REVIEW

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Optimizing oocyte competence and embryo quality: advances in media composition and culture strategies for bovine *in vitro* production

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Abstract

In vitro production (IVP) of bovine embryos includes oocyte retrieval, *in vitro* maturation (IVM), *in vitro* fertilization (IVF), somatic cell nuclear transfer (SCNT), *in vitro* culture (IVC), and embryo transfer (ET). It is a foundation of modern cattle breeding and germplasm conservation. Nevertheless, the efficiency of IVP is hampered by an inefficient system of culture that cannot replicate the reproductive physiological environment. This review is based on the recent progress in bovine-specific IVM and IVC media, combining functional supplementation with macromolecules, amino acids, antioxidants, and the ability to precisely control the physicochemical parameters. We emphasize optimization of energy sources, hormonal signals, and redox balance at the stages of nuclear and cytoplasmic maturation, blastocyst development, and enhanced implantation. Although new dynamic environments, such as microfluidics and extracellular vesicle (EV) supplementation, offer physiologically viable and responsive culture environments, the transition to chemically defined environments leads to diminished epigenetic perturbations and variability. These strategies aim to function by matching the conditions of media composition and culture with the specific metabolic demands of bovines and bring IVP closer to the generation of embryos with *in vivo*-like developmental capability. This is favorable toward higher pregnancy success rates and sustainable bovine genetic improvement plans.

Keywords: Oocyte, Embryo, Bovine, In vitro, Reproduction

Introduction

Anthropogenic stressors, particularly suboptimal culture systems and environmental pressures, damage the reproductive health of animals, extend the generation intervals, and pose a risk to the sustainability of natural breeding, thereby leading to biodiversity loss[1]. The surging global demand of meat and milk to cater to a rising population of more than 11 billion by 2100 has accelerated the relevance of advanced reproductive strategies[2]. One of them, *in vitro* production (IVP) of bovine embryos, offers a scalable and fortified biotechnology with securing germplasm and rapid-growing genetic gain.

The IVP continuum, which encompasses oocyte retrieval, *in vitro* maturation (IVM), *in vitro* fertilization (IVF), somatic cell nuclear transfer (SCNT), *in vitro* culture (IVC), and embryo transfer (ET), among others, have become a feasible system maximizing reproductive efficiency in bovines[3]. In juxtaposition with conventional multiple ovulation and embryo transfer (MOET), IVP yields more embryos per year (72 IVP versus 40 MOET), at lower cost, while reducing disease transmission risks[4]. In 2023, global bovine embryo production reached a new milestone in cattle breeding strategies, with 1.87 million IVP embryos produced, more than five times the number of in vivo-derived embryos[3].

In spite of its immense potential, IVP still encounters relative bottlenecks of natural biological constraints that result in suboptimal reproductive outcomes. Only 20%–40% of fertilized bovine zygotes develop into blastocysts under typical *in vitro* circumstances[5]. The composition of the culture media is a critical determinant that results in inadequate outcomes[6]. Poor formulations, including nutrient imbalances, growth factors, hormones, fetal bovine serum (FBS), amino acids (AAs), or antioxidants, can disrupt protein synthesis, redox systems, metabolism, and competent development. These inefficiencies result in reduced pregnancy rates, abnormal growth of the fetus, an increased rate of chromosomal abnormalities, and large offspring syndrome[7]. Addressing these problems requires a cattle-specific tailored culture medium that can closely recapitulate the maternal reproductive tract to accommodate the optimal processes of oocyte maturation, fertilization, and embryo development, which is a desperate need.

This review aims to explore culture media formulations and physicochemical parameters, especially at the IVM and IVC stages, as these have a significant impact on oocyte competence, fertilization efficiency, and embryo's viability. It identifies specific interventions as the key to bridging *in vivo* and *in vitro* developmental potential. By optimizing these media systems, it is possible to improve the blastocysts' quality and pregnancy success rates, leading to desirable genetic gain and helping to preserve valuable or endangered germplasm in cattle[5].

IVM of bovine oocytes

In this section, the plans of bovine oocyte collection, maturation and competence assessment are summarized, and the link between intrinsic

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quality determinants and optimized IVM is connected to enhance developmental potential.

Oocyte collection in IVP

Oocytes for IVP can be obtained through oocyte retrieval methods such as ovum pick-up (OPU) from prepubertal calves, open-cycling heifers, postpartum cows, subfertile cows, or from abattoir-derived ovaries to avoid exploitive breeding practices in bovines[8]. Abattoir-derived ovaries are readily accessible but often originate from animals of unknown donor status, which contributes to heterogeneity in developmental competence[8]. Moreover, the interval between slaughtering and ovary processing of animals can subject oocytes to a suboptimal temperature and O_2 environment, diminishing cumulus–ooctye complexes' (COCs') viability and developmental potential[9]. Conversely, OPU enables live donors to be directly sampled with prior knowledge of their reproductive status, age, and management history, enabling the process to be more consistent in terms of the quality of oocytes[10]. Irrespective of origin, intrinsic quality during collection is a major determinant of maturation efficiency, fertilization success, and embryo viability.

IVM in bovines: Physiological basis, metabolic requirements, and media optimization

IVM is an integral component of bovine *in vitro* embryo production, involves the culture of immature cumulus–oocyte complexes (COCs) differentiated from Prophase I to the Metaphase II (MII) stage under controlled conditions[11]. IVM, first reported in cattle in 1965[12], is now crucial to both conventional IVF and SCNT[11]. Moreover, it eliminates the risk of ovarian hyperstimulation syndrome, minimizes hormonal stimulation, lowers the cost of medication, and facilitates rapid oocyte retrieval and maturation[13], which is particularly valuable for commercial breeding and fertility preservation. The success of IVM relies on the synchronization of nuclear and cytoplasmic maturation[9]. Ovarian follicles shift from primordial to Graafian follicle growth in a dynamically regulated intrafollicular microenvironment, where oocyte, granulosa, and cumulus cell (CC) signaling is bidirectional and coordinates growth and follicle maturation[14]. Moreover, the ovarian environment provides endocrine signals, such as gonadotropins and paracrine growth factors, which induce meiotic differentiation and cytoplasmic reprogramming[11]. However, to recreate this *in vivo* environment *in vitro*, media containing hormonal stimulation and specialized metabolic support are necessary.

Bovine oocytes rely on pyruvate, supplied via glycolysis in the surrounding CCs, and AAs such as glutamine for adenosine triphosphate (ATP) production, redox balance, and protein synthesis[15]. Similarly, lipids contribute to ATP production and membrane remodeling[15]. Insufficient provision of these substrates disrupts maturation-promoting factor (MPF) activity, spindle assembly, and subsequent developmental competence. Notably, MPF activity is inherently lower in prepubertal oocytes[16], further reducing maturation competence. Most bovine IVM systems use tissue culture medium 199 (TCM-199) as a basal medium, supplemented with FBS and gonadotropins such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E_2) to mimic the ovarian milieu[17], as shown in Table 1. However, they are typically excluded from IVC media, as their role is limited to pre-fertilization maturation. Growth factors, such as epidermal growth factor (EGF), insulin-like growth factor (IGF1), fibroblast growth factor 2 (FGF2), transforming growth factor-alpha, transforming growth factor-β1, and basic fibroblast growth factor[17,18], improve maturation and developmental competence. Consequently, these physiological and metabolic requirements underpin targeted IVM media design in bovine IVP. Such pertinent stage-specific formulations must be able to meet the energy and biosynthetic requirements without compromising on the integrity of the epigenome to achieve the highest oocyte competence and embryo viability[19].

Assessing oocyte quality for IVM

The quality of oocytes is a primary predictor of the success of IVP and is affected by donor-specific variables, including age, estrous cycle, nutrition, genetic potential, and reproductive health[9]. These variables are uncontrollable under routine practices, so, noninvasive evaluation techniques are necessary to select developmentally competent oocytes.

Morphological evaluation of the COCs is the most widely used method in bovine IVM. CCs play a central role in supporting oocytes by supplying essential metabolites such as pyruvate from glucose, cholesterol and AAs that oocytes cannot efficiently acquire alone[34]. These metabolic interactions are controlled by oocyte-secreted signals such as growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), and fibroblast growth factor 8[14]. According to the conventional grading system, COCs are divided into three grades, namely Grade I = multiple compact layers of CCs and uniform cytoplasm; Grade II = partially intact layers of CCs with some expansion; Grade III = expanded or darkened CCs with granular cytoplasm, indicative of advanced atresia and reduced competence[34], as indicated in Fig. 1. Although morphological grading provides a quick screening method, the grading criteria are not standardized across studies, particularly for cumulus expansion and the degree of atresia[35–38]. Some studies reported that darker and less compact COCs showed faster meiotic resumption and higher blastocyst rates[35,36]. However, others associate reduced CC layers with lower cleavage rates, higher DNA damage, and reduced developmental competence[37]. In another grading scheme, COCs classified as Grade III on the basis of slight cumulus expansion showed blastocyst rates similar to Grade I[38], further indicating that grade labels should be interpreted according to their specific morphological criteria.

Additional information on nuclear and cytoplasmic maturation can be obtained with the help of complementary intrinsic markers, including polar body extrusion, cytoplasmic granularity, spindle imaging, and the integrity of the zona pellucida (ZP)[39]. However, these parameters are inherently subjective and offer limited predictive value in isolation. Their reliability improves substantially when combined with morphological grading using intrinsic and molecular markers, or refined systems like the five-grade cumulus expansion scale[40], which can improve predictions of oocytes' competence and guide targeted IVM media optimization.

Donor-specific tailored IVM media

The success of IVM is highly connected with the physiological conditions of the oocyte donors, which influence the metabolic, hormonal, and developmental environment of COCs[9]. Different groups of donors, including prepubertal calves, postpartum cows, and subfertile or high-performance females, have very different needs regarding optimal maturation, which require tailored IVM protocols that cannot be covered by standardized media[8].

Prepubertal donors, such as calves or early heifers, have low meiotic competence, disturbed MPF activity, and inefficient cytoplasmic maturation due to immature hypothalamic–pituitary–ovarian axes[41]. These oocytes are typically subjected to gonadotropic stimulation protocols, primarily FSH and, in a few instances, progesterone (P4), and allowed to complete the peripubertal follicular period to augment COCs' competence[41]. Moreover, such hormonal priming improves follicular growth, raises the count of antral follicles, and modestly increases blastocyst rates, although complete developmental competence is only achieved after puberty[13,41]. On the other hand, postpartum lactating cows, particularly those that have a negative energy balance, yield oocytes with a high lipid content, mitochondrial

Table 1. IVM media formulations and maturation outcomes of bovine oocytes.

Year	Species/ breed	Key supplements					%MII	Key findings	Ref.
		H	MM	AAs	AOX	GFs			
2025	Buffalo	0.5 μg/mL FSH, 2 IU hCG, 1 μg/mL E2	10% FBS	–	12 μg/mL cysteine	–	71	hCG yielded 71% MII vs. 80.5% with LH (0.5 μg); cleavage and blastocyst rates were similar with ↓SOD1 expression in hCG group	[20]
2023	Cattle	0.01 UI/mL FSH, 50 ng/mL E_2, 150 ng/mL P4	4 mg/mL BSA	–	1 mM cysteamine	10 ng/mL IGF-1, 100 ng/mL AREG	84–87	BSA-based IVM medium achieved 84%–87% MII. NRG1 had no impact on maturation but modulated cumulus–oocyte gene expression (↓EGFR, ↑NPR2/PTX3) and post-IVF blastocyst quality	[21]
2022		1 μg/mL E2	10% heat-inactivated FCS	–	0.8 mM cysteine	–	75.3	L-cysteine improved MII, and cytoplasmic maturation (mitochondria, cortical granules) with ↑BMP15/GDF9 expression, supporting oocyte–cumulus signaling	[22]
		–	10% FCS	L-glutamine	–	–	83	FCS supported higher MII, antioxidant defense (↑GPx), and IGF2/IGF1R expression. BSA was beneficial but less effective than FCS	[7]
		6 μg/mL FSH, 6 μg/mL LH, 1 μg/mL E_2	10% inactivated FBS	–	–	10 ng/mL EGF, 100 ng/mL IGF1, or 500 ng/mL FGF2	90	Chemically defined IVM without serum, supplemented with gonadotropins and EGF/IGF1/FGF2, supported MII rates comparable to FBS (~88%–90%); gonadotropins were essential	[17]
2021		10 μg/mL FSH, 1 μg/mL E_2	10% FBS	–	1 mM NAC	10 ng/mL EGF	83.1	NAC improved MII by increasing GSH levels, decreasing ROS and apoptosis–surpassing the effects of L-cysteine	[23]
2019	Yak	5 μg/mL FSH, 5 μg/mL LH, 1 μg/mL E_2	20% FCS	–	0.6 mM cysteine	100 ng/mL IGF-1, and 10 ng/mL EGF	84.4	Cysteine + IGF-1 + EGF synergistically enhanced yak oocyte maturation (84.4%), outperforming single or dual additives	[24]
2017	Cattle	0.5 μg/mL FSH, 5 μg/mL LH	10% FBS	–	100 μM cysteamine	–	91.9	Cysteamine supplementation achieved the highest MII among tested antioxidants, supporting nuclear maturation	[25]
		0.5 μg/mL FSH, 100 IU/mL hCG	10% FCS	–	–	–	91.4	FCS yielded the highest MII (91.4%). Co-supplementation with antioxidants maintained comparable MII (89.7%) and reduced ROS under stress.	[26]
2016		0.5 μg/mL FSH, 100 IU/mL hCG	10% FCS	–	0.6 mM cysteine, 100 μM cysteamine, and 100 IU catalase	–	78.2	Antioxidant tri-complex reduced ROS, increased GSH and ΔΨm while keeping MII comparable to individual antioxidant.	[27]
2015		1 μg/mL FSH, 50 μg/mL hCG, 1 μg/mL E_2	5% FCS	–	–	–	89.6	FBS improved nuclear maturation and cortical granule migration vs. BSA, with no adverse effect on oocyte quality	[28]
2010		2 mg/mL FSH, 10 μg/mL LH, 1 μg/mL E_2	100 μL/mL heat-inactivated FBS	Glutamax-I	–	–	83	Serum-free FAF-BSA supported nuclear maturation similar to FBS, whereas complete serum/hormone deprivation significantly impaired maturation	[29]
2000	Holstein cattle	0.5 μg/mL FSH, 0.06 IU hCG, l μg /mL E_2	20% heat-inactivated ECS	0.1 mg/mL L-glutamine	–	–	84.5	High-quality oocytes showed superior maturation and development; serum presence further enhanced IVM outcomes	[30]
1989	Cattle	5 μg/mL FSH, 1 μg/mL E_2	20% heat-treated FCS	–	–	–	87.9	Serum and gonadotropins improved MII to 87.9% vs. 59.2% in the control, confirming the synergistic effect	[31]
1986		10 μg/mL FSH	10% FCS	–	–	–	67	FCS in Tyrode's medium supported CCs' viability and MII, essential for FSH-induced expansion. In contrast, BSA failed to support maturation	[32]
–	Key trends	FSH + LH + E_2 combination improves IVM outcomes	10% FBS/FCS-based media consistently support high MII rates	Limited use; L-glutamine is most reported	Cysteamine and NAC are the most consistent in enhancing MII	IGF-1 and EGF are beneficial	80–91	Multi-component strategies (NAC, cysteamine, serum) reliably enhance MII	–

Most studies applied a 24 h IVM protocol, with minor variations: 22 h[7,23,25,27], 22–24 h[17], or 24–25 h[31]. TCM-199 was the standard basal medium; only one study used Tyrode's solution[32]. Antibiotics such as gentamicin (10–50 μg/mL)[18,25,31], amikacin (50 μg/mL)[26,27], high-dose amikacin (75–83.4 mg/mL)[21,28], and penicillin/streptomycin (varied combinations)[20,22–24,29,30,33] were routinely included to ensure aseptic conditions. One study applied a 1% antibiotic-antimycotic solution[7]. Abbreviations: MII, metaphase II; H, hormones; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; E_2, estradiol; GnRH, gonadotropin-releasing hormone; MM, macromolecules; FBS, fetal bovine serum; FCS, fetal calf serum; BSA, bovine serum albumin; FAF-BSA, fatty acid-free bovine serum albumin; AAs, amino acids; AOX, antioxidant; NAC, N-acetyl cysteine; GFs, growth factors; IGF, insulin-like growth factor-1; EGF, epidermal growth factor; AREG, amphiregulin; NRG-1, neuregulin-1; PVA, polyvinyl alcohol.

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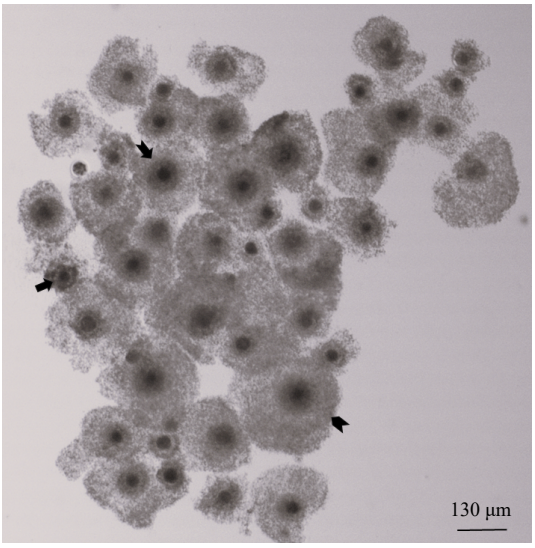

Fig. 1 Representative grading of bovine cumulus–oocyte complexes (COCs) based on cumulus cells' (CCs') morphology. Grade I (pentagon), compact, multi-layered CCs; Grade II (notched arrow), partially expanded CCs; Grade III (arrow), atretic oocytes with sparse CCs. Scale bar = 130 µm. The image was botained by the authors.

dysfunction, and high levels of oxidative stress[9]. Lipid-targeted interventions, including a decrease in FBS, the substitution of BSA as the macromolecule, and the addition of a modulator, such as L-carnitine, have been demonstrated to restore fatty acid oxidation in these donors, which enhances maturation success and blastocysts' outcomes[28].

Subfertile donors who have a high genetic merit may present special IVM issues because of an abnormal endocrine pattern or previous reproductive interventions. These oocytes can be assisted by modification in the LH–E_2 ratio and specific hormonal stimulation to restore the endocrine equilibrium. Although fewer studies directly focus on these cases, the selective application of supplementation with antioxidants such as N-acetyl-L-cysteine (NAC) or amino acid support has been applied under conditions of metabolic stress, but more evidence should be gathered in order to support precise tailoring[21]. Even though IVM has been widely successful in various donor systems, Table 1 indicates a void in formulations specifically tailored to the donors' physiology. The integration of donor-specific IVM techniques is an imperative move towards the standardization of oocyte competence and better reproducibility of IVP across diverse populations of cattle.

Functional supplementation strategies to optimize IVM

The strategic application of macromolecules, AAs, and antioxidants during the IVM process can mimic the follicular microenvironment and promote nuclear and cytoplasmic maturation and the subsequent developmental potential, as shown in Fig. 2.

Role and impact of macromolecule supplementation in bovine IVM

Macromolecular supplements, most notably FBS, remain the standard in bovine IVM because of their multifactorial support of cumulus expansion, nuclear and cytoplasmic maturation, and overall developmental competence[7]. FBS is a complex mixture of beneficial substances, including peptides, hormones, lipids, carbohydrates, growth factors, vitamins, pH buffers, and chelators[42]. These substances facilitate protein biosynthesis and transcript activity, and maintain intercellular communication[7], modulating cells' junctional integrity by regulating proteins such as ZO-1, Na/K-ATPase, E-cadherin, and connexins[43]. Multiple studies demonstrated that FBS is better than bovine serum albumin (BSA) in its effects on oocytes' maturation and blastocysts' development[29,32], partly because it inhibits ZP hardening, which facilitates sperm penetration[19]. However, BSA-based systems can also support high MII rates[21]. The optimal concentrations typically range between 10% and 15% but 5%–20% has been reported[44]. Nevertheless, although FBS has proven to be efficient, it is undefined in composition and is characterized by variability from batch to batch. Such inconsistencies may affect lipid metabolism, gene expression (e.g., DNA methyltransferase 3a, heat shock protein 70, interferon tau, IGF1, IGF2, and desmosomal glycoprotein desmocollin III), and epigenetic regulation, which affects reproducibility[17,45].

To counter such issues, serum-free alternatives have been considered including BSA-based systems, hormone supplementation, and defined protein alternatives such as growth factors, plant-origin proteins, sericin, and vegetal serum fractions[42,46]. More unconventional bioresources, such as coelomic fluid from earthworms, and mealworms (*Tenebrio molitor*)[47], are emerging candidates. Moreover, their application and mechanistic efficacy in bovine IVM remain limited. Although FBS still shows consistent performance in enhancing oocytes' competence, its undefined nature supports the need to develop chemically defined, serum-free formulations with the ability to replicate its multifactorial benefits. Importantly, chemically defined systems should not be regarded as inherently more developmentally efficient than FBS; rather, their principal advantages are improved compositional control, standardization, and reproducibility, provided developmental competence is maintained[17,29,45]. In addition, the effects of FBS during IVM are carried over to later culture stages. Its composition and the time of exposure may affect embryo quality during IVC, emphasizing the need to optimize coordination across both the maturation and culture phases.

Role and impact of AA supplementation in bovine IVM

AAs play an essential role in the regulation of bovine oocytes' maturation, which facilitates nuclear and cytoplasmic maturation[48], expansion of CCs, redox balance, and further embryo maturation[49]. They are crucial elements of protein synthesis, energy metabolism, osmotic equilibrium, intracellular pH (pHi) control[50], and gene expression modulation[51]. Depending on their metabolic origin, AAs are categorized into essential AAs (EAAs, which are supplied exogenously, and nonessential AAs (NEAAs), which may be synthesized *de novo*[52]. Three AAs, namely glutamine, proline, and histidine, are particularly relevant for IVM media. Glutamine maintains nucleotide synthesis and antioxidant defense via glutathione precursors and promotes CCs' expansion[53]. Proline is an osmoprotectant and stress-response modulator of oocytes[27], whereas histidine has a pH-buffering action and metal ion chelation, which ultimately affects the microenvironment during IVM[54].

Supplementation with glutamine, proline, and isoleucine enhances meiotic progression and zygote production in cattle, and a combination of glutamine, proline, and 12 EAAs also can restore maturation rates similar to comparable complex formulations like TCM-199[53]. Previous studies indicated that glutamine alone had the capacity to sustain maturation, but subsequent studies promote the concept of extensive supplementation to ensure uniform oocyte competence[55]. Furthermore, species-specific differences are evident: Glutamine alone impaired germinal vesicle breakdown and reduced oocyte viability in mice[56], whereas histidine improved buffalo oocytes' maturation[54]. AA metabolism also provides functional biomarkers of oocyte quality.

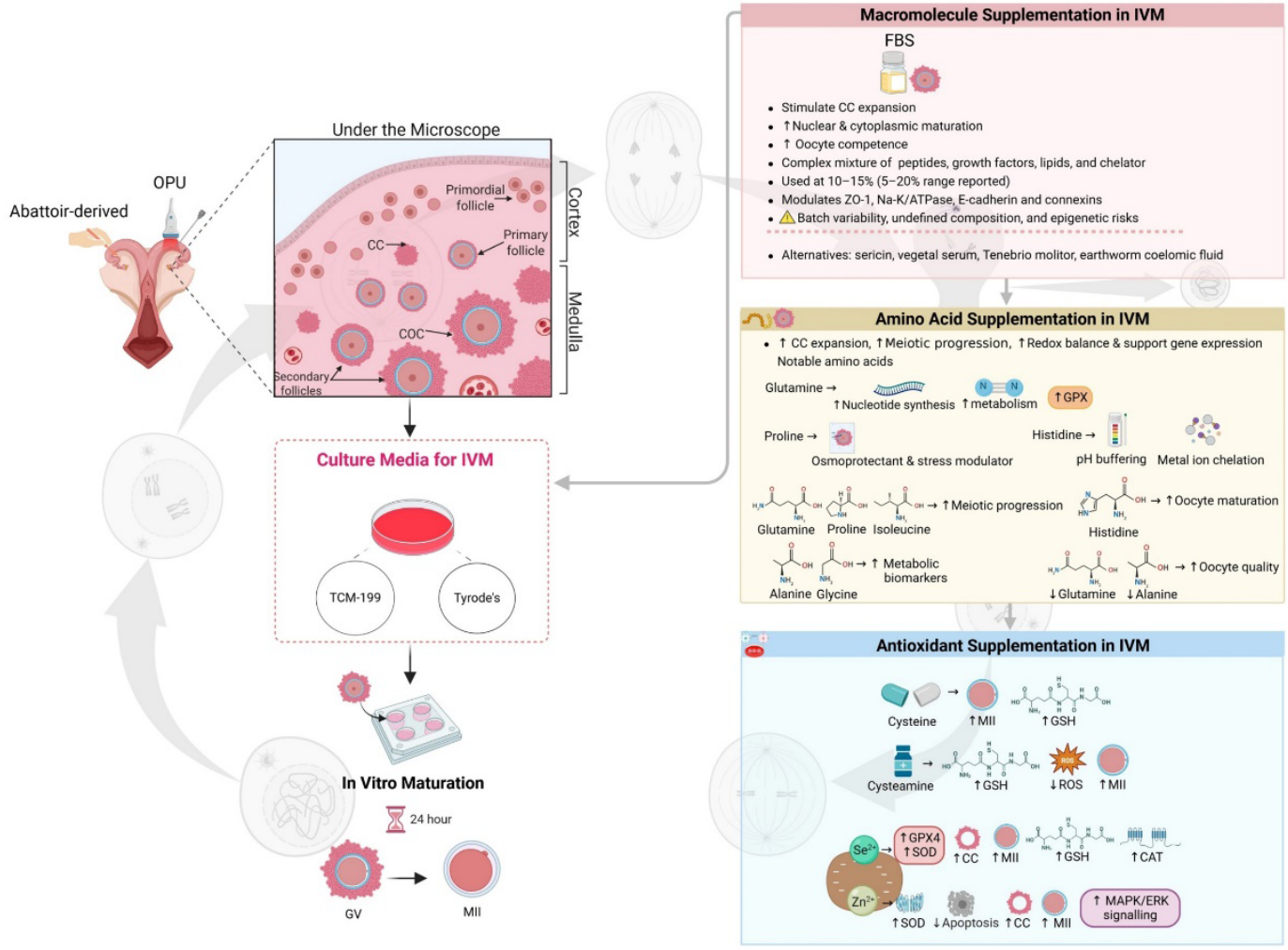

Fig. 2 Schematic overview of bovine *in vitro* maturation. Oocytes are collected either by OPU or from abattoir-derived ovaries. COCs are cultured in defined media (e.g., TCM-199, Tyrode's solution) for ~24 h, progressing to MII. Supplementation strategies, including macromolecules (e.g., FBS), amino acids, and antioxidants, modulate CCs' expansion, nuclear/cytoplasmic maturation, redox balance, and oocytes' developmental competence. Created by the authors using BioRender.

It is reported that alanine and glycine concentrations in follicular fluid correlate with cleavage and blastocyst potential, reflecting enhanced energy metabolism, glutathione synthesis, and redox stability[57,58]. In the final hours of IVM, metabolically competent oocytes show particular AA turnover, which results in less glutamine and less alanine released into the medium than their incompetent counterparts[57]. Taken together, these results indicate that supplementation with selective AAs, in nature and in concentration, is critical to maintaining metabolic homeostasis, synchronizing nuclear and cytoplasmic maturation, and increasing the early embryonic development potential of bovine IVP systems.

Role and impact of antioxidant supplementation in bovine IVM

Oxidative stress is one of the major limitations in bovine IVM, which is mainly caused by a rise in the accumulation of reactive oxygen species (ROS), which outstrips the capacity for endogenous detoxification. Excessive accumulation of ROS leads to the disruption of DNA, lipids, and proteins in a manner that alters epigenetic reprogramming and reduces developmental ability[59]. The addition of antioxidant supplementation to the culture medium increases the redox balance, preserves organelles, promotes cellular homeostasis[23], and enhances oocytes' quality and embryos' viability. This section focuses on five antioxidants with the most reported benefits, including cysteine, NAC, cysteamine, selenium (Se), and zinc (Zn). Cysteine, NAC, and cysteamine have beenthe most actively researched antioxidants because of their central position as precursors in the intracellular production of glutathione (GSH), which plays a key role in ROS detoxification.

Cysteine supplementation enhances bovine IVM by elevating the MII oocytes[22]. However, its effects on blastocyst yield are inconsistent: The research results show no enhancement[26,27], but some studies show a positive effect, especially in the presence of heat stress[60]. A negative shift in the rates of blastocysts has also been noted in particular contexts[60,61].

A stable derivative of cysteine, NAC, has been reported to improve oocyte maturation when used during maturation procedure[23]. It has been suggested that cysteamine supplementation is especially effective in patients with high levels of intracellular GSH, reduced ROS, and improved oocyte maturation[62]. Se is a cofactor of glutathione peroxidase (GPx) isoforms and plays a central role in cellular peroxidase detoxification[63]. Limited research has been conducted to investigate Se supplementation during IVM, either separately or in conjunction with other trace elements such as copper (Cu), manganese (Mn), and Zn[64]. Moreover, it has become evident that Se can be administered during IVM, enhancing CCs' viability and intracellular GSH levels[64]. A second analysis that engineered nuclear maturation by including Se

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under heat stress revealed improved nuclear maturation and regulated antioxidant-related genes, including GPx4, superoxide dismutase SOD, and catalase (CAT)[65]. The Zn supplementation in IVM was related to oocyte maturation and later cleavage percentages[66]. Zn-treated COCs had a lower rate of apoptosis and higher levels of SOD activity in CCs[67]. Zn also facilitated oocyte maturation and embryogenesis in buffalo[68]. In addition to its antioxidant activity, Zn also affects signal transduction pathways and the metabolism of secondary messengers more generally, such as the regulation of MAPK/ERK pathways, which are implicated in cumulus expansion and oocyte competence[69,70]. Additionally, Zn regulates thymidine kinase (TK1), a Zn-dependent enzyme involved in DNA synthesis during the G1/S phase, further supporting its role in cellular proliferation during early embryogenesis[71]. Hypotaurine, though present in some conventional maturation media, has received limited investigation in bovine IVM. One study reported no change in intracellular GSH concentrations when provided individually to each oocyte per culture drop during IVM[72], which has limited further investigation. These results indicate that antioxidant supplementation during bovine IVM may be effective, especially when it is related to stress or suboptimal culture conditions. Nevertheless, the responses vary, depending on the type of antioxidant, the concentration, and the environmental conditions, which suggests that a particular application, with mechanistic reasoning, can yield the most consistent enhancements in oocytes' competence.

IVC of bovine embryos

IVC supports embryos' development from the zygote stage via IVF, SCNT, or parthenogenesis to the blastocyst stage and has been proven to produce viable calves through IVF and SCNT, as demonstrated in Fig. 3. On the other hand, parthenogenetic embryos can develop to the blastocyst stage *in vitro* but fail to produce viable offspring because of genomic imprinting defects[73]. However, *in vitro*-derived embryos tend to be different from *in vivo* embryos in terms of gene expression, cryotolerance, metabolism, and pregnancy outcomes[74]. These shortcomings highlight the need for culture conditions that more closely reflect the dynamic environment of *in vivo* development.

IVP systems for bovine embryos

Various systems can be applied to produce bovine embryos *in vitro*, such as IVF or SCNT. In all cases, IVP embryos require IVC conditions that recapitulate those found *in vivo* and satisfy stage-specific metabolic requirements, as shown in Table 2.

IVF

IVF enables the controlled fusion of *in vitro*-matured oocytes with capacitated spermatozoa[82], producing zygotes in a reproducible and scalable manner[62]. The fertilization process is also highly dependent on the quality of oocytes, which is mainly achieved during IVM, and the

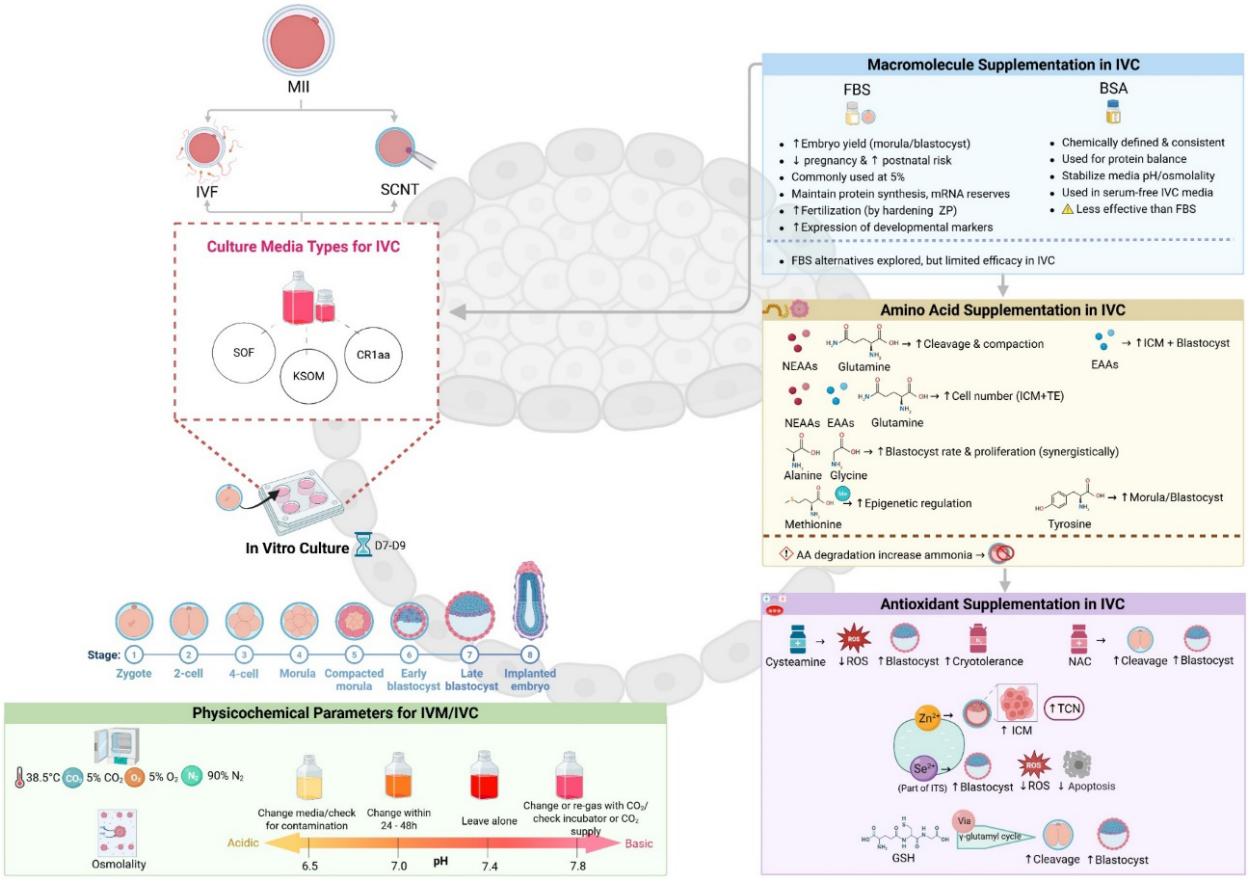

Fig. 3 Bovine *in vitro* culture (IVC) workflow and supplementation strategies. After maturation, oocytes are fertilized by IVF or activated by SCNT and cultured in defined media (SOF, KSOM, CR1aa) until the blastocyst stages (D7–D9). Supplementation strategies during IVC include macromolecules (FBS, BSA), amino acids (NEAAs, EAAs), and antioxidants (e.g., cysteamine, NAC, and GSH), which modulate cleavage, compaction, blastocyst quality, and redox balance. Physicochemical parameters, including pH, osmolality, gas composition, and temperature, are critical for maintaining embryos' viability and developmental competence. Abbreviations: SOF, synthetic oviductal fluid; KSOM, potassium simplex optimization medium; NEAAs, nonessential amino acids; EAAs, essential amino acids; NAC, N-acetyl cysteine; GSH, glutathione. Created by the authors using BioRender.

Table 2. IVC media formulations and developmental outcomes of bovine embryos.

Year	Species/source	Key supplements			CC %	Blastocyst % (days)	Key findings	Ref.
		MM	AAs	AOX				
2025	Bovine/IVF	5 mg/mL BSA	–	–	81	31.7 (Days 7–9)	Day 7 embryos supported pregnancy rates, whereas Day 9 blastocysts showed ↑apoptosis (↑BAX/↓BCL2) and ↓ICM, indicating reduced competence with prolonged culture.	[75]
2024		Undisclosed composition			84.9	35.8 (Day 8)	Confined individual culture (~70 nL) yielded 35.8% blastocysts with ↓apoptosis and smaller lipid droplets vs. group culture.	[76]
	Bovine/SCNT, ICSI, parthenogenesis	–	–	50 µg/mL L-ascorbic acid	82.8	33.2 (Day 7)	Pre-IVM with L-ascorbic acid improved maturation (88.5%), blastocyst quality (↑cell number and H3K9ac intensity), and enhanced SCNT/ICSI reprogramming.	[77]
	Bovine/IVF	10% FBS	750 mg NEAAs	Unspecified	51.4 (AAs) 63.3 (AOX)	42.1 (AAs) 42.1 (AOX)	750 mg NEAAs + antioxidants increased cleavage and supported Day 7 blastocyst formation, underscoring their complementary roles during bovine IVC.	[78]
2022	Bovine/IVF	–	–	ITS–5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium	87.4	43.3 (Day 8)	Lycopene (0.2 µM) during IVM increased Day 8 blastocyst rate (56% vs. 43.3% in the control), with improved embryo quality (↑TCN, ICM/TCN; ↓ROS, ↓apoptosis), despite identical IVC conditions.	[44]
2016		–	1 mM glycine	–	71.1	34.6 (Day 7)	Early IVC glycine under low osmolarity improved bovine blastocyst and hatching rates by Day 7, confirming its effective osmolyte and metabolic role in defined serum-free IVC systems.	[79]
2015		8 mg/mL BSA-FAF	–	–	91.1	36.2 (Day 8)	FBS (used in IVM) increased blastocysts' lipid content despite all groups using 8 mg/mL BSA during IVC. Reducing FBS (5%) lowered lipid accumulation without affecting development.	[28]
2008	Bovine/ parthenogenesis (PLCZ1)	3 mg/mL BSA	–	–	92	33.6	PLCZ1-activated parthenotes in KSOM + BSA achieved higher cleavage and blastocyst rates, outperforming IVF and indicating the efficacy of defined serum-free IVC systems.	[80]
2007	Bovine/IVF	SSS	–	–	75.6	27.8 (Day 8)	Replacing FCS with defined SSS + EGF during IVM/IVC media slightly improved blastocyst rates and altered gene expression without compromising embryo quality.	[45]
2006		10% FBS	–	–	80.1	32.8 (Day 8)	SOFaa yielded greater blastocyst formation over KSOMaa and CR1aa, with similar cell counts and apoptotic rates, while keeping gene expression profiles more similar to *in vivo* embryos, underscoring its physiological relevance.	[81]
1997		5% FCS	–	–	–	90.4 (Day 9)	Among morula selected at 120 hours post-insemination replacing BSA with addition of FCS increased blastocyst formation (90.4% by Day 9) and hatching vs. BSA.	[43]
–	Key trends	FBS/FCS more consistently supports blastocyst formation.	NEAAs and glycine are occasionally used.	ITS improves developmental outcomes.	51.4–92	27.8–43.3	FBS/BSA support IVC; defined AOX improves outcomes.	–

SOF/mSOF served as the base medium in most studies, whereas the study by Herrick et al.[79] employed bovine oviduct epithelial cell co-culture. Standard antibiotic supplementation e.g., amikacin (84.3 mg/mL)[28], penicillin (100 U/mL), streptomycin (100 µg/mL)[45], and gentamicin sulfate (50 µg/ml)[43] was applied to ensure aseptic conditions. MM, macromolecule; AAs, amino acids; AOX, antioxidant; CC, cumulus cells; ECS, estrous cow serum; SSS, serum substitute supplement; SOF, synthetic oviductal fluid; mSOF, modified synthetic oviductal fluid; KSOMaa, potassium simplex optimization medium with amino acids; CR1aa, Charles Rosenkrans 1 with amino acids; SOFaa, synthetic oviductal fluid with amino acids; PLCZ1, phospholipase C Zeta 1; SCNT, somatic cell nuclear transfer; ICSI, intracytoplasmic sperm injection.

functional competence of the spermatozoa[83]. Standard IVF procedures support capacitation, hyperactivation, and the acrosome reaction[83], with optimization geared toward reducing polyspermy during fertilization[84]. In addition, the IVF media offer a transient microenvironment for the interaction of the gametes[84], producing a zygote with synchronized cytoplasmic and nuclear maturity, which is also vital for subsequent IVC success[85]. Successful IVM–IVF coupling is a direct determinant of the developmental capabilities that embryos would be able to achieve under favorable IVC conditions.

SCNT

SCNT produces cloned embryos by transferring a donor somatic cell nucleus into an enucleated MII oocyte[86]. This method facilitates the propagation of genotypes, rescue of endangered breeds, and precise livestock breeding[86]. It involves enucleation of an oocyte matured *in vitro*, transfer and fusion of the donor cell, and artificial activation to trigger embryogenesis[87]. Nevertheless, the efficiency of SCNT is limited by the insufficiency of nuclear reprogramming, which often leads to the emergence of an altered epigenetic environment, the disruption of gene regulation, and reduced post-transfer survival[77]. In bovine SCNT, the

donor cells are most commonly fibroblasts derived from ear skin biopsies, and their choice relies on their availability, astable karyotype, and their ease of culture[87]. The quality of the recipient oocytes, shaped by the IVM conditions, is a decisive factor in the success of reprogramming[88]. SCNT embryos typically need prolonged IVC (e.g., 8–9 days compared with 7 days for IVF) to accommodate delayed cleavage dynamics. Moreover, 0.1 mM of trichostatin, which is a histone deacetylase inhibitor, is typically added to optimize IVC media at the early stages of development to improve nuclear reprogramming and epigenetic remodeling[77,81]. In contrast to IVF embryos, SCNT embryos also benefit from lowered glucose levels during early IVC to alleviate metabolic stress and enhance the quality of the blastocysts[77,89]. The results highlight the fact that flexible and stage-sensitive IVC systems are needed that can accommodate the customized metabolic and developmental characteristics of the cloned embryos.

Metabolic demands of embryos regarding the media's composition

According to the developed metabolic background during IVM, there is a profound shift in bovine embryos' energy consumption and biosynthetic rates in the context of IVC. Cross-sectional measurements of in vivo-derived bovine oocytes and embryos showed mean protein contents of 0.126 µg in oocytes, 0.367 µg in expanded blastocysts, and 946.6 µg in Day 16 embryos[90]. This stage-associated increase underlines the significance of AAs, energy sources, and complex macromolecules, including FBS in the culture media. In the early growth stages, bovine embryos actively oxidize pyruvate and the AAs of choice (e.g., glutamine) through the Krebs cycle, but the efficiency of oxidizing glucose is low, since pyruvate inhibits pyruvate kinase[89]. After the formation of the morula, a change in metabolism to oxidative metabolism takes place to facilitate the growth and hatching of the blastocyst because of the high ATP demand[89]. Moreover, at the blastocyst phase, an evident pattern of metabolic segmentation can be observed, in which inner cell mass (ICM) cells are more dependent on glycolysis, whereas trophectoderm (TE) cells prefer oxidative phosphorylation[91]. These dynamical and stage-specific energy signatures of IVC stage-adapted formulations, where the substrate supply is customized to alter according to changing metabolic needs, thus promote intensive growth and implantation ability.

Assessment of embryos

Morphological assessment, which is commonly done on Days 2 to 3, depending on the criterion of symmetry of the blastomere, fragmentation, multinucleation, and cleavage patterns, has been shown to lack precision[92]. More precise selection usually needs prolonged culture to the blastocyst stage[92]. Pursuing a standard of morphological scoring or other molecular markers has proven difficult becuase of interlaboratory variability as well as the biological complexity of embryo development[93].

Noninvasive technologies such as time-lapse systems, spent culture media metabolomics, and AA turnover are emerging as potential predictors of developmental competence without interfering with embryos' viability[94]. These methods provide real-time, biochemical, and kinetic information, which may supplement conventional morphological scoring. Instead, the combination of morphological and metabolic analysis with specific individualized IVC preparations in the form of antioxidant indicators, energy sources, and growth factors could be an effective integrated approach to indicate the deduced physiological state of the embryo. This synergy could prevent biological variability, improve blastocyst yield, and enhance embryo quality in bovine IVP.

Design and optimization of bovine IVC systems

Embryonic metabolism and morphology change throughout IVC and are associated with changing nutrient and stress-response requirements. These stage-specific changes provide the foundation for media formulation and selection, as indicated in Table 2.

Media design approaches

Embryo culture media have traditionally followed two conceptual approaches: The "let the embryo choose" approach, which focuses on minimal intervention using single-step media, and the back-to-nature approach, which recreates the reproductive tract environment[17]. Notable examples include SOFs, human tubal fluid, and porcine zygote medium, each based on the oviduct fluid composition of specific species[95].

Among the widely used bovine media, such as Charles Rosenkrans medium with AAs (CR1aa)[96], and potassium simplex optimized medium (KSOM)[17], each has strengths and context-specific performance advantages. CR1aa and KSOM are designed as simplified single-step systems that minimize handling yet support blastocyst development. Despite their conceptual differences, no single system has consistently outperformed the others[97]. However, blastocyst rates reported across studies are not directly comparable because the developmental stage at the start of culture and the assessment time can differ[43,47]. The outcomes of performance are determined by the laboratory workflows, the embryo stage at the onset of the culture, and the supplementation strategy. Modification of SOF, initially designed using sheep oviduct fluid[98], often fails to optimally support one-cell bovine embryos; frequently, most of these embryos arrest by Day 6 of culture. This limitation has led to successive modifications, including the addition of specific supplements, which have improved its role as a sequential medium for bovine embryos, with performance in some contexts surpassing that of KSOM and CR1aa[81]. The media's design philosophy directly influences how well IVC systems can accommodate stage-specific metabolic shifts, a critical factor in enhancing blastocysts' quality and yield. Choosing between these systems should be guided by the metabolic stage's needs, operational constraints, and empirical performance within each laboratory.

Advances in culture systems

Advances in embryo culture devices have aimed to simulate the *in vivo* environment more closely. Time-lapse incubators allow for continuous monitoring of embryonic development and for strictly controlling the physicochemical parameters[99]. Recently, microfluidic "embryo-on-a-chip" platforms have provided continuous perfusion for nutrient delivery and waste removal, which has been associated with improved blastocyst development and quality in mice, cattle, and even human embryos[100]. Additionally, three-dimensional (3D) biomimetic culture systems such as hydrogels, scaffolds, and organ-on-chip platforms mimic extracellular matrix-like conditions, allowing for more physiological cell–cell and cell–matrix interactions[101]. Similarly, co-culture with maternal cell types can also provide paracrine signals and growth factors, reducing fragmentation and improving developmental rates[102]. These innovations illustrate how the physical and biochemical aspects of IVC systems can be engineered to complement the composition of the media to diminish the quality gap between *in vitro* and *in vivo* embryos.

Epigenetic regulation in IVP

Embryonic developmental potential, imprinting fidelity, and the long-term health of offspring are based on epigenetic regulation[103]. Controlled *in vitro* conditions subject gametes and embryos to unstable

O_2 stress and nutrient levels which may disrupt DNA methylation and histone modifications and cause transcriptional instability[30]. The use of supplementation of both methyl donors and antioxidants to optimize the epigenetic landscape has thus become a useful method to enhance the quality of IVP embryos.

Methionine is the central amino acid in the one-carbon (1C) metabolic pathway, which is the precursor of S-adenosylmethionine (SAM), the universal donor of methyl in DNA and histone methylation[104]. The availability of methionine regulates the activity of DNA methyltransferase (DNMT) and methionine adenosyltransferase (MAT2A) in bovine oocytes and embryos[104,105]. Remarkably, certain key enzymes in the complete methionine–folate cycle, including betaine-homocysteine methyltransferase (BHMT) and MAT1A, are minimally expressed in early bovine embryos, indicating that developing embryos rely on external or maternal sources of folate to provide methyl groups[106]. This reliance demonstrates the metabolic immaturity of early embryos engaged in methyl group cycling and provides a justification for supplementing the culture media with methyl donors. Normal physiological levels of methionine can maintain the SAM–S-adenosylhomocysteine (SAH) ratio and support both *de novo* methylation and the appropriate imprinting of genes[104]. On the other hand, methionine depletion leads to global hypomethylation, especially in the trophectoderm, and dysregulation of developmental and placental genes, indicating a failure to maintain epigenetic programming during early embryogenesis[104]. Similarly, methyl-donor flux involving choline, folate, and vitamin B_{12} is sustained by homocysteine demethylation, ensuring methylation homeostasis[105]. Complementary evidence from bovine embryonic fibroblasts shows that a balanced supply of these modifiers stimulates mitochondrial respiration, cell proliferation, and DNA methylation, with cellular energy and epigenetic stability associated with 1C metabolism[105]. Individually, these results indicate that methyl donor supplementation in IVM/IVC media partially counteracts the low endogenous 1C capacity of the embryo, such that SAM synthesis is sufficient and epigenetic programming is highly stable *in vitro*.

Excessive ROS production resulting from high O_2 tension or metabolic stress is disruptive to these finely-tuned mechanisms in IVC[59]. Oxidative stress perturbs redox-sensitive methylation enzymes and interferes with SAM-mediated reactions, resulting in aberrant DNA methylation and chromatin remodeling. In bovine embryos, high O_2 tension promotes an oxidative load and universal DNA hypermethylation, alongsidereduced developmental quality[60,107]. These effects can be mitigated by antioxidant supplementation, which is able to restore the redox balance and maintain the catalytic activity of methylation and histone-modifying enzymes. Therefore, the oxidative–epigenetic equilibrium that is maintained during IVM and IVC contributes to the preservation of gene expression and the developmental competence of IVP embryos.

Functional supplementation strategies to optimize IVC of bovine embryos

Targeted supplementation with macromolecules, AAs, and antioxidants can align IVC conditions with stage-specific metabolic demands, thereby improving developmental competence and embryo quality, as depicted in Fig. 3.

Role and impact of macromolecule supplementation in bovine IVC

Macromolecular supplements, as described above, support both IVM and IVC, but vary in their optimum concentration and developmental performance. These macromolecules stabilize the culture conditions, eliminate mechanical stress, and regulate metabolic activity during IVC[7]. FBS concentrations of 2%–10% are desired, with 5% being the one that sustains development the best[21,28,43]. Additionally, absence of serum during earlier stages of IVM/IVF has been linked to retarded first and fourth cell cycles in early embryos within 4–5 h, possibly affecting development during IVC[71]. Even though FBS positively influences blastocyst production *in vitro*, its addition during IVC has been linked with reduced embryo survival, reduced post-transfer pregnancy rates, and reduced rates of embryo survival[108]. High risk of postnatal abnormalities has also been reported by other studies, indicating that earlier, better development may not necessarily enhance higher reproductive success[108,109]. FBS and BSA have the capacity to promote bovine embryogenesis in IVC, which is well documented[45]. Embryos' protein content could be taken as a developmental indicator, and it suggests that metabolism and biosynthesis are active under these supplementation conditions[90].

Several macromolecule alternatives previously described for IVM have also been tested in IVC, including established options such as growth factors[17] and animal or plant-derived protein fractions[42]. Emerging sources, such as sericin protein[42], mealworms (*Tenebrio molitor*), coelomic fluid from earthworms, and vegetetable serum[42], have limited bovine IVC data. Although many of these have shown promise in IVM, no universally effective alternative has yet replicated the unique composition and consistent performance of FBS in IVC. These findings indicate that there is a need to develop serum-free, chemically defined IVC systems to enhance standardization, reproducibility and embryo quality.

Role and impact of AA supplementation in bovine IVC

A number of AAs continue to assist oocytes' maturation in IVM. Nevertheless, they change functional priorities during IVC and align with the structural and metabolic demands of cleavage, compaction, and blastocyst development[49]. Their significance is increased following compaction, as metabolic activity intensifies[110]. The requirement for AAs changes with development. NEAAs and glutamine, which are relatively abundant in oviduct fluid, stimulate cleavage and cell viability in cleavage-stage embryos[52]. Following compaction, NEAAs and glutamine induce blastocoel formation and hatching, and EAAs play a critical role in the development of the ICM[111]. A combination of NEAAs, EAAs, and glutamine supplementation has been reported to enhance embryo quality in terms of total cell number (TCN), total cell number in TE, and ICM[52,57,96]. Conversely, EAAs alone have been linked with lower blastocyst rates[49].

Specific AAs also display the desired effects. Alanine and glycine have a synergistic effect on the formation of the blastocyst, and they can complement each other, increasing the proliferation of cells and blastocyst formation[112]. Methionine also influences the epigenetic control through folate-mediated methylation pathways[106]. Moreover, the development of morula and blastocysts in buffalo embryos has been enhanced by L-tyrosine supplementation in the SOF medium[54]. Glutamine peaks occur in the initial cleavage phases, decline in the blastocyst formation phase, and rise once more in the blastocyst growth phase (applied dynamically according to metabolic considerations)[113]. The AA-depleted media of mouse embryos altered gene expression and interfered with the imprinting of H19, which demonstrated their role outside of metabolism[114].

Despite all these, AA supplementation has caveats. Among the significant issues, the spontaneous disintegration of some AAs could produce a toxic ammonium that impairs the survival of the

blastomeres and influences the formation of the blastocysts[115]. Therefore, to reduce this risk, media with AAs should be refrigerated at 4 °C and exposure to 37 °C should be reduced to only before the embryos are added[54]. With appropriate handling of the media, stage-specific AA supplementation confers enormous metabolic and homeostatic advantages on the developing embryo, particularly when progressing with serum-free or chemically defined IVC systems.

Role and impact of antioxidant supplementation in bovine IVC

Despite their established role in IVM, only a few studies have explored antioxidant supplementation during bovine IVC. Most have focused on oxidative stress arising under challenging conditions such as heat stress[116]. During IVC, the effects of thiol antioxidants appear to depend on the specific compound, concentration, developmental stage, and culture conditions. Cysteine supplementation has been associated with reduced blastocyst and hatching rates under some IVC conditions[60]. Cysteamine has shown beneficial effects on subsequent embryo development when used during IVM[62,117], although evidence for its direct use during IVC is limited. NAC also shows stage-dependent effects during embryo culture, with early exposure reducing cleavage and later exposure improving blastocyst development and hatching[23]. These findings suggest that the effects of thiol antioxidants during IVC cannot be explained only by their relationship with GSH.

There have been a few studies that have identified positive outcomes from supplementing GSH during IVC. GSH supplementation has improved blastocyst development, TCN, and ICM numbers[118], whereas isotope-labeled GSH (GSX) increased cleavage and blastocyst rates via the γ-glutamyl cycle[119]. Additionally, there have been efforts to explore Se and Zn for their abilities to improve the success of IVC. Multiple studies evaluated that Se is most often tested in conjunction with an insulin–transferrin–Se (ITS) complex, a serum substitute that has been shown to significantly improve blastocyst development[63,120]. Insulin plays an important role in the uptake of AAs and provides antiapoptotic and growth-promoting effects[63]. Transferrin reduces ROS formation via iron sequestration[63]. Se, as a trace element, contributes to protecting cells from oxidative damage through the GPx pathway[64]. Although many selenides (Se, Cu, Mn, and Zn) did not impact the cleavage rate, they improved blastocyst formation and TCN, and reduced ROS and lipid content, indicating augmented IVC embryo quality measures[64]. Furthermore, Zn supplementation during IVC did not affect blastocyst yield but improved ICM and TCN, indicating improved embryo quality[59]. In the case of IVM, Zn has been linked to higher subsequent cleavage rates, improved blastocyst formation, and a faster increase in blastocyst cell numbers[66], making the timing of exposure the most important variable of efficacy. Hence, antioxidant supplementation during IVC is still emerging. The current findings show greater potential for embryo quality parameters than for blastocyst yield. The outcomes are largely qualitative, with efficacy depending on the match between the antioxidant's type, dose, and timing, and the metabolic state of the embryo during culture. Further studies are needed to define stage-specific mechanisms and to assess whether these improvements translate into higher post-transfer success rates.

Optimization of physicochemical parameters for IVM and IVC

The precise control of pH, osmolality, temperature, and gas composition is essential in the application of bovine IVM and IVC systems to mimic the reproductive tract microenvironment, reduce cellular stress. and promote stage-specific developmental competence[121], as shown in Fig. 3.

pH

The physiological uterine pH of bovines is 7.22 to 7.35, which increases marginally after ovulation[122]. *In vitro*, the media's pH is primarily determined by bicarbonate buffering and the CO_2 levels in the incubators[73]. Precompaction, embryos are vulnerable to changes in pHi[123], which may hinder spindle formation, MPF activity, and the formation of the blastocoel[124]. After compaction, embryos are more resilient, and it is important to maintain the external pH conditions during the early culture phases[50]. Various media formulations differ in their buffering capacity and ionic composition, which may have an indirect effect on pHi[124]. To this end, the pH must be adjusted to each formulation, rather than being set to a global value.

Osmolality

Osmolality influences embryos' development by regulating fluid balance and cell volume, with optimal osmolality ranges of 255–265 mOsm/kg. Values above 300 mOsm/kg, often caused by evaporation, can impair blastocysts' development[97]. NEAAs act as compatible osmolytes, improving cellular resilience under osmotic stress in both oocytes and embryos *in vitro*[125]. To maintain stable osmolality, humidified incubators and proper use of oil overlays are recommended.

Temperature

Temperature control is a critical variable of IVP systems, influencing both oocytes' maturation and embryonic development. It is well known that suboptimal temperatures can disrupt the meiotic spindle's stability, alter embryonic metabolism, and affect lineage allocation[126]. Although both 36.5 and 38.5 °C support maturation, most bovine IVM/IVC systems favor 38.5–39 °C for higher maturation and blastocyst rates[17,23,127]. Heat stress at 41 °C impairs CCs' function, disrupts gene expression, and reduces cleavage rates[128]. Even minor fluctuations can destabilize the components of the medium, reducing embryos' viability[128].

Gas composition (O_2, CO_2, and N_2)

Low oxygen tension, i.e., 5% O_2, better reflects the environment of the oviduct and uterus, where the levels are approximately 5%–7%, compared with atmospheric conditions (20%)[129]. The benefits of low O_2 likely stem from reduced ROS generation, improved blastocyst yield, and higher air quality[130]. It is known that CO_2 (5%) interacts with bicarbonate to regulate extracellular pH in the culture medium[124], whereas N_2 acts as a carrier to stabilize the overall gas composition. SOF-based media under 5% O_2 consistently outperform those at atmospheric levels, underscoring the importance of hypoxic culture conditions[130].

Physicochemical troubleshooting

- Osmolality > 300 mOsm/kg: Dilute the media with sterile and filtered water (10 mL/mL media) and retest. Do not overdilute (range 255–265 mOsm/kg).
- pH < 7.2: Add a buffering solution (5%–5.5%) to the incubator for 30 min; ensure the bicarbonate buffer is fresh (expired buffers have no buffering capacity).

Embryo transfer: from laboratory to herd

Optimized IVM and IVC programs are the cornerstone of developing developmentally competent embryos, which can be transferred to fertilize the recipient female either as an acute transfer or following

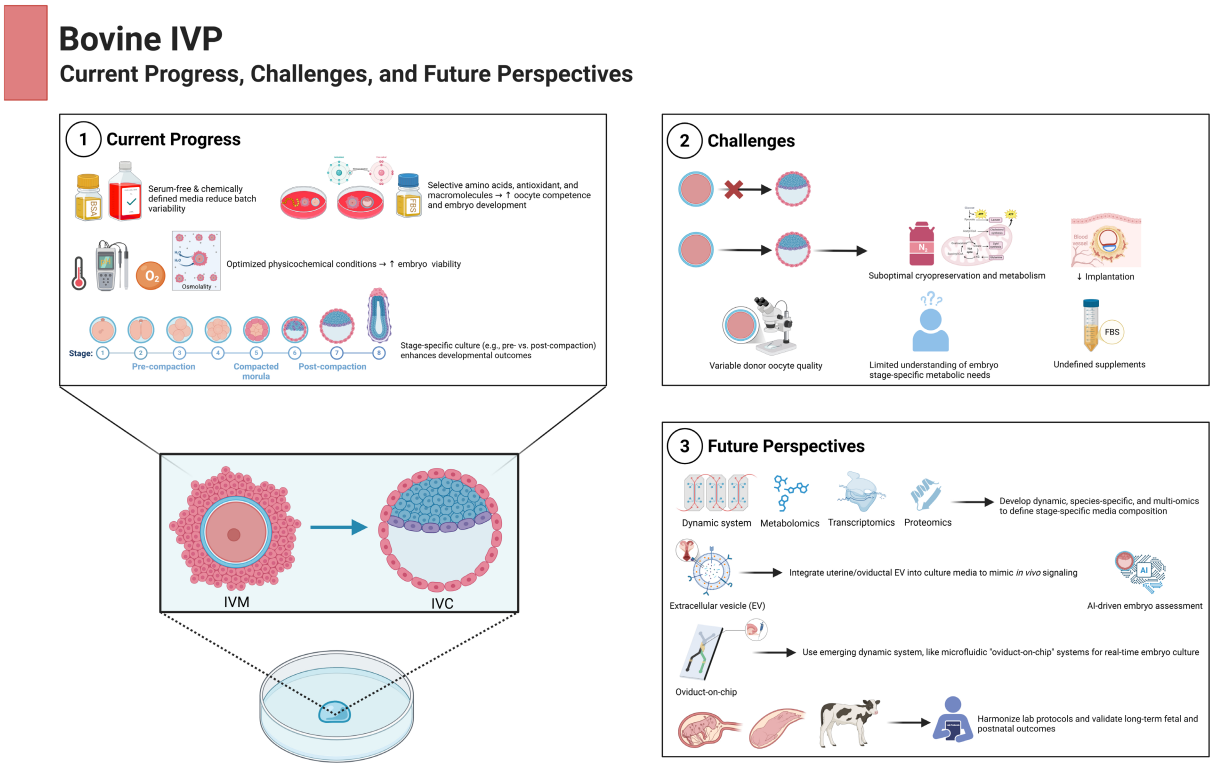

Fig. 4 Current progress, challenges, and future perspectives in bovine IVP. (1) Current progress: Advances in bovine *in vitro* production (IVP) include serum-free, chemically defined media, optimized physicochemical conditions, and selective supplementation strategies. (2) Challenges: Key limitations remain, including donor variability, undefined supplements, limited understanding of metabolic demands, and continued reliance on undefined supplements. (3) Future perspectives: Emerging approaches emphasize dynamic, species-specific media informed by multi-omics (metabolomics, transcriptomics, and proteomics), integration of extracellular vesicles, microfluidic "oviduct-on-a-chip" platforms, artificial intelligence-driven embryo assessment, and long-term validation of the offspring's health. Created by the authors using BioRender.

cryopreservation for use at a later time. In both cases, synchronization protocols are mandatory to match the uterine environment of the recipient with the stage of development of the embryo so as to optimize receptivity and the capacity of implantation[131]. Fresh ET often yields slightly higher pregnancy rates under commercial conditions because of the absence of cryoinjury[131]. However, advances in vitrification techniques, cryoprotectant formulations, and post-thaw handling have enabled pregnancy outcomes in some settings that are similar to those achieved with fresh embryos[98]. Cryopreservation offers logistical flexibility, facilitates international germplasm exchange, and enables herd improvement programs without immediate recipient availability[132].

Future directions

Further development of bovine IVP will necessitate the replacement of empirically developed static culture models with dynamically data-driven and species-specific media systems. To enhance reproducibility and mechanistic understanding, the most urgent priority is the development and validation of chemically defined IVM and IVC media that reduce reliance on undefined supplements, including serum, without compromising developmental outcomes[17]. The design of future media ought to incorporate stage-specific supplementation with AAs, energy sources, and antioxidants more precisely, based on bovine-specific multi-omics datasets to define developmental demands, such as metabolomics to determine nutrient fluxes and metabolic biomarkers, transcriptomics to determine changes in gene regulation, and proteomics to describe the profiles of enzyme activity[133,134].

A combination of these omics techniques will permit the rational design of culture media that more closely mimic the follicular and oviduct environment *in vivo*. Comprehensive metabolomic profiling of follicular and oviduct fluids of high-quality donors should be prioritized to seek species-specific AA and lipid signatures that can guide the next-generation culture media formulations. In addition to the individual additives, the synergistic effects of the supplements should be evaluated through interlaboratory comparisons using common reporting criteria. These data could also support an open-access repository of optimized IVP formulations and metadata for reproducibility and meta-analyses. Of interest, integrating physiological factors, including EVs made of oviduct or uterine fluids, has demonstrated the possibility of enhancing developmental competence and molecular quality[135]. Nonetheless, standardization of EV isolation procedures, dose determination, and storing protocols is of paramount importance to guarantee uniformity among studies. After these procedures are standardized, collaborative studies could evaluate the feasibility, cost and scalability of EV manufacturing systems for commercial IVP workflows.

Physiologically relevant microenvironments can be offered by emerging dynamic systems, such as microfluidic oviduct-on-a-chip systems, which have dynamic nutrient exchange and waste clearance. Future development should focus on device scalability, media compatibility, and affordability in existing IVP workflows in order to support the translation between research and commercial environment. Combining these systems with real-time monitoring tools for pH, O_2, and metabolism, as well as artificial intelligence (AI)-based embryo assessment algorithms[136], could thus enable precision culture with predictive scoring of embryos' competence[20,24,31,33,75,137].

Further assessments of IVP embryos should not be restricted to blastocyst formation. The critical point is to evaluate long-term validation, such as fetal development, epigenetic stability, and postnatal

health indicators[103], ensuring laboratory gains and transforming the embryos into healthy and productive progeny in commercial practice. Lastly, the bovine IVP must be aligned with global animal welfare and biotechnology standards through the sustainable sourcing of media components and adherence to ethical considerations for FBS. The innovations, as shown in Fig. 4, including integrated defined media, omics-informed optimization, standardized EV and microfluidic systems, and AI-assisted evaluation, can be taken as a roadmap toward recapitulating bovine IVP systems that are reproducible, efficient, and fulfill ethical, commercial, and scientific objectives.

Conclusions

The optimization of IVM and IVC media is central to improving bovine IVP. Precise stage-specific inclusion of the identified energy substrate, AAs, antioxidants, growth factors, and hormones can enhance oocytes' competence and embryos' viability, while reducing batch variability and epigenetic perturbations. In spite of this progress, *in vivo* benchmarks continue to be lower because of donor variability, limited knowledge of metabolic requirements, and continued reliance on undefined supplements. This given review highlights the need to switch to dynamic, species-specific, and chemically defined systems to improve reproducibility while maintaining developmental potential. Such advancements strengthen cattle breeding, germplasm exchange, and conservation programs.

Ethical statements

Not applicable.

Author contributions

The authors confirm their contributions to the paper as follows: Saeed HA was responsible for study design and drafting the manuscript. Cao D, Wang Z, He Y, Li H, Lionel LH, Sabir R, Avais M, and Cai Y were involved in data collection. Li J contributed to supervision and manuscrip revision. All authors reviewed the results and approved the final version of the manuscript.

Data availability

Data sharing is not applicable to this review, as no original datasets were generated or analyzed; the data summarized in Tables 1 and 2 were obtained from the cited literature.

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Conflict of interest

The authors declare that they have no conflict of interest.

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