comparing two commercial culture media reported higher levels of relative mRNA expression in groups cultured in Ménézo B2 media indicating that the embryos were able to develop faster compared to those cultured in COOK media [94].

The emergence of microfluidic technology combining engineering and biology knowledge has led to a promising and innovative option for the in vitro culture of gametes and embryos in both static and dynamic ways. One of the obvious benefits of this technology would be the reduction of the "human factor" which would imply less manipulation of embryos and gametes, minimal fluctuations in the in vitro microenvironment and provision of a more physiological nutrient delivery in the culture media as the embryo develops. Consequently, embryonic stress would be substantially reduced and the embryo viability could be enhanced [43]. Microfluidic approaches attempt to replicate more closely the early events of embryogenesis normally occurring during in vivo conditions [103]. The principle of this technology is basically a very small working volume of medium, in the nano-to-micro liter range. Two categories, static and dynamic, are recognized: in the dynamic system, the embryo remains stationary in a microwell in which media can be slowly changed. Several prototypes of microfluidic systems, either static or dynamic, have yielded overall positive results (equal to or better than control groups) [103]. However, in some cases it appeared there was decreased embryo development and these inconsistencies could not be explained. Current challenges to be overcome are the material selection and the flow rate [43, 104]. Microfluidic technology is progressing rapidly as it seems that imagination is the only limiting factor when designing microfluidic devices [103]. One microfluidic in vitro culture system for mechanical stimulation of bovine embryos emulated the peristaltic constriction that normally occurs in vivo in the oviduct by designing microchannels with constricted areas [105]. The results were positive and suggested that this constriction contributes to the early development of bovine embryos in ART systems.

Static microfluidic models using WOW systems have been used by several research groups and in most cases cleavage and blastocyst rates were not different compared to controls cultured in the usual groups. Improved blastocyst rate of ICSI-derived swine embryos cultured in small groups (n=4–5/WOW) in WOW of 100 nm diameter compared to other groups (24.6 % vs. 13 %) has been reported [106]. It has been concluded that a microenvironmental embryo density of 1:0.269 μL and a macroenvironmental embryo density of 1:30 μL were most successful for culturing bovine embryos in WOWs [107]. The use of the WOW system to produce monozygotic twin bovine calves using the blastomere

separation technique resulted in higher pregnancy rates compared to the monozygotic twin blastocysts obtained by conventional bisection of in vivo derived blastocysts (78.9 vs. 40 %) [108]. This may have a significant impact for the livestock industry and for research purposes as progeny would be derived from genetically valuable animals that could also be used for comparative studies by providing animals with an identical genetic background, thereby reducing the need for higher numbers of experimental units to reach statistical power.

Polydimethysilixane (PDMS) is widely used for manufacture of microfluidic devices due to its physical properties and low cost. Bovine embryos cultured in PDMS microwell plates had similar rates of development to blastocyst and similar ICM and TE cell number to embryos cultured in groups of 20 in conventional systems [109]. Novel approaches using microfluidic devices capable of manipulating oocytes, which also enhanced embryo development, have been reported [110, 111]. These positive results suggest that microfluidic devices simplify the overall IVP by reducing the human factor and also by tracking single embryo development.

Microfluidic technology allows the addition of additional tools such as time lapse cinematography (TLC) which could facilitate selection of the top quality embryos monitored in real time. TLC has led to better understanding of fertilization and early preimplantation embryonic development that may have an impact later on pregnancy rates [112, 113]. Recently, pregnancy and birth of a healthy infant in a single human IVF case using TLC in combination with WOW to select a single embryo for transfer has been reported [114]. Although a single case is not sufficient to validate the usefulness of this approach, the authors encouraged application of TLC in combination with WOW. In bovine embryos, TLC in combination with polystyrene based-microwell culture resulted in pregnancy rates higher (51.7 %) than control (21.9 %) at day 60 post embryo transfer [115]. This approach allowed the tracking and selection of the healthier bovine embryos for transfer. In addition, the risk of pregnancy loss was minimized. The authors did suggest that PDMS might absorb medium components such as embryotrophic factors, making the novel polystyrene-WOW embryo culture system preferable.

The development of the "IVP lab-on-a-chip" would have a significant impact especially for human IVF since it would allow integrating oocyte positioning, sperm selection, fertilization medium replacement, embryo culture and monitoring of embryo development. Optimizing single embryo culture conditions will play a pivotal role in the development of this new technology. In addition, a current challenge is integrating all the phases of the IVP into an "IVP-lab-on-a-chip" with in vitro maturation and cumulus removal included on the device. Equal or higher quality standards than the current conventional methods and reliable results would be required for adoption by clinicians and embryologists.

Uterine receptivity and embryo survival

A healthy endometrium is critical for pregnancy to occur especially in individuals undergoing repetitive ART. Fertility has been reported to be higher for cows with an endometrial thickness >10 mm after ovulation [116]. Thus, an adequate thickness of the endometrium is recommended before embryo transfer. Neuromuscular electrical stimulation has been used in women to improve endometrial thickness and such treatment has resulted in a tendency for increased pregnancy rates compared to untreated controls [117]. A thicker endometrium is associated with increased blood flow which may contain embryotrophic factors and substrates for a successful pregnancy and enhance immunotolerance processes at the maternal-fetal interface. In a bovine study, a nonclassical MHC-I gene was reported to be responsive to cytokines that positively regulate pregnancy and suggests that these cytokines are not only involved in the preimplantation embryo survival per se, but also function as regulators at this interface [118, 119]. Reduced overall cell number (particularly reduced trophectoderm cell number) in bovine embryos exposed to endometrial fluid recovered from a cow with uterine inflammation has been reported [120]. The embryo-maternal environment interaction during pregnancy is complex and dynamic and future experiments toward single embryo culture in more defined conditions may benefit from studies integrating new knowledge from such interactions.

Conclusions and further remarks

In the field of assisted reproductive technologies, in vitro derived embryos are still less developmentally competent than their in vivo counterparts. Several steps from in vitro maturation to fertilization and culture are not yet fully optimized in vitro. There is an increased awareness of the spatio-temporal requirements at several stages of preimplantation embryo development and the different requirements for achieving optimal rates of development and quality. Although much can be learned from embryo co-culture systems that provide important insights, completely defined and optimized media is the goal. To obtain serum-free media for culturing embryos (especially single embryo culture) with minimum risk of disease transmission, there is a need to know the most critical nutrients, metabolic regulators, cytokines and hormones for each stage of embryo development. Animal models such as bovine IVP could be better exploited and results extrapolated to other species including the human. Important tools such as the non-invasive evaluation approaches for selection of elite quality embryos either for transfer or cryopreservation might be routinely used for minimizing the number of embryos transferred, assuring embryonic competence, and resulting in the birth of healthy offspring. Nevertheless,

the use of invasive approaches such as transcriptome evaluation will continue to be a reliable tool to discover more relationships between embryonic markers and the maternal environment.

Conflict of interest The authors declare no conflicts of interest.

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