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Comparative Analysis of Human GV-Oocyte Maturation Under Cumulus-Supported and Denuded Culture Conditions

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Die in dieser Arbeit angegebenen Experimente, einschließlich der Kultivierung und Behandlung der Cumuluszellen, der Durchführung der AMPK- und ROS-Analysen, der Immunfluoreszenzfärbungen sowie der konfokalen Mikroskopie, sind von mir selbstständig ausgeführt worden.

Die Aufarbeitung der Bilddaten, die Quantifizierung der Fluoreszenzsignale und die statistische Auswertung wurden von mir eigenständig mit der entsprechenden Software vorgenommen.

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ABBREVIATION

Abbreviation	Full Term
AMH	Anti-Müllerian Hormone
AMPK	AMP-activated protein kinase
ART	Assisted Reproductive Technology
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CAPA-IVM	Capacitation In Vitro Maturation
CCs	Cumulus Cells
CCs-O	Cumulus–Oocyte co-culture
CDK1	Cyclin-dependent Kinase 1
CNP	C-type Natriuretic Peptide
COC	Cumulus–Oocyte Complex
COS	Controlled Ovarian Stimulation
DCF	2′,7′-Dichlorofluorescein
DCFH-DA	2′,7′-Dichlorodihydrofluorescein Diacetate
DMEM/F-12	Dulbecco's Modified Eagle Medium / Nutrient Mixture F-12
EGF	Epidermal Growth Factor
EGF-like ligands	Epidermal Growth Factor-like ligands (e.g., amphiregulin, epiregulin)
FBS	Fetal Bovine Serum
FSH	Follicle-Stimulating Hormone
GV	Germinal Vesicle

GVBD	Germinal Vesicle Breakdown
GVBD rate	Percentage of oocytes that underwent Germinal Vesicle Breakdown
H_2DCFDA	2′,7′-Dichlorodihydrofluorescein Diacetate (fluorescent ROS probe)
H_2O_2	Hydrogen Peroxide
HSD	Honestly Significant Difference (Tukey's post-hoc test)
ICC	Immunocytochemistry
ICSI	Intracytoplasmic Sperm Injection
IVF	In Vitro Fertilization
IVM	In Vitro Maturation
LH	Luteinizing Hormone
MAPK	Mitogen-Activated Protein Kinase
MI	Metaphase I
MII	Metaphase II
MPF	Maturation-Promoting Factor
mTOR	Mechanistic Target of Rapamycin
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
O_2	Oxygen
OC	Oocyte-Only Culture
PBS	Phosphate-Buffered Saline
PCOS	Polycystic Ovary Syndrome
PDE3A	Phosphodiesterase 3A

PGC-1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha
ROS	Reactive Oxygen Species
SIRT	Sirtuin
SPSS	Statistical Package for the Social Sciences
TGF-β	Transforming Growth Factor Beta

1. ABSTRACT

Background:

In-vitro maturation (IVM) of human oocytes provides a safer, hormone-sparing alternative to conventional in-vitro fertilization (IVF), particularly for patients at risk of ovarian hyperstimulation syndrome (OHSS) or with contraindications to gonadotropin use. Despite its clinical appeal, IVM efficiency remains limited, which is thought to result partly from disruption of the somatic microenvironment normally maintained by cumulus cells. This study examined whether preserving the cumulus–oocyte complex (COC) during culture improves meiotic resumption and modulates oxidative and metabolic states in surrounding cumulus cells.

Methods:

Primary cumulus cells and GV-stage oocytes were obtained from follicular aspirates collected during ultrasound-guided ovarian puncture at the Women's Clinic, University of Cologne. For the oocyte experiments, immature GV oocytes were allocated to two culture conditions: (i) only oocytes, where oocytes were cultured without cumulus cells; and (ii) oocyte with cumulus, where oocytes were cultured together with primary cumulus cells. After 48 hours, germinal vesicle breakdown (GVBD) was evaluated as an indicator of nuclear maturation (Fisher's exact test). For the cumulus-cell experiments, primary cumulus cells were maintained under two conditions: (i) only cumulus; and (ii) cumulus with oocyte. Intracellular reactive oxygen species (ROS) levels were quantified using the H_2DCFDA fluorescence probe, with hydrogen peroxide (H_2O_2)-treated cells as the positive control (one-way ANOVA with Tukey's HSD). AMP-activated protein kinase (AMPKα1) expression was evaluated by immunocytochemistry under identical imaging settings, with a no-primary-antibody control verifying staining specificity (two-group comparison).

Results:

Oocytes cultured together with cumulus cells showed a significantly higher rate of GVBD (66.7%) than oocytes cultured without cumulus cells (18.2%; $P = 0.036$). Cumulus–Oocyte co-culture exhibited markedly lower intracellular ROS levels (12.11% vs. 21.87% of the H_2O_2 reference; $P < 0.01$) and significantly higher AMPK expression (228.98% relative to cumulus cells cultured alone; $P < 0.0001$). Morphologically, co-cultured cumulus cells displayed enhanced expansion and tighter intercellular connections.

Conclusions:

Maintaining oocytes with cumulus during culture establishes a low-oxidative, energy-efficient microenvironment that promotes meiotic resumption in human GV-stage oocytes. These findings highlight the critical role of somatic–germ cell communication and provide a mechanistic basis for microenvironment-centered refinements in human IVM protocols, particularly in patients with PCOS or undergoing fertility preservation.

2. Introduction

2.1 Background and Clinical Context

Infertility is a prevalent and growing public-health concern with medical, psychosocial, and economic consequences for individuals and healthcare systems alike. Within assisted reproductive technologies (ART), the developmental potential of the oocyte is a pivotal bottleneck that constrains embryo quality and ultimately live-birth rates, regardless of the sophistication of downstream laboratory procedures. In physiological reproduction, the oocyte must complete a tightly choreographed sequence of nuclear and cytoplasmic events—progressing from prophase I arrest through germinal vesicle breakdown (GVBD) to metaphase II (MII), while concurrently reorganizing organelles, transcripts, and metabolic circuits—to acquire competence for fertilization and early embryogenesis[1]. Any departure from this orchestration, whether due to endocrine pathology, microenvironmental stress, or suboptimal laboratory handling, diminishes the likelihood of establishing an ongoing pregnancy. The clinical and scientific impetus for optimizing oocyte maturation, therefore, is both obvious and urgent[2–4].

Conventional in vitro fertilization (IVF) relies on controlled ovarian stimulation (COS) to recruit multiple dominant follicles[5], improving the chance of retrieving several mature MII oocytes in one cycle and enabling modern practices such as blastocyst culture, single-embryo transfer, and elective cryopreservation[6]. Despite these strengths, COS is not universally benign. Pharmacologic stimulation may precipitate ovarian hyperstimulation syndrome (OHSS), imposes repeated injections and intensive monitoring, and increases the time and cost of care. For specific patient populations—most notably those with polycystic ovary syndrome (PCOS), very high antral follicle counts, or medical contraindications to supraphysiologic estrogen—these liabilities can outweigh the benefits of standard protocols[7]. Even in well-tolerated cycles, oocyte retrievals frequently yield a heterogeneous cohort spanning GV, metaphase I, and MII stages; immature oocytes are often discarded[8], representing a lost therapeutic opportunity if safe and efficient maturation could be completed ex vivo. These practical considerations motivate strategies that either minimize stimulation or recover value from the immature fraction without additional patient burden.

In-vitro maturation (IVM) directly addresses this space by retrieving immature oocytes and completing maturation under controlled culture conditions, with the aim of generating MII oocytes suitable for ICSI and embryo development[9]. IVM fills at least three clinically meaningful niches. First, as a primary treatment pathway for patients in whom gonadotropin exposure is undesirable or risky, IVM can reduce or eliminate stimulation while preserving access to ART outcomes—an approach particularly relevant to PCOS, where the propensity for exuberant follicular responses elevates OHSS risk[10]. Second, as a rescue strategy, IVM can mature the immature fraction inadvertently collected during conventional IVF cycles, thereby increasing usable oocyte yield without additional stimulation, anesthesia, or delay[11]. Third, in oncofertility contexts, IVM offers a time-sensitive option when gonadotoxic therapies are imminent or when hormonally driven stimulation is contraindicated, allowing fertility preservation without the delays inherent to COS[12]. Across these indications, IVM aligns with a patient-centered ethos: it reduces pharmacologic exposure, contains costs, and expands access to treatment in clinical scenarios that would otherwise be constrained.

The biological premise of IVM is to recreate, as faithfully as feasible, the follicular microenvironment that naturally coordinates the timing and quality of oocyte maturation. In vivo, the cumulus–oocyte complex (COC) integrates physical connectivity through gap junctions with paracrine signaling to maintain meiotic arrest via cyclic nucleotides until the luteinizing hormone (LH) surge triggers GVBD[13]. Cumulus cells provide metabolic and redox support—trafficking substrates, shaping glycolytic flux, buffering reactive oxygen species (ROS), and modulating lipids—while the oocyte reciprocally instructs cumulus gene expression and function through oocyte-secreted factors such as GDF9 and BMP15[14]. When oocytes are removed from the follicle, the abrupt shift in oxygen tension, oxidative load, and nutrient milieu can fragment this intercellular dialogue. If the somatic companion network is stripped away or inadequately reconstructed, nuclear events may proceed out of synchrony with cytoplasmic maturation, compromising spindle architecture, mitochondrial efficiency, and downstream embryo development[15]. These mechanistic considerations underpin contemporary IVM strategies that preserve or engineer somatic support rather than treating the oocyte as an isolated unit.

Notwithstanding its rationale and clinical appeal, historical IVM outcomes in unselected populations—measured by MII yield, fertilization, blastocyst development, and live birth—have trailed those of conventional IVF[16]. A substantial component of this performance gap reflects

legacy protocols that used single-phase culture of denuded oocytes under ambient oxygen in generic media, without explicit attention to the metabolic and redox needs of the COC. In recent years, multiple advances have converged on a microenvironment-first paradigm. Biphasic protocols that temporarily preserve meiotic arrest before initiating maturation—for example, pre-IVM exposure to C-type natriuretic peptide and EGF-like ligands, followed by an IVM step—seek to synchronize nuclear and cytoplasmic programs more closely with in vivo physiology[17,18]. Adjustments in oxygen tension and the development of engineered somatic supports, including granulosa-like or ovarian support cells derived from pluripotent sources, further aim to restore cumulus-mediated cues that are difficult to replicate with media alone[19,20]. Clinical experiences suggest that these refinements narrow the outcome gap in selected cohorts—particularly PCOS—by aligning the timing and quality of maturation with a healthier somatic context. Yet heterogeneity in patient selection, denudation timing, oxygen settings, and culture composition continues to obscure best practice, reinforcing the need for studies that pair nuclear endpoints with quantitative readouts of the surrounding microenvironment.

PCOS provides a particularly instructive clinical and biological context for IVM. The syndrome is characterized by hyperandrogenism, oligo-/anovulation, and polycystic ovarian morphology, and is frequently accompanied by insulin resistance, dyslipidemia, and low-grade inflammation[21]. Clinically, PCOS amplifies the risk of OHSS during COS and increases the likelihood of harvesting immature oocytes; biologically, it is associated with altered follicular fluid composition, oxidative stress, and mitochondrial dysfunction that may undermine oocyte competence[22]. IVM can mitigate both safety and quality concerns by minimizing gonadotropin exposure while leveraging somatic support during culture. Importantly, PCOS foregrounds redox and energy-sensing pathways—including AMP-activated protein kinase (AMPK)—as plausible mediators of somatic–germ cell cross-talk. The cumulus compartment's capacity to buffer oxidative stress and modulate metabolic flux may be especially consequential in this diagnosis, making PCOS an ideal setting to interrogate how preserving the COC alters the oocyte's probability of timely meiotic resumption.

Oxidative stress is a central constraint in vitro. ROS at physiologic levels participate in signaling pertinent to cumulus expansion and meiotic progression, but supra-physiologic loads damage lipids and nucleic acids, distort spindle geometry, and impair embryo development[23]. Culture conditions commonly expose gametes to oxidative challenges: higher oxygen tensions than in the follicle, photo-oxidation during imaging, and media components that may not fully

recapitulate in vivo antioxidant capacity. Cumulus cells act as a protective redox buffer, scavenging ROS and channeling antioxidants and substrates that stabilize mitochondrial and cytoskeletal dynamics within the oocyte[24]. Early or complete denudation removes this shield, potentially raising the threshold for GVBD and desynchronizing nuclear and cytoplasmic maturation. For these reasons, evaluating maturation outcomes with and without cumulus support, while directly quantifying the somatic redox state, is essential for disentangling structure–function relationships within the COC during IVM.

Cellular energy sensing intersects with redox control through nodal regulators such as AMPK. Activated by shifts in adenylate charge and diverse stresses, AMPK reprograms metabolism to conserve ATP, enhances mitochondrial quality control, and modulates autophagy and cytoprotective pathways[25]. In germ cells and their supporting somatic neighbors, AMPK activity influences gap-junctional communication, substrate utilization, and resistance to oxidative injury—processes directly relevant to oocyte maturation and competence. Experimental data across species indicate that perturbations in AMPK signaling alter meiotic timing and spindle integrity, whereas physiologic activation can enhance the supportive functions of cumulus cells and improve the oocyte's micro-niche during culture[26]. Integrating ROS readouts with measures of AMPK expression or activity within cumulus cells therefore provides a mechanistic window into how somatic integrity—or its engineered substitutes—affects maturation trajectories in human IVM.

These converging clinical needs and mechanistic insights explain both the promise of IVM and the persistent variability that has limited its broader adoption. Safety advantages are clear in PCOS, oncofertility, and rescue settings, but reproducible optimization requires that culture design be guided by mechanism rather than tradition. Two gaps are particularly salient. First, many studies report nuclear endpoints such as GVBD or MII attainment without concurrent characterization of the somatic microenvironment that the oocyte experiences during culture; as a result, protocol refinement lacks biologically anchored feedback loops. Second, relatively few human datasets have directly compared cumulus-enclosed and denuded GV oocytes under standardized conditions while simultaneously quantifying cumulus ROS and AMPK signals. Addressing these gaps can clarify whether preserving the COC confers measurable redox and energy-sensing advantages that translate into higher probabilities of meiotic resumption and, ultimately, better developmental potential.

The practical implications for ART programs are immediate. If cumulus retention demonstrably lowers oxidative stress and engages adaptive energy signaling in support cells, then maintaining or reconstructing the somatic compartment should be treated as a foundational design choice in IVM—on par with media formulation and oxygen settings. In concrete terms, this may involve delaying complete denudation until after a defined pre-IVM window, implementing autologous or engineered co-culture systems that supply EGF-like and metabolic cues, and adopting laboratory quality-control biomarkers—such as plate-internal ROS normalization and standardized AMPK immunofluorescence pipelines—to detect unfavorable microenvironments before they manifest as poor maturation outcomes. Such practices shift IVM from a one-size-fits-all procedure to a microenvironment-engineered platform tailored to diagnosis- and protocol-specific constraints.

Against this background, the present thesis situates human GV-oocyte maturation within the context of the somatic milieu by comparing culture with versus without cumulus cells under standardized laboratory conditions. Nuclear maturation (GVBD) is evaluated as the primary endpoint, while the surrounding cumulus microenvironment is interrogated through intracellular ROS quantification and AMPK immunocytochemistry, complemented by morphological appraisal of cumulus organization during culture. By connecting what the oocyte does—resume meiosis—with how its somatic partners behave—manage oxidative load and energy signaling—the work seeks to ground IVM protocol design in measurable biology and to provide a rationale for systematic microenvironment optimization in clinical practice.

2.2 Physiology of Oocyte Maturation and the Cumulus–Oocyte Complex (COC)

The maturation of the mammalian oocyte is one of the most precisely orchestrated processes in reproductive biology. It encompasses both nuclear and cytoplasmic changes that must occur in perfect temporal coordination to ensure the acquisition of developmental competence. Nuclear maturation refers to the resumption and completion of meiosis, progressing from the germinal vesicle (GV) stage to metaphase II (MII), while cytoplasmic maturation involves extensive reorganization of organelles, the cytoskeleton, and the molecular machinery required for fertilization and early embryogenesis. These two dimensions are tightly interdependent; disturbances in one invariably impair the other. Understanding this coordination is fundamental for any attempt to reproduce oocyte maturation in vitro[27,28].

In vivo, the mammalian oocyte is arrested at the diplotene stage of prophase I until a specific hormonal signal—the luteinizing hormone (LH) surge—triggers meiotic resumption. The oocyte is not an isolated unit during this prolonged arrest but is intimately surrounded by somatic companion cells that form the cumulus–oocyte complex (COC). These cumulus cells, together with mural granulosa cells, provide the structural and biochemical support that sustains oocyte growth and preserves meiotic arrest through paracrine and gap-junctional communication. Small molecules, such as cyclic nucleotides and metabolites, continuously shuttle through these intercellular bridges to maintain the oocyte's intracellular environment in a state that favors stability rather than progression. When the LH surge occurs, these intercellular channels are reorganized or disrupted, second messengers are redistributed, and the oocyte resumes meiosis, culminating in GVBD and subsequent progression to MII[29,30].

At the biochemical level, meiotic arrest is maintained by high intra-oocyte levels of cyclic adenosine monophosphate (cAMP). This cAMP is derived both from the oocyte's own adenylate cyclase activity and from cumulus cells that supply cyclic guanosine monophosphate (cGMP) through gap junctions. The cGMP acts by inhibiting the oocyte's phosphodiesterase PDE3A, thereby preventing cAMP degradation and keeping maturation-promoting factor (MPF) inactive. This delicate equilibrium maintains the GV state until the LH surge induces the expression of epidermal growth factor (EGF)-like peptides, such as amphiregulin (AREG) and epiregulin (EREG), in mural granulosa and cumulus cells[31]. These signals activate mitogen-activated protein kinase (MAPK) cascades in the cumulus layer, leading to rapid closure of gap junctions, a decline in intra-oocyte cAMP, and activation of MPF. The morphological manifestation of this cascade is GVBD, marking the oocyte's irreversible commitment to meiotic progression.

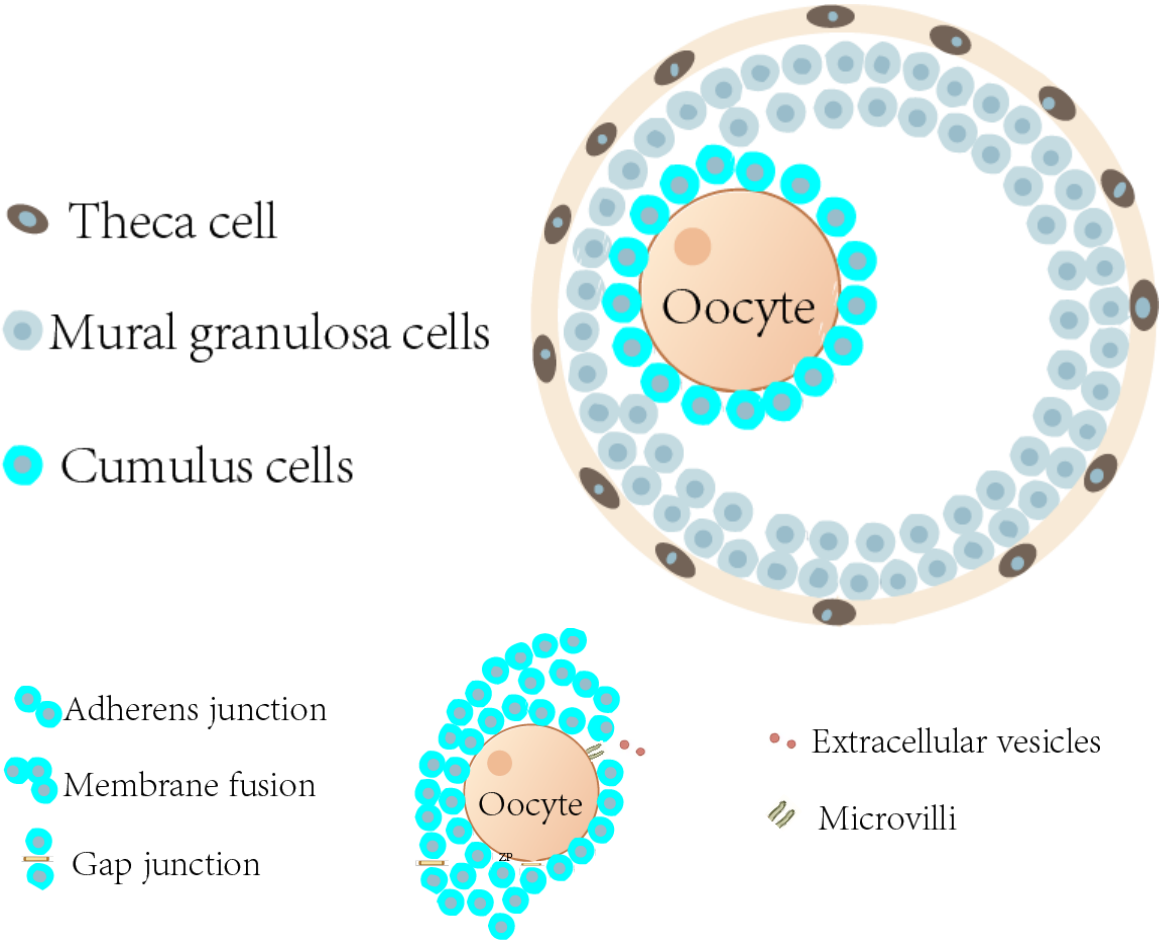

Figure I. Structural organization and intercellular communication within the human cumulus–oocyte complex (COC). The oocyte is enclosed by cumulus and mural granulosa cells connected by transzonal projections and junctional complexes (gap, tight, and adherens junctions). These contacts mediate bidirectional transfer of metabolites and signals essential for meiotic regulation.

Beyond transmitting signals, cumulus cells regulate the oocyte's microenvironment through complex metabolic cooperation. The oocyte itself has limited ability to utilize certain substrates—particularly glucose—due to low phosphofructokinase activity. Cumulus cells metabolize glucose via glycolysis and the pentose phosphate pathway to produce pyruvate and other intermediates, which are then transferred to the oocyte through gap junctions and paracrine routes. These substrates fuel mitochondrial oxidative phosphorylation, ATP generation, and biosynthetic processes essential for organelle reorganization during maturation. Similarly, cumulus cells participate in lipid metabolism, amino-acid exchange, and redox homeostasis, ensuring that the oocyte's energy and signaling requirements are met dynamically as it transitions from quiescence to activity.

The bidirectional nature of oocyte–cumulus communication is a hallmark of follicular physiology. While cumulus cells nurture the oocyte metabolically, the oocyte in turn releases paracrine factors that guide cumulus cell differentiation and function. Two members of the transforming growth factor β (TGF-β) superfamily—growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15)—are particularly well characterized in this regard. These oocyte-secreted factors modulate the transcriptional programs of cumulus cells, stimulating glycolytic enzymes, promoting extracellular matrix production, and regulating the expression of hyaluronan synthase and prostaglandin-endoperoxide synthase 2 (PTGS2), all of which are essential for cumulus expansion. Disruption of either GDF9 or BMP15 signaling, as shown in several animal models, results in abnormal cumulus differentiation, failed cumulus expansion, and compromised oocyte maturation. Thus, the oocyte–cumulus unit operates as a highly integrated system in which reciprocal signaling ensures the synchronization of metabolic and developmental events[32,33].

Morphologically, the COC undergoes striking changes during the peri-ovulatory period. Under the influence of LH and EGF-like factors, cumulus cells secrete a hyaluronan-rich extracellular matrix that drives cumulus expansion. This process is more than a passive swelling; it represents a functional remodeling of intercellular architecture that facilitates oocyte release and optimizes interactions with spermatozoa after ovulation. Expansion depends on coordinated upregulation of several genes, including HAS2, PTX3, TNFAIP6, and TSG6, whose expression is under the combined control of FSH, LH, and oocyte-derived signals. In the in vitro environment, cumulus expansion is often used as a morphological indicator of cumulus health and responsiveness. Its extent and pattern reflect not only the activation of signaling pathways but also the metabolic and oxidative state of the cells involved.

The metabolic interplay between the oocyte and its cumulus cells also extends to the regulation of oxidative stress. Reactive oxygen species (ROS) are continuously generated as by-products of cellular metabolism, particularly in mitochondria. At physiological concentrations, ROS serve as signaling molecules that modulate kinase pathways, transcription factors, and even meiotic resumption. However, excessive ROS accumulation leads to oxidative damage, lipid peroxidation, and mitochondrial dysfunction, all of which are detrimental to oocyte competence. Cumulus cells act as the primary antioxidant barrier by expressing scavenging enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, as well as by maintaining intracellular pools of reduced glutathione (GSH). The oocyte itself has

relatively limited antioxidant capacity, relying on these somatic defenses to maintain redox balance. Disruption of cumulus–oocyte communication, whether by premature denudation or suboptimal culture conditions, compromises this protective buffering system and predisposes the oocyte to oxidative injury.

The interplay between energy metabolism and redox control is further exemplified by the role of AMP-activated protein kinase (AMPK). AMPK functions as a cellular energy sensor that responds to fluctuations in the AMP/ATP ratio and to oxidative stress by promoting catabolic processes that restore energy homeostasis. In the context of oocyte maturation, AMPK activation has been associated with improved mitochondrial function, enhanced ATP production, and increased resilience against oxidative stress. Experimental activation of AMPK in oocytes or cumulus cells has been shown to stimulate meiotic resumption in several mammalian species. Conversely, inhibition of AMPK impairs gap-junctional communication and delays GVBD, suggesting that AMPK activity within the COC contributes to the coordination of metabolic and developmental signals. The cumulus compartment, being more metabolically active and responsive to environmental stimuli, may act as a relay through which AMPK-mediated signaling indirectly influences the oocyte's fate.

Recent transcriptomic and proteomic studies have revealed that the COC is not a static entity but a dynamic signaling hub. Single-cell RNA sequencing of human and animal follicles has identified distinct cumulus subpopulations specialized in metabolism, extracellular matrix organization, and redox regulation. These functional domains are spatially distributed around the oocyte, indicating that the somatic microenvironment is spatially as well as biochemically heterogeneous. Communication between these domains and the oocyte ensures the fine-tuning of nutrient delivery, ROS scavenging, and hormonal responsiveness. Such insights underscore the complexity of reproducing the in vivo niche in vitro, where physical and biochemical gradients are inevitably simplified.

In the clinical laboratory, these physiological principles translate into several operational considerations for IVM. Maintaining the COC intact during early culture stages preserves gap-junctional communication and allows the natural exchange of metabolites and signaling molecules to continue. Denudation, while necessary for precise assessment of nuclear status or ICSI, should be timed to minimize the loss of these interactions. The choice of medium, oxygen concentration, and supplementation with factors such as FSH, EGF-like peptides, or

antioxidants are all aimed at mimicking aspects of the native follicular milieu. Yet, despite these efforts, current in vitro systems often fall short of replicating the spatial and temporal complexity of the in vivo follicle. Consequently, oocytes matured in isolation tend to exhibit reduced cytoplasmic maturity, aberrant spindle morphology, and lower developmental potential compared with those matured within an intact COC.

The cumulus–oocyte complex therefore represents more than a structural association; it is a functional ecosystem in which biochemical, mechanical, and redox signals converge to determine oocyte quality. The disruption of this ecosystem—whether through denudation, oxidative imbalance, or metabolic insufficiency—can decouple nuclear and cytoplasmic maturation, resulting in oocytes that appear mature morphologically but are functionally incompetent. Understanding the physiology of this partnership provides the conceptual foundation for improving IVM. By respecting and reproducing the essential features of the COC—its communication channels, metabolic interdependence, and adaptive redox regulation—researchers can move closer to achieving maturation outcomes that match the efficiency and developmental integrity of the natural process.

2.3 The Challenge of Oxidative Stress in IVM

One of the most critical challenges in establishing efficient in-vitro maturation (IVM) systems lies in maintaining redox equilibrium within an environment that inherently differs from the physiological follicular niche. In vivo, the developing oocyte resides within a relatively hypoxic and biochemically buffered microenvironment, where the coordinated activity of granulosa and cumulus cells tightly regulates oxygen gradients, nutrient flow, and antioxidant protection. The follicular fluid contains a complex antioxidant network—including glutathione, superoxide dismutase (SOD), catalase, and non-enzymatic scavengers such as vitamins C and E—that counterbalances the reactive oxygen species (ROS) produced as natural by-products of aerobic metabolism. These subtle regulatory mechanisms ensure that ROS remain within a physiological range, allowing them to act as signalling mediators without causing cellular injury.

During IVM, this delicate balance is easily disturbed. Laboratory culture typically exposes oocytes and their surrounding somatic cells to atmospheric oxygen levels of about 20%, which far exceed the 2–8% oxygen concentration measured in human preovulatory follicles[34]. Elevated oxygen tension accelerates electron leakage from the mitochondrial respiratory chain, increasing the formation of superoxide radicals and hydrogen peroxide. Additional ROS arise

from the spontaneous oxidation of culture-medium components, trace-metal catalysis, temperature fluctuations, and even illumination during microscopic inspection. Together, these sources create an oxidative environment that is foreign to the oocyte's physiology. Without the fine-tuned feedback of in-folliculo metabolism, the cumulative oxidative burden during culture can surpass the limited antioxidant capacity of the gamete and its companion cells.

The biological consequences of excessive ROS are multifaceted. At low levels, ROS act as essential secondary messengers that participate in cumulus expansion and trigger germinal-vesicle breakdown (GVBD). However, beyond a threshold, oxidative stress induces molecular lesions that compromise cellular structure and function. Lipid peroxidation alters membrane integrity and mitochondrial cristae morphology; protein carbonylation and thiol oxidation disrupt cytoskeletal elements and enzymes critical for spindle assembly; and oxidative DNA damage, particularly the formation of 8-oxoguanine, interferes with replication and chromosomal segregation[35]. These subcellular injuries manifest as spindle disorganization, abnormal chromosome alignment, and fragmentation of mitochondrial networks—all detrimental to the oocyte's developmental competence. Furthermore, persistent oxidative imbalance disturbs kinase signalling cascades, including mitogen-activated protein kinase (MAPK) and cyclin-dependent kinase 1 (CDK1), desynchronizing the progression of nuclear and cytoplasmic maturation.

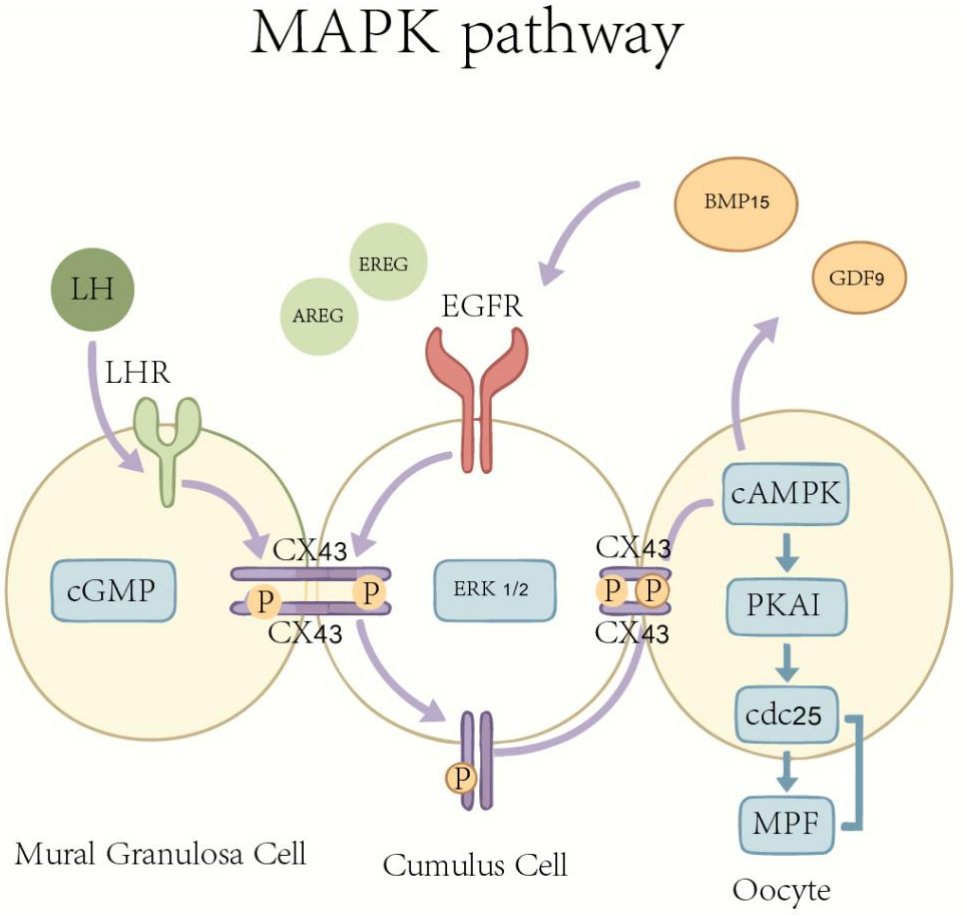

Figure II. Signaling network coordinating cumulus–oocyte communication and meiotic resumption under physiological conditions. LH stimulation of mural granulosa cells activates the MAPK pathway, inducing connexin-43 (Cx43) phosphorylation in cumulus cells and closure of gap junctions. This cascade reduces oocyte cAMP levels, activates maturation-promoting factor (MPF), and triggers germinal-vesicle breakdown (GVBD). Oocyte-secreted factors such as GDF9 and BMP15 reciprocally regulate cumulus cell metabolism, ensuring synchronized maturation within the cumulus–oocyte complex (COC).

Cumulus cells function as the oocyte's first line of defence against such oxidative insults. Through enzymatic and non-enzymatic systems, they neutralize ROS and recycle oxidized antioxidants. The pentose-phosphate pathway within cumulus cells generates NADPH, which fuels the reduction of glutathione disulphide (GSSG) back to its active form, reduced glutathione (GSH). This mechanism sustains high intracellular GSH levels that in turn protect the oocyte by diffusion or through gap-junctional exchange. Cumulus cells also convert glucose to pyruvate and lactate, thereby modulating the oocyte's substrate preference and reducing mitochondrial overload—an indirect yet crucial means of lowering ROS production at source. When the cumulus layer is stripped away by denudation, this protective system collapses. The oocyte, possessing relatively few antioxidant enzymes, must cope alone in a hyperoxic medium. Empirically, such conditions have been associated with elevated

intracellular ROS, abnormal spindle morphology, and reduced rates of GVBD and subsequent fertilization[36].

Recent human and animal studies provide quantitative evidence for these phenomena. Von Mengden et al. (2020) analysed redox profiles in human cumulus cells and demonstrated a positive correlation between antioxidant capacity and embryo developmental success. Their synthesis proposed that cumulus redox markers could serve as functional indicators of oocyte quality and as practical quality-control tools for ART laboratories. Morimoto et al. (2024) extended these observations by linking metabolic dysregulation—particularly in obesity and advanced maternal age—to excessive ROS generation and impaired cumulus–oocyte communication[37]. These findings underscore that oxidative stress during IVM is not merely an artefact of culture but a continuation of physiological stressors amplified under in-vitro conditions. Managing oxidative load is therefore both a biological and a technical imperative.

Efforts to mitigate oxidative stress in IVM have pursued several complementary strategies. Lowering oxygen tension to 5% more closely mimics intrafollicular conditions and can reduce ROS formation, although outcomes depend on the specific media and pre-IVM protocols employed[38]. Supplementation with antioxidants such as melatonin, resveratrol, and vitamin E has been investigated to restore redox balance, with variable success. While these compounds can scavenge free radicals and up-regulate endogenous defences, excessive supplementation may inadvertently suppress the physiological ROS signalling required for GVBD. A structurally more robust approach involves preserving the cumulus–oocyte complex intact during the early culture phase or re-establishing somatic support through granulosa-like co-culture systems. Such strategies maintain the natural antioxidant buffer and help preserve the dynamic equilibrium between ROS production and removal.

Ultimately, the challenge of oxidative stress in IVM lies in achieving—not eliminating—oxidation. The goal is to sustain a controlled, signalling-permissive redox state that promotes maturation without inflicting damage. Because this balance depends heavily on the integrity and metabolic competence of cumulus cells, understanding and preserving the somatic microenvironment is indispensable for successful human IVM. The cumulative literature suggests that protecting the oocyte from oxidative excess through somatic buffering, oxygen optimization, and targeted antioxidant strategies can substantially enhance its developmental

potential. In this context, oxidative stress represents both a measurable biomarker of culture quality and a mechanistic target for improving the physiological authenticity of IVM systems.

2.4 AMPK Signaling: Energy Sensor and Stress Regulator

AMP-activated protein kinase (AMPK) is a conserved serine/threonine kinase that couples cellular energy status to adaptive metabolic and stress-response programmes. Functionally, AMPK senses the adenylate charge through cooperative binding of AMP/ADP versus ATP and is activated by phosphorylation at Thr172 on the catalytic α-subunit by upstream kinases, principally LKB1 and CaMKKβ[39]. Once engaged, AMPK restrains ATP-consuming anabolic pathways while promoting ATP-generating catabolic routes, orchestrating changes that encompass glucose uptake and glycolysis, fatty-acid oxidation via inhibitory phosphorylation of acetyl-CoA carboxylase (ACC), autophagy initiation through ULK1, and mitochondrial biogenesis in concert with sirtuin-PGC-1α signalling[40]. In parallel, AMPK attenuates mTORC1 activity and interfaces with redox control, positioning it as a nodal integrator of metabolism, proteostasis, and oxidative stress[41].

Within the ovarian follicle, AMPK's relevance extends beyond generic bioenergetics to the specific choreography of oocyte maturation and cumulus support. Cumulus cells are metabolically active guardians of the oocyte's microenvironment; their ability to buffer reactive oxygen species (ROS), channel substrates, and sustain gap-junctional communication depends on flexible energy management. AMPK activation in cumulus cells enhances glycolytic flux and fatty-acid oxidation, stabilizes mitochondrial function, and can up-regulate antioxidant capacity—each of which reduces electron leak and basal ROS generation[42]. By modulating connexin trafficking and cytoskeletal dynamics, AMPK has also been implicated in maintaining gap-junctional intercellular communication (GJIC), a prerequisite for the exchange of cAMP/cGMP and metabolites that restrain or release meiotic arrest[43]. These actions collectively support a microenvironment in which meiotic resumption proceeds with preserved synchrony between nuclear and cytoplasmic events.

Evidence from mammalian models indicates that AMPK activity within the oocyte itself influences meiotic timing and spindle integrity. Pharmacologic or genetic enhancement of AMPK signalling has been associated with more timely germinal-vesicle breakdown (GVBD), improved spindle morphology, and stabilized mitochondrial membrane potential (ΔΨm), whereas inhibition delays meiotic resumption and increases spindle abnormalities[44]. Although

species-specific differences exist, convergent data suggest that AMPK balances the energetic cost of chromatin remodelling, spindle assembly, and organelle redistribution with the need to prevent redox-induced injury during the brief but demanding transition from GV arrest to MII[45]. Importantly, several of these effects appear to be indirect consequences of improved cumulus support: when somatic buffering is intact, the oocyte experiences lower oxidative burden and more predictable substrate delivery, reducing the likelihood that energy shortfalls or ROS spikes derail maturation.

The AMPK axis is particularly salient in clinical contexts where metabolic stress is prevalent, notably polycystic ovary syndrome (PCOS). PCOS features insulin resistance, altered lipid handling, and low-grade inflammation that reshape follicular metabolism and redox tone[46]. In such settings, cumulus cells may enter culture with a pre-existing energetic and oxidative mismatch. Adjusting AMPK tone—either physiologically through co-culture with healthy somatic partners or experimentally via metabolic cues—could improve resilience, sustain GJIC, and help restore a milieu permissive for GVBD. Translational observations that metformin exposure modulates granulosa/cumulus metabolic pathways and lowers oxidative indices align with this view, although direct causal links to human IVM efficiency remain to be definitively established and likely depend on timing, dosage, and the integrity of the cumulus–oocyte complex[47].

Mechanistically, AMPK's integration with redox biology provides a plausible bridge between oxidative stress management and maturation outcomes described in the preceding section. Oxidative stress can activate AMPK via rises in AMP/ADP and through CaMKKβ-mediated calcium signals; in turn, AMPK activation promotes mitophagy and mitochondrial quality control, increases NAD+ availability and SIRT1 activity, and enhances transcriptional programmes that expand antioxidant capacity. This bidirectional relationship predicts a virtuous cycle when cumulus cells are preserved: lower ROS facilitates efficient ATP production and AMPK-guided metabolic routing; AMPK then further constrains ROS by improving mitochondrial coupling and antioxidant defences. Conversely, denudation removes the somatic substrate and redox governance, making AMPK-mediated adaptation more difficult and increasing the probability that maturation proceeds under energetically constrained, oxidizing conditions.

From an IVM-design perspective, these considerations argue for a “structure-first, signalling-aware” strategy. Maintaining or reconstructing somatic support provides the architecture in which AMPK can operate productively; only thereafter should targeted modulation—oxygen tension, substrate composition, or carefully chosen adjuncts—be contemplated to fine-tune energy and stress responses. This hierarchy helps reconcile inconsistent reports about universal benefits of antioxidants or AMPK-modulating agents: in the absence of the cumulus micro-niche, pharmacologic interventions may have muted or paradoxical effects because the spatial and temporal organization of signalling is missing. It also clarifies why biphasic approaches (e.g., CAPA-IVM) that stage meiotic resumption while preserving COC integrity often outperform single-phase protocols in cohorts prone to metabolic stress.

In practical terms, assessing AMPK biology in human material requires methodological humility. Total AMPKα1 abundance—assayed by immunocytochemistry (ICC)—is an informative, but indirect, proxy for pathway engagement. True kinase activity is more closely reflected by Thr172 phosphorylation and by downstream substrate phosphorylation (e.g., p-ACC); however, ICC for total protein can still reveal biologically coherent differences when acquisition parameters are strictly standardized and negative controls define background. Interpreting elevated AMPK signal in cumulus cells co-cultured with oocytes as part of a convergent pattern—coincident lower ROS, healthier morphology, and improved GVBD—respects these limitations while leveraging the strength of multi-endpoint alignment. In small human datasets, such triangulation often yields more robust inference than any single biomarker alone.

Finally, AMPK’s crosstalk with sirtuins and mTOR positions it as a systems-level coordinator of the maturation microenvironment. By promoting PGC-1α-dependent mitochondrial biogenesis and restraining mTORC1-driven anabolism during stress, AMPK helps match biosynthetic demand to energetic capacity at precisely the moment the oocyte’s requirements surge. In cumulus cells, the same logic preserves extracellular-matrix production and hyaluronan-rich expansion without collapsing ATP reserves, thereby maintaining the structural and paracrine conditions that support oocyte competence. Conceived this way, AMPK is not a narrow “switch” but a scaffold upon which successful IVM can be built: its activity reflects, and helps enforce, a state of metabolic poise and oxidative restraint that allows meiosis to resume on time with minimal collateral damage.

In summary, AMPK operates at the intersection of energy management, redox control, and intercellular communication within the cumulus–oocyte unit. Its activation supports a low-ROS, high-resilience microenvironment in which meiotic resumption is more likely to succeed. Because AMPK's beneficial effects depend on the presence and health of the somatic compartment, preserving the cumulus architecture is a logical first step in IVM optimization; targeted modulation of AMPK-linked pathways is best viewed as a secondary, context-dependent lever. This framework motivates the present study's combined use of cumulus ROS quantification and AMPK immunofluorescence, analysed alongside GVBD outcomes under co-culture versus denudation, to ground protocol design in measurable cell biology.

2.5 Advances in In-vitro Maturation Technologies

Over the past decade, human IVM has shifted from a single-phase, oocyte-centric procedure toward a microenvironment-engineered platform that stages meiosis, preserves somatic support, and tunes redox–metabolic conditions with increasing precision[48]. This evolution reflects a broad consensus that culture design must emulate the follicular niche rather than simply provide permissive media. Contemporary innovations span biochemical staging of meiotic arrest and resumption, structural preservation or reconstruction of the cumulus–oocyte complex (COC), control of oxygen tension and substrates, targeted antioxidant strategies, and the emergence of engineered ovarian support systems. Together, these developments have narrowed the performance gap to conventional IVF in selected cohorts while clarifying protocol dependencies that previously produced inconsistent results[49,50].

A central breakthrough has been the adoption of biphasic or "capacitation-IVM" (CAPA-IVM) concepts. In these protocols, a brief pre-IVM period maintains nuclear arrest ex vivo—most commonly using C-type natriuretic peptide (CNP) to sustain cGMP in cumulus via NPR2, thereby inhibiting oocyte PDE3A and preserving intra-oocyte cAMP—followed by an IVM phase in which EGF-like ligands (e.g., amphiregulin, epiregulin) trigger meiotic resumption in a manner that better recapitulates the LH cascade. The practical consequence is improved temporal coupling between nuclear and cytoplasmic maturation: the oocyte has time to realign organelle, redox, and mRNA translation programmes before GVBD is permitted, and the cumulus layer remains functionally competent to buffer oxidative and metabolic stress during the transition. Pre-IVM "holding" media that modulate cAMP/cGMP with phosphodiesterase inhibitors (e.g., cilostamide, milrinone) or natriuretic peptides have produced similar

synchronizing effects, although timing windows are narrow and excessive arrest prolongation can blunt responsiveness to EGF-like signals[51,52].

Parallel attention to the physical state of the COC has yielded practical guidance that now underlies many laboratories' standard operating procedures. Excessive or early denudation deprives the oocyte of somatic buffering and perturbs gap-junctional exchange precisely when ROS and energetic demand rise; conversely, delaying complete denudation until after the pre-IVM window preserves intercellular cAMP/cGMP trafficking, substrate governance, and antioxidant protection. Even seemingly small details—enzyme concentration and exposure time during hyaluronidase treatment, shear stress during pipetting, and the geometry of culture drops—have measurable consequences for cumulus integrity and, by extension, maturation probability. In this sense, "mechanics are biology": minimizing mechanical and enzymatic insults functions as an upstream antioxidant and energy-sparing intervention.

Control of the gaseous and nutritional milieu has also matured beyond rule-of-thumb heuristics. Lowering oxygen tension toward physiologic levels ($\approx 5\%$ O_2) can reduce mitochondrial electron leak and ROS generation, but benefits are protocol-dependent and interlock with pre-IVM staging, media composition, and the health of the somatic compartment. Reports that low oxygen improves maturation and embryo development coexist with studies in which 5% O_2 underperforms 20% O_2 within specific biphasic designs—likely reflecting differences in carbon source balance, lipid availability, and cumulus functionality. These observations have reframed oxygen as a tunable variable rather than a universal constant: when robust somatic support is present, oxygen becomes a secondary fine-tuning knob; when support is weak (e.g., early denudation), oxygen exerts larger effects because the oocyte confronts oxidative load more directly. Substrate tailoring follows the same logic. Media that prioritize pyruvate and lactate supply—aligned with the oocyte's substrate preference—and that provide pentose-phosphate pathway capacity in cumulus can simultaneously stabilize ATP production and maintain glutathione recycling, thereby lowering basal ROS at its source.

Pharmacologic antioxidation remains attractive but is now applied with greater restraint. Agents such as melatonin, resveratrol, N-acetylcysteine, and coenzyme Q10 can lower reporter signals of oxidative stress and induce endogenous defences; nonetheless, indiscriminate ROS suppression risks blunting physiological oxidant pulses that participate in cumulus expansion and GVBD timing. The emerging consensus is "structure-first, adjunct-

second”: retain or reconstruct somatic protection, then deploy antioxidants in dose-timed regimens that preserve signalling while curbing sustained oxidative load. This stance is reinforced by meta-analyses showing heterogeneous clinical benefits that appear contingent on patient selection (e.g., PCOS, advanced age), dosing, and co-interventions rather than on antioxidants per se.

A particularly fast-moving frontier is the reconstruction of somatic support using engineered ovarian support cells (OSCs). Several groups have derived granulosa-like cells from pluripotent or adult sources that secrete EGF-like factors, steroids, and metabolites, partially restoring the paracrine and metabolic functions of natural cumulus when COCs are unavailable or insufficient. Early translational reports describe improved maturation to MII and encouraging blastocyst quality metrics in selected human cohorts, with xeno-free and GMP-inclined manufacturing beginning to address implementation and safety barriers. While long-term offspring data remain limited, initial safety signals are reassuring; critically, OSC approaches mechanistically align with the thesis that microenvironment, not merely media formulation, determines oocyte readiness. In the same vein, 3D culture matrices—hyaluronan-rich hydrogels, collagen, fibrin, or alginate systems—have been used to preserve COC architecture, maintain diffusion gradients, and dampen mechanical stress. Microfluidic platforms add flow-controlled delivery of oxygen and substrates, enabling more physiological gradients and reducing accumulation of reactive by-products that occur in static drops. Although these technologies are not yet routine, they illustrate the trajectory toward niche engineering where geometry, flow, and paracrine signalling are jointly specified.

Methodological advances are also reshaping quality control (QC). Time-lapse imaging is being repurposed from embryo assessment to oocyte/COC monitoring, capturing morphokinetic correlates of cumulus expansion and cytoplasmic changes without repeated light exposure. Simple, portable biochemical readouts—plate-internal normalization of ROS with defined H_2O_2 references, standardized immunofluorescence pipelines for stress/energy markers such as AMPK or p-ACC, and calibration-locked imaging settings—allow small human datasets to produce reproducible, cross-site signals. Single-cell and spatial transcriptomics of cumulus and mural granulosa have identified subpopulations specialized for glycolysis, extracellular-matrix organization, and antioxidant defence, offering candidate biomarkers that could stratify oocyte support capacity before culture begins. Integrating these tools shifts maturation from a black-box outcome to a monitored process with actionable intermediate checkpoints.

Clinically, these innovations have coalesced into pragmatic workflows. Many centres now preserve COCs through a short pre-IVM hold (CNP±PDE inhibitor), initiate IVM with EGF-like ligands, delay complete denudation until after resumption signals are engaged, and operate under oxygen and substrate conditions tuned to their media and patient mix. Where feasible, laboratories add co-culture with autologous cumulus or validated OSCs to reinforce somatic buffering. Adjuncts such as melatonin are layered cautiously, typically within defined time windows. Importantly, diagnosis-specific tailoring has emerged: PCOS and advanced-age oocytes, which enter culture with metabolic and redox liabilities, appear to benefit most from microenvironment-first designs, whereas low-reserve cohorts may require different staging and substrate strategies. Regulatory considerations—xeno-free components, traceability, and documentation of long-term outcomes—are increasingly integrated from the outset to support clinical translation.

Taken together, the state of the art in human IVM supports a unifying principle: maturation success depends on engineering a somatic-supported, signalling-permissive, low-noise microenvironment. Biphasic staging aligns nuclear timing with cytoplasmic readiness; preservation or reconstruction of the COC supplies antioxidant and metabolic governance; oxygen and substrates are tuned to minimize electron leak while sustaining ATP; and targeted adjuncts are reserved for closing residual gaps without erasing necessary signals. As these elements are combined, the field is moving from empirical recipes toward mechanism-anchored protocols with embedded QC. This conceptual consolidation provides the rationale for the present work's focus on the cumulus microenvironment—quantifying ROS and AMPK alongside GVBD under co-culture versus denudation—to connect modern IVM design principles with measurable biology and, ultimately, with clinically meaningful endpoints.

2.6 Rationale and Research Gap

Despite decades of methodological refinement, human in-vitro maturation (IVM) remains variably effective across centres and patient groups. A recurrent theme in this variability is the limited attention paid to the somatic microenvironment that the oocyte experiences during culture. Many studies enumerate nuclear endpoints—germinal vesicle breakdown (GVBD) or attainment of metaphase II (MII)—without concurrently characterizing the biochemical state of the surrounding cumulus cells that, in vivo, govern redox balance, metabolic routing, and intercellular signalling. As a result, protocol optimization often proceeds by trial and error rather

than by feedback from mechanistically informative biomarkers. A rationale for the present work is that linking nuclear maturation to quantitative readouts of the cumulus milieu can convert IVM from an outcome-only procedure into a monitored, tunable process[53,54].

Several specific gaps motivate a focused human study. First, there is a paucity of datasets that directly compare cumulus-enclosed versus denuded GV oocytes under matched laboratory conditions while simultaneously quantifying cumulus oxidative status. Although it is widely appreciated that culture under ambient oxygen and repeated handling elevates reactive oxygen species (ROS), few reports measure intracellular ROS in cumulus cells and relate those measurements to the oocyte's meiotic trajectory within the same experiment. This leaves unresolved whether the presence of cumulus cells merely correlates with, or actively establishes, a lower-ROS state that is permissive for timely GVBD[55,56].

Second, energy sensing—particularly AMP-activated protein kinase (AMPK) signalling—has been implicated in cumulus resilience and oocyte competence in animal models, yet human IVM studies rarely include any index of AMPK biology. Where AMPK is mentioned, measurements often rely on heterogeneous platforms or are separated from functional readouts by differences in specimen, timing, or acquisition settings, making cross-study synthesis tenuous. A practical gap therefore exists for standardized imaging pipelines that can report AMPK signal in primary human cumulus cells with sufficient consistency to support between-group comparisons within small clinical datasets[57].

Third, literature heterogeneity obscures causal interpretation. Reports differ in the timing and extent of denudation, oxygen tension, media formulation, and handling mechanics; many lack plate-internal controls that would normalize fluorescent readouts across runs. These differences plausibly explain why some studies find modest or no advantage to preserving the cumulus–oocyte complex (COC), while others report sizeable gains. Without harmonized acquisition and normalization (for example, in-plate H_2O_2 references for ROS assays and matched microscope settings for immunocytochemistry), real biological effects can be confounded by technical variability. Addressing this gap requires simple, portable quality-control elements built into the assay design[58].

Fourth, contemporary IVM innovations—including biphasic CAPA-IVM, oxygen tuning, and engineered ovarian support cells—derive their rationale from microenvironment engineering,

yet there is little human evidence connecting these macro-level design choices to concrete somatic biomarkers measured during the same culture. If cumulus integrity truly lowers oxidative load and engages adaptive energy signalling, this should be experimentally observable as a “low-ROS / high-AMPK” phenotype that co-occurs with improved nuclear outcomes. Demonstrating such convergence would supply the missing mechanistic bridge between protocol components and maturation success[59].

Finally, the clinical case for microenvironment-aware IVM is strongest in diagnoses characterized by metabolic and redox stress, such as polycystic ovary syndrome (PCOS), obesity, and advanced maternal age. Yet most human studies pool heterogeneous indications or under-report diagnostic strata, limiting the ability to infer who stands to benefit most from somatic preservation. Even when sample sizes are modest—as is inevitable in carefully phenotyped human experiments—designs that align endpoints across the same specimens can yield interpretable signals that generalize to protocol decisions in these high-need cohorts[60].

In view of these gaps, the present thesis adopts a deliberately simple but mechanistically anchored approach: compare human GV-stage oocytes cultured with versus without cumulus cells under standardized conditions; quantify intracellular ROS in matched cumulus populations using a plate-internal normalization scheme; assess AMPK signal by acquisition-matched immunocytochemistry with negative controls; and relate these somatic readouts to GVBD at 48 hours. By triangulating nuclear outcomes with redox and energy-sensing markers, the study aims to determine whether preserving the COC establishes a measurably different microenvironment that plausibly drives maturation. This rationale sets the stage for the next chapter, which states the aims and hypotheses explicitly and links them to the methods used to generate comparable, quality-controlled data[61].

2.7 AIM

This thesis investigates whether preserving the cumulus–oocyte complex (COC) during culture improves meiotic resumption of human germinal-vesicle (GV) oocytes and how somatic microenvironmental features relate to this outcome.

Primary aim.

Compare the 48-hour germinal-vesicle breakdown (GVBD) rate between denuded oocytes and oocytes co-cultured with primary cumulus cells under matched laboratory conditions.

Secondary aims.

1) Quantify intracellular reactive oxygen species (ROS) in cumulus cells with and without oocyte co-culture using a standardized H_2DCFDA assay with in-plate normalization.
2) Assess AMPKα1 immunofluorescence in cumulus cells (acquisition-matched; no-primary negative control) as a surrogate of energy-sensing/stress response.
3) Describe cumulus morphology (expansion, cell–cell continuity) at predefined time points.

Working hypotheses.

H1: Cumulus co-culture yields a higher GVBD rate than denudation alone.

H2: Co-culture is associated with lower cumulus ROS.

H3: Co-culture is associated with higher cumulus AMPK signal.

H4: A “low-ROS/high-AMPK” cumulus phenotype aligns with higher GVBD.

Design overview.

Human GV oocytes are allocated at the oocyte level to two culture arms with identical media, gas, handling, and imaging settings. GVBD is compared by Fisher’s exact test; ROS by one-way ANOVA with plate-internal reference; AMPK by two-group testing on ROI-based intensities. Analyses are blinded and emphasize convergence across endpoints.

3. Materials and Methods

3.1 Materials and major equipment

3.1.1. Clinical specimens / cells

The immature GV-stage oocytes and cumulus cells used in this study were obtained from patients participating in ovarian tissue cryopreservation at the Women's Clinic of the University of Cologne, North Rhine-Westphalia, Germany. Period: August 2024 – June 2025. All participants provided informed consent prior to the procedure. Donor data recorded: age (years), BMI (kg/m²), AMH (ng/mL), baseline FSH (IU/L), antral follicle count (AFC), stimulation protocol.

3.1.2. Media, reagents and antibodies

Reagent	Manufacturer
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12)	Gibco
Fetal Bovine Serum (FBS)	Gibco
Penicillin-Streptomycin	Gibco
Hyaluronidase	Sigma Aldrich
2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA)	Sigma Aldrich
Hydrogen Peroxide (H_2O_2)	Sigma Aldrich
Paraformaldehyde	Sigma Aldrich
Triton X-100	Sigma Aldrich
Bovine Serum Albumin (BSA)	Sigma Aldrich
Anti-AMPK alpha 1 antibody [Y365]	Abcam
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 350	Thermo Fisher Scientific

3.1.3. Equipment

Apparatus	Manufacturer
Biosafety cabinet	Thermo Fisher Scientific

Apparatus	Manufacturer
Laminar flow clean bench	Heraeus
CO_2 Incubator	Thermo Fisher Scientific
Centrifuge	Eppendorf
Inverted microscope	Nikon
Confocal laser scanning microscope (LSM 700)	Zeiss
Sterile 10 mm culture dishes / 4-well plate	Sigma Aldrich
Sterile specimen container	Sigma Aldrich
Denudation pipettes	Vitromed GmbH
Analytical balance	Ohaus Corporation

3.2 Methods

3.2.1. Oocyte and cumulus-cell collection after ovarian puncture

Under transvaginal ultrasound guidance, a 17-gauge needle was used to aspirate follicular fluid (FF) from visible follicles. FF was transferred to sterile conical tubes and processed in a Class II biosafety cabinet. Each sample was labeled with a coded ID to ensure de-identification. For descriptive baseline comparability, age (years), FSH (IU/L), and LH (IU/L) were recorded for donors whose oocytes entered the culture experiments. Allocation to experimental arms occurred at the oocyte level; there were no significant differences in these variables between oocytes assigned to Oocyte-Only Culture and Cumulus–Oocyte co-culture (t-test, $P>0.05$). Cumulus–oocyte complexes (COCs) were identified under a stereomicroscope and transferred to DMEM/F-12 supplemented with 10% FBS and 1% penicillin–streptomycin (Gibco, Thermo Fisher). Cultures were maintained at 37 °C in a humidified incubator at 5% CO_2 in ambient O_2 (~20%).

3.2.2. Isolation of Cumulus Cells and GV-stage Oocytes

Following ovarian puncture and initial handling, FF was centrifuged at 1000 rpm for 10 min (room temperature) to pellet cells. The supernatant was discarded and the pellet gently

resuspended. COCs were confirmed under a stereomicroscope and placed into DMEM/F-12 + 10% FBS + 1% penicillin–streptomycin at 37 °C, 5% CO_2 (± 5% O_2, as above).
After 24 h of equilibration, the outer cumulus layers typically exhibited visible expansion. Denudation was then performed in gamete buffer containing hyaluronidase 80 IU/mL for 30–60 s, followed by gentle, repeated pipetting with denudation tips of increasing inner diameters until granulosa/cumulus cells were removed. This procedure yielded two fractions: (i) denuded oocytes for subsequent culture and nuclear-status assessment, and (ii) primary cumulus cells (CCs) for downstream assays.

Nuclear stage was determined under an inverted microscope. Oocytes with a clearly visible germinal vesicle were classified as GV (no GV breakdown). Oocytes without a visible GV were recorded as GVBD (i.e., progression to MI). All subsequent culture steps and assessments used these isolated fractions under identical environmental conditions unless otherwise specified.

3.2.3. In Vitro Maturation of Immature Oocytes

Immature oocytes, defined as germinal-vesicle (GV) or metaphase-I (MI) at baseline, were cultured in DMEM/F-12 supplemented with 10% FBS and 1% penicillin–streptomycin (Gibco, Thermo Fisher) at 37 °C in a humidified incubator with 5% CO_2 in ambient O_2 (~20%). Oocytes were randomly assigned at the oocyte level to one of two conditions under otherwise identical settings:

(i) only oocytes culture: immature oocytes cultured without cumulus cells.

(ii) oocytes with cumulus: immature oocytes co-cultured with primary cumulus cells, isolated from the same follicular material whenever available.

Cultureware, drop volumes/plate format, media lot numbers, and gas settings were kept identical between conditions. Handling time outside the incubator was minimized and matched. After 48 h of culture, nuclear status was assessed on an inverted microscope by an assessor blinded to allocation. Oocytes with a clearly visible germinal vesicle (GV) were recorded as GV (no GVBD); oocytes without a visible GV were recorded as GVBD. The primary endpoint was the proportion of GVBD at 48 h in Oocyte-Only Culture versus Oocyte–Cumulus Co-culture. Given the total sample size (N = 23), Fisher's exact test (two-sided, $\alpha = 0.05$) was used for

between-group comparison, and results are reported with odds ratios and 95% confidence intervals.

3.2.4. Morphological Observation of Cumulus Cells

The morphological appearance of cumulus–oocyte complexes (COCs) and the associated cumulus cells was examined during in-vitro culture. Observations were carried out under the same environmental conditions used for maturation experiments (37 °C, humidified incubator, 5% CO_2 in ambient O_2 ~20%) to ensure consistency. Imaging was performed at 24 h and 48 h after culture initiation, which were chosen as the time points most representative of early and late changes in cumulus–oocyte interactions.

Observation groups:
Morphology was recorded separately under two cell-culture contexts:

(i) cumulus with oocyte — primary cumulus cells maintained together with denuded oocytes obtained from the same follicular material whenever available.

(ii) only cumulus — primary cumulus cells maintained without oocytes.

3.2.5. Measurement of Intracellular ROS Levels of Cumulus Cells

Principle:
ROS analyses were performed on cumulus cells fixed after 48 h of culture, corresponding to the time point used for oocyte GVBD evaluation. The intracellular levels of reactive oxygen species (ROS) in cumulus cells were assessed using the cell-permeant probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), which is widely employed as a sensitive indicator of oxidative stress in mammalian cells. DCFH-DA itself is non-fluorescent and readily diffuses across the plasma membrane. Once inside the cell, it is cleaved by intracellular esterases to form the non-fluorescent intermediate dichlorodihydrofluorescein (DCFH), which is membrane-impermeable and thus retained in the cytoplasm. In the presence of ROS, DCFH is oxidized to form the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Measurement of DCF fluorescence intensity therefore provides an indirect, semi-quantitative readout of intracellular ROS burden. This method is characterized by high sensitivity and

reproducibility, and has been widely adopted in studies of oxidative stress, apoptosis, and cellular metabolism[62,63].

Groups.

Cumulus cells were evaluated under three conditions:

(i) cumulus with oocytes — cumulus cells maintained together with denuded oocytes;

(ii) only cumulus — cumulus cells maintained without oocytes;

(iii) positive control (H_2O_2-treated) — cumulus cells treated with 0.5 mmol/L (0.5 mM) hydrogen peroxide for 30 minutes after probe loading, to verify assay responsiveness and provide a normalization reference[64–66].

Probe loading and handling.

Intracellular ROS was detected using DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate). DCFH-DA was freshly diluted to 10 μM in serum-free DMEM/F-12 immediately prior to use. Cumulus cells were seeded in six-well plates, allowed to adhere (~24 h), and maintained at 37 °C in a humidified incubator with 5% CO_2 in ambient O_2 (~20%). Culture medium was replaced with the DCFH-DA working solution; cells were incubated for 30 min at 37 °C in the dark, then washed three times with pre-warmed serum-free medium to remove extracellular probe. For the positive control, H_2O_2 (0.5 mmol/L) was applied for 30 min immediately prior to imaging. All steps were performed under low light to minimize probe photo-oxidation and leakage.

Imaging and acquisition parameters.

Probe-loaded cells were imaged immediately on a laser-scanning confocal microscope. Excitation/emission were λ_ex = 488 nm and λ_em = 525 nm. Identical acquisition settings (objective lens, laser power, detector gain, exposure) were maintained across groups and time points. Images were saved as uncompressed TIFF files with embedded scale bars, labeled with anonymized IDs and timestamps, and archived on a write-protected institutional server. Acquisition and file naming were performed blinded to group assignment.

Quantification.

Fluorescence intensity was analyzed in ImageJ (Version 1.47; NIH, Bethesda, MD, USA). Regions of interest were drawn around individual cumulus cells to obtain per-cell mean intensity, which was averaged to a per-well mean for analysis. To enable between-run

comparability without a vehicle group, values were expressed as normalized arbitrary units and/or as percent of the H_2O_2 positive-control response on the same plate.

Statistical analysis.

Analyses were conducted using SPSS (Version 28.0; IBM Corp.) and GraphPad Prism (Version 9.0). Because three groups were analyzed (Cumulus–Oocyte co-culture, Cumulus Alone, H_2O_2 positive control), one-way ANOVA followed by Tukey's HSD was used for pairwise multiple comparisons. A P-value < 0.05 was considered statistically significant.

3.2.6. Immunocytochemical staining for AMPK in cumulus cells

Principle.

AMPK immunocytochemistry was performed on cumulus cells fixed after 48 h of culture, corresponding to the time point used for ROS analysis and oocyte GVBD evaluation. Immunocytochemistry (ICC) was used to visualize AMPKα1 in primary cumulus cells. After chemical fixation to preserve cellular architecture and permeabilization to allow antibody access, nonspecific binding sites were blocked. Cells were then incubated with a primary antibody directed against AMPKα1; bound primary antibody was detected by a fluorophore-conjugated secondary antibody (Alexa Fluor™ dye), enabling fluorescence imaging of AMPKα1 distribution. A no-primary (negative) control was processed in parallel to verify staining specificity and to define background. ICC provides an indirect, semi-quantitative readout of protein presence and subcellular localization rather than absolute abundance or enzymatic activity. Accordingly, fluorescence intensity can be compared across conditions when identical acquisition settings are maintained, while AMPK kinase activity per se typically requires phospho-epitope assays (e.g., p-AMPK Thr172 or downstream p-ACC) and is not inferred from total AMPKα1 ICC[10].

Groups.

Cumulus cells were processed under three conditions for AMPK immunocytochemistry:
(i) cumulus with oocyte — primary cumulus cells maintained together with denuded oocytes;
(ii) only cumulus — primary cumulus cells maintained without oocytes;
(iii) control (no primary antibody) — cells processed identically to (i) or (ii) but omitting the AMPKα1 primary antibody to verify staining specificity.

All groups underwent the same fixation, permeabilization, blocking, and imaging settings; the negative-control preparations were used to confirm specificity/define background and were not included in inferential statistics.

Fixation, permeabilization, and blocking.

Cells were fixed in 4% paraformaldehyde at room temperature for 30 min. Permeabilization was performed with 0.5% Triton X-100 in culture medium for 20 min at room temperature. Cells were then incubated in PBS containing 1% bovine serum albumin (BSA) and blocking buffer for 1 h.

Primary and secondary antibodies.

Following blocking, cells were incubated with the primary anti-AMPK α1 antibody for 4 h at room temperature (no primary antibody in the negative-control group). After three washes with PBS, cells were incubated with the Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 350 for 1 h at room temperature, then rinsed in PBS before imaging.

Imaging and acquisition parameters.

AMPK immunofluorescence was recorded on a Zeiss LSM 700 confocal microscope (Jena, Germany) using appropriate laser/filter settings for the fluorophore. Identical acquisition settings (objective, laser power, detector gain, exposure) were maintained across groups and time points. Images were saved as uncompressed TIFF files with embedded scale bars, labeled with anonymized IDs and timestamps, and archived on a write-protected institutional server. Image acquisition and file naming were performed blinded to group assignment.

Quantification.

Fluorescence intensity was analyzed in ImageJ (Version 1.47; NIH, Bethesda, MD, USA). Regions of interest (ROIs) were drawn around individual cumulus cells to obtain per-cell mean intensity, which was averaged to generate a per-well mean for statistical analysis. Negative-control images were used to confirm specificity and establish background, but were not entered into between-group comparisons.

Statistical analysis.

Statistical analyses were conducted using SPSS (Version 28.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism (Version 9.0; GraphPad Software, San Diego, CA, USA). Because the

quantitative comparison involved two experimental groups (cumulus with oocyte co-culture vs cumulus alone), we applied a two-sample test (Student's t-test for normally distributed data or Mann–Whitney U for non-normal data). A P-value < 0.05 was considered statistically significant.

4. Results

4.1 GVBD occurrence under different culture conditions

After 48 h of in-vitro culture, oocytes were classified as GV-intact (visible germinal vesicle) or GVBD occurred (no visible germinal vesicle with homogeneous cytoplasm) by a blinded assessor under an inverted microscope, as prespecified in Methods. Across the study (total N = 23 oocytes), the only oocyte group arm yielded 2/11 GVBD (18.18%), whereas the oocyte with cumulus group arm yielded 8/12 GVBD (66.67%). The between-group difference was significant by Fisher's exact test (P = 0.036), indicating a markedly higher likelihood of nuclear maturation (GVBD) when cumulus cells were present during culture (Fig. 1; Table 1). On a descriptive scale, the observed odds ratio favored co-culture (OR ≈ 9.0), consistent with a biologically meaningful effect size under the standardized conditions described in §4.2.2–4.2.3.

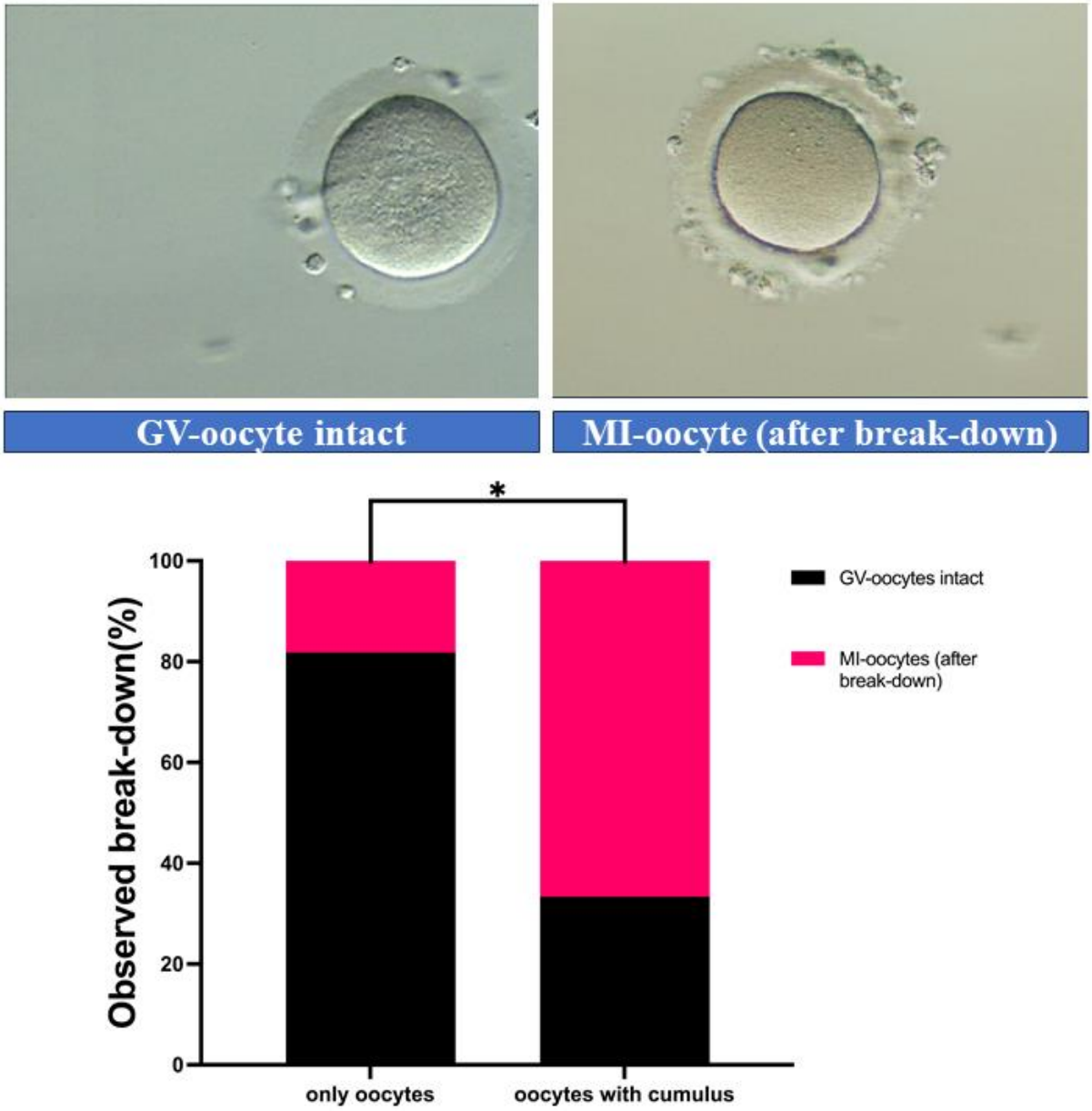

Figure 1. Representative images of human GV-stage oocytes after 48 hours of in vitro culture

(Left) GV-intact oocyte with a clearly visible germinal vesicle.

(Right) GVBD occurred, as evidenced by the absence of a visible GV and homogeneous cytoplasm.

(Below) Comparison of GVBD Occurrence between only oocytes and oocytes with cumulus groups. Percentages represent the proportion of oocytes classified as 'GV-intact' (black) and 'GVBD occurred' (pink) in each group. *P < 0.05, Fisher's Exact Test.

Table 1. Comparison of GVBD Rates Between only oocyte group and oocytes with cumulus group

Group	GV-intact (n, %)	GVBD (n, %)	Total (n)	P-value
only oocytes	9(81.82%)	2 (18.18%)	11	
oocytes with cumulus	4(33.33%)	8 (66.67%)	12	0.036(*)
Total	13	10	23	

4.2 Morphological appearance of cumulus cells under matched culture conditions

Cumulus-cell morphology was recorded at 24 h and 48 h under two predefined contexts: cumulus with oocytes and only cumulus.

At 24 h, fields categorized as cumulus with oocytes typically showed compact central aggregates with peripheral spread, where cells formed short, contiguous cords radiating from the cluster. Individual cells displayed polygonal to ovoid bodies and short cytoplasmic processes linking adjacent neighbors. In contrast, only cumulus cells at the same time point more often exhibited smaller, discontinuous colonies interspersed with isolated, spindle-like cells, and the intercellular gaps between neighboring cells were visibly wider.

By 48 h, images from cumulus with oocytes co-culture more frequently demonstrated broader sheet-like expansion with denser cell–cell apposition and continuous borders delineating the margins of expanding clusters. Along the periphery, cells displayed lamellipodia-like protrusions and short intercellular bridges, giving the field a tessellated appearance. In only cumulus group at 48 h, cells remained more sparsely distributed; discrete micro-colonies persisted alongside elongated, bipolar cells, and cell-free intervals between colonies were still apparent at this readout.

Across both time points, nucleus–cytoplasm contrast, outline sharpness, and background levels were comparable under the shared acquisition parameters. Overall, cumulus with oocyte co-culture characteristically displayed greater lateral spread of contiguous cells and tighter

local packing by 48 h, whereas only cumulus group commonly retained patchier coverage with inter-cluster spacing that remained evident at the same time point.

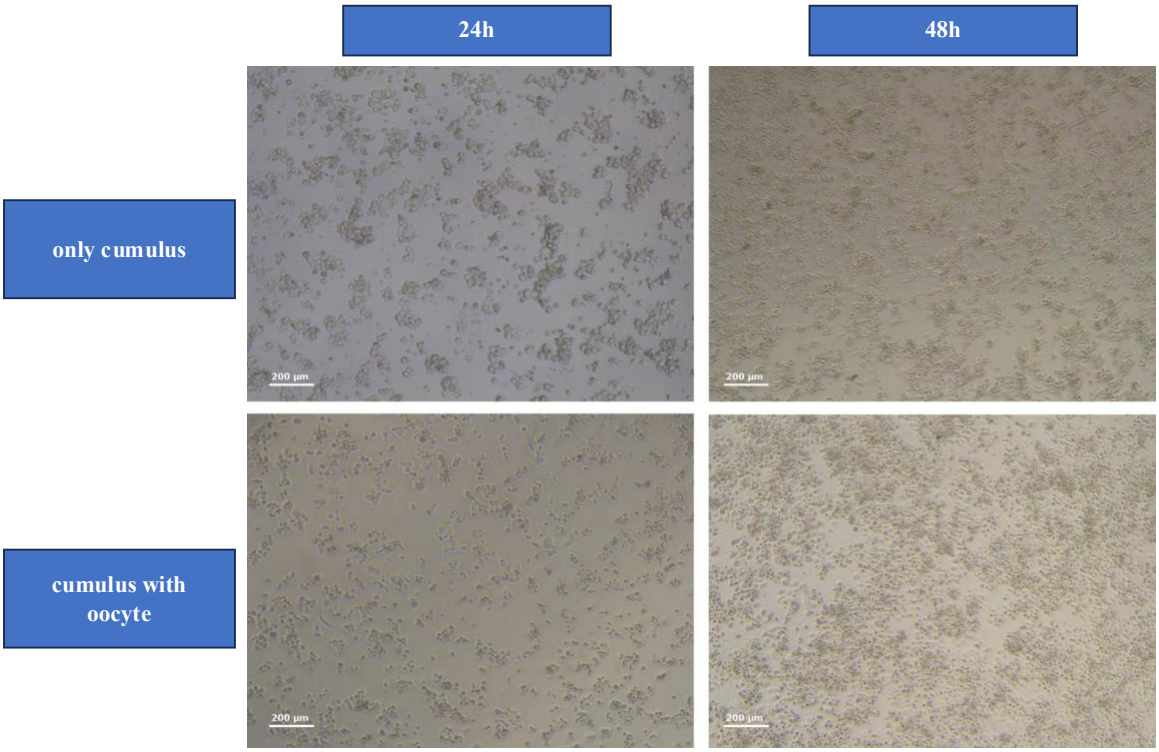

Figure 2. Morphological observation of Cumulus Cells (CCs) at 24 hours and 48 hours under different culture conditions.

4.3 Intracellular ROS levels in cumulus cells

Intracellular reactive oxygen species (ROS) were quantified with H_2DCFDA under three pre-defined conditions: Cumulus Cells with Oocyte Co-culture, Cumulus Cells Alone, and Positive Control (H_2O_2-treated cumulus cells), using identical acquisition settings and the same normalization scheme described in Methods (§3.2.5). The Positive Control was used as the within-plate reference and assigned 100% for relative comparison.

When expressed as a percentage of the positive control, only cumulus group reached 21.87% of the reference signal, whereas cumulus with oocyte co-culture were 12.11% of the reference. One-way ANOVA across the three conditions indicated a group effect, and Tukey's HSD

showed that cumulus with oocytes co-culture had lower ROS than only cumulus ($P < 0.01$). Post-hoc comparisons versus the positive control confirmed that both experimental conditions were below the reference level; individual post-hoc P-values are annotated alongside bars in the figure. These outcomes are summarized in Figure 3.

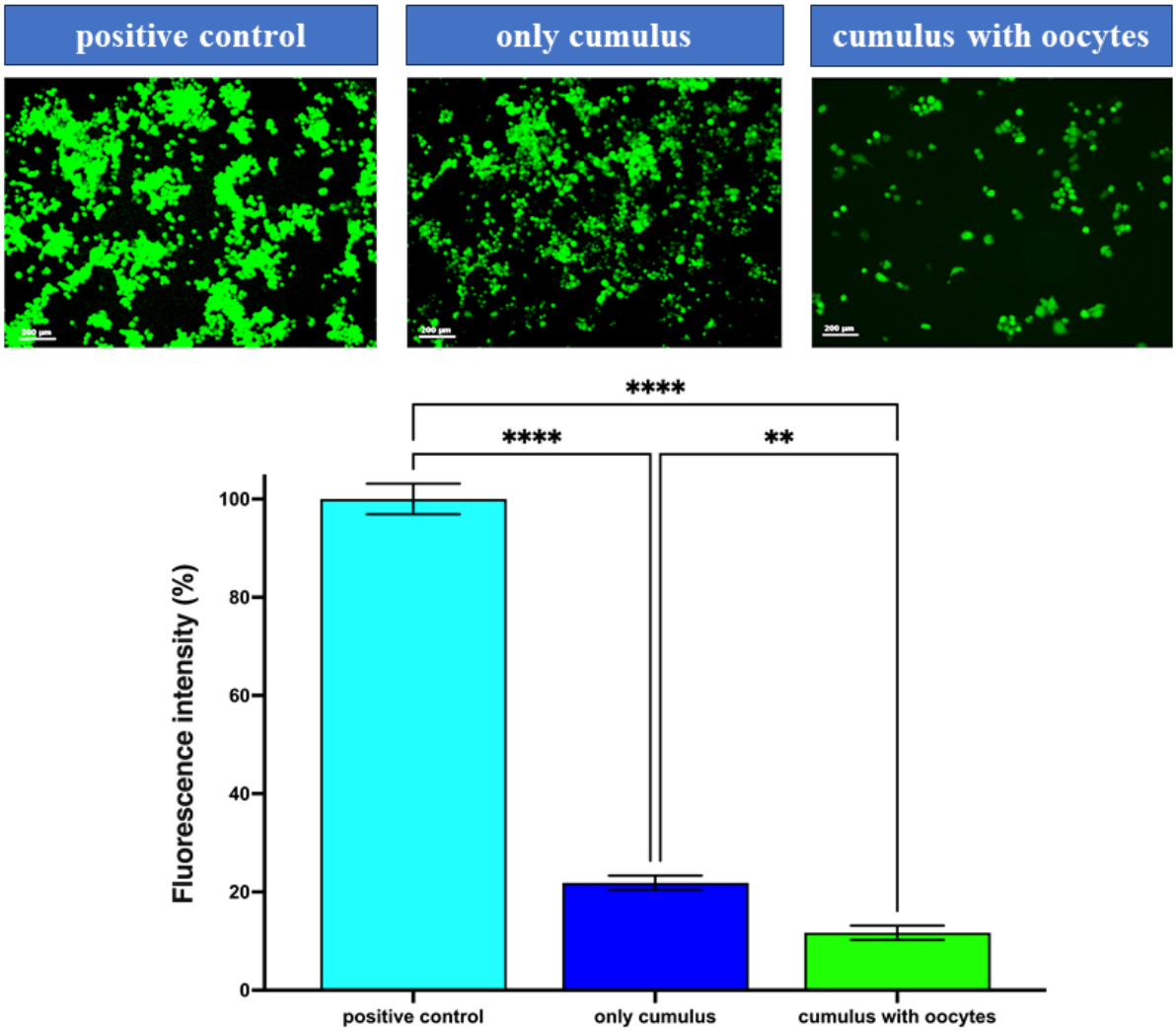

Figure 3. Intracellular ROS levels in cumulus cells under three culture conditions.
(Upper panel) Representative fluorescence micrographs acquired with identical settings (λ_ex 488 nm / λ_em 525 nm), shown for positive control (H_2O_2), only cumulus, and cumulus with oocyte co-culture. Scale bars embedded.
(Lower panel) Quantification of fluorescence intensity reported as % of the positive control on the same plate. Bars display group means ± SEM; One-way ANOVA with Tukey's HSD for multiple comparisons. **P < 0.01, ***P < 0.001, ****P < 0.0001.

4.4 AMPK immunocytochemistry in cumulus cells

AMPKα1 was assessed by immunocytochemistry under three predefined conditions: cumulus with oocyte co-culture, only cumulus, and a negative control (no primary antibody) processed in parallel to verify staining specificity. Image acquisition used the same microscope, optics, and detector settings across conditions, and intensity quantification followed the ROI-based workflow described in Methods (per-cell means aggregated to a per-well mean for analysis). Negative-control images served to confirm specificity/background and were not entered into between-group statistics.

For the quantitative comparison, signals were reported relative to only cumulus cells (set as 100%). Under these reporting conventions, cumulus with oocytes group reached 228.98% of only cumulus group ($P < 0.0001$, two-group comparison as specified in Methods). The negative control (no primary antibody) showed background-level fluorescence, consistent with assay specificity. These outcomes are presented as representative micrographs and a corresponding quantification panel (Figure 4).

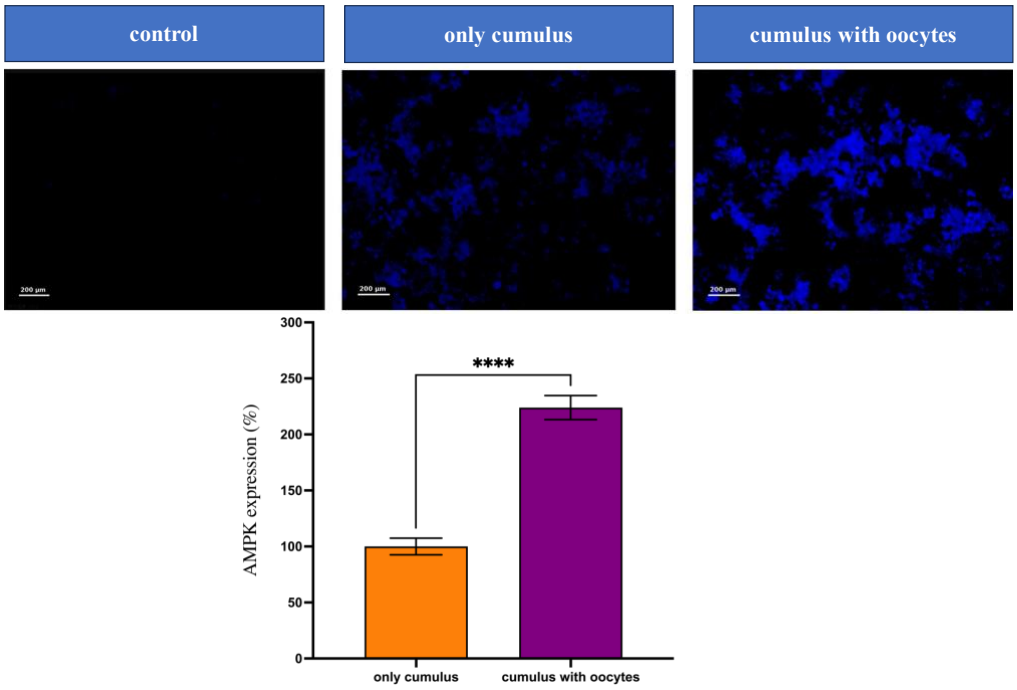

Figure 4. AMPK immunocytochemistry in cumulus cells under two experimental conditions, with a negative control.

(Upper panel) Representative fluorescence images: negative control (no primary antibody), only cumulus, and cumulus with oocytes; identical acquisition parameters and embedded scale bars.
(Lower panel) Quantification of AMPK expression levels. AMPK expression in cumulus with oocytes co-culture is significantly higher than in only cumulus group, shown as a percentage of only cumulus group. Data are presented as mean ± SEM (****P < 0.0001).

5. Discussion

This study demonstrates that preserving the cumulus–oocyte complex (COC) during early culture coincides with a substantially higher likelihood that human GV-stage oocytes initiate meiotic resumption and with a somatic microenvironment that is measurably more favorable to maturation. Under rigorously matched conditions, the co-culture arm yielded a higher GVBD rate at 48 h (66.67% vs. 18.18%; P = 0.036), while the surrounding cumulus cells exhibited a lower intracellular oxidative burden (12.11% vs. 21.87% of the H_2O_2 reference) and a markedly stronger AMPK immunofluorescence signal (228.98% relative to only cumulus group; P < 0.0001). Morphologically, co-cultured cumulus cells displayed more cohesive, expansion-like organization, consistent with preserved junctional continuity and active matrix remodeling. Considered together, these nuclear, biochemical, and morphological findings converge on the same direction of effect: the intact oocyte–cumulus unit functions as a redox–metabolic buffer that lowers the barrier to meiotic resumption in vitro[67–69].

The biological plausibility of this pattern rests on well-described features of oocyte–somatic reciprocity. Through gap junctions and paracrine mediators, cumulus cells supply substrates (notably pyruvate and lactate), recycle glutathione, and relay EGF-like cues, while the oocyte reciprocally instructs cumulus transcriptional programs via oocyte-secreted factors such as GDF9 and BMP15. Within this circuitry, AMPK operates as a nodal sensor of adenylate charge and stress, coordinating glycolysis, fatty-acid oxidation, mitochondrial quality control, and autophagy, and, through crosstalk with mTOR/SIRT pathways, aligning biosynthetic demand with energetic capacity. A “low-ROS / high-AMPK” cumulus phenotype in co-culture is therefore not an incidental association but a coherent readout of a healthier, more resilient micro-niche. In practical terms, when the somatic shield is preserved, ROS production is dampened at source and antioxidant recycling more efficient; AMPK activation then further stabilizes mitochondrial coupling and gap-junctional communication, supporting timely GVBD without pharmacologic escalation[70].

These data also align with the contemporary evolution of human in-vitro maturation (IVM) from single-phase, oocyte-centric recipes toward microenvironment-aware designs. Biphasic or “pre-IVM + IVM” approaches (e.g., CNP-based CAPA-IVM) seek to temporize nuclear resumption ex vivo and then trigger it in a manner closer to the peri-ovulatory cascade, a strategy that implicitly assumes an intact, functional COC to carry the paracrine and metabolic conversation. Likewise, oxygen tension is increasingly treated as a tunable variable rather than

a dogma: while lower O_2 can lessen mitochondrial electron leak, its net benefit is protocol-dependent and interacts with the state of somatic support, media composition, and timing of denudation. Engineered ovarian support-cell platforms extend the same logic by reconstructing granulosa-like signals when natural COCs are limited. Against this backdrop, our results provide direct human evidence that simply keeping the unit intact measurably shifts somatic redox and energy-sensing biology in the right direction and that these shifts co-occur with improved nuclear outcomes[71,72].

Differences between studies that report modest or absent co-culture benefits can be partly explained by design heterogeneity that obscures true biology. Early or aggressive denudation removes the somatic buffer at precisely the moment oxidative load and energetic demand rise. Oxygen settings vary across laboratories, with some comparisons confounded by differences in media and pre-IVM staging. Importantly, fluorescence-based assays for ROS or protein abundance are sensitive to acquisition settings, batch effects, and plate-to-plate drift. Our use of (i) plate-internal normalization for ROS (H_2O_2 set as a reference) and (ii) acquisition-matched imaging with a no-primary negative control for AMPK minimized these sources of technical variance in a small human dataset and likely increased sensitivity to detect biologically meaningful differences. The internal consistency linking higher GVBD to lower cumulus ROS and higher cumulus AMPK strengthens causal plausibility, even though causality is not proven without pathway perturbation[73,74].

The translational implications for assisted reproduction are straightforward. First, maintaining COCs during an initial culture window is a low-complexity, low-cost intervention that can be adopted without new pharmacology or equipment. It is particularly attractive for cohorts in which oxidative and metabolic stress are prevalent—PCOS, advanced maternal age, and diminished ovarian reserve—where a somatic buffer may be most consequential. Second, laboratories can elevate quality control by complementing nuclear endpoints with simple, portable somatic biomarkers. Expressing cumulus ROS as a percentage of a within-plate positive control and quantifying AMPK immunofluorescence under standardized acquisition provides early, device-agnostic readouts of microenvironment health that can guide protocol tuning before clinical outcomes accrue. Third, our findings argue for integration rather than substitution when adopting contemporary refinements: intact COC as the foundation, with optional pre-IVM staging (e.g., CNP), protocol-specific oxygen tuning, and carefully timed adjuncts (antioxidants or AMPK-linked modulators) layered on top. Conceptually, this stacks

orthogonal benefits: structure (somatic integrity), timing (synchronized nuclear/cytoplasmic programs), atmosphere (controlled electron leak), and targeted chemistry (preserving signaling while restraining stress)[75–77].

Several limitations should temper over-interpretation. Sample size was modest and powered for categorical contrasts rather than multivariable adjustment; effect-size precision is therefore limited. We evaluated nuclear resumption at 48 h (GV→GVBD) but did not extend observation to MI/MII attainment, spindle integrity, fertilization, embryo development, or pregnancy outcomes; whether the microenvironmental advantages observed here propagate downstream remains to be established. ROS and AMPK were assessed in cumulus cells, not within oocytes; while mechanistic inferences are strengthened by convergence across endpoints, causality along the AMPK–redox axis is inferred rather than demonstrated. Finally, our culture used ambient oxygen and did not include explicit CAPA or engineered-support arms; thus, direct benchmarking against state-of-the-art biphasic or OSC-based protocols is not possible within this dataset[78,79].

These boundaries define clear next steps. Short-exposure, dose-timed perturbations of the AMPK–redox pathway (e.g., AICAR/metformin analogs, melatonin or other antioxidants) should be tested within an intact-COC framework, with readouts spanning GVBD → MI/MII, spindle architecture, and mitochondrial membrane potential. Multi-omic profiling of matched oocyte–cumulus pairs—single-cell transcriptomics and targeted metabolomics—can yield a compact “COC health” signature that predicts maturation and embryo competence and that can be used as a cross-laboratory QC anchor. Pragmatic, diagnosis-stratified trials that layer structural preservation with pre-IVM staging, oxygen tuning, and/or engineered support cells will be necessary to map boundary conditions and verify that the microenvironmental gains documented here translate into embryo-level and clinical outcomes. Within such designs, oxygen should be treated as a planned contrast (e.g., 5% vs. 20%) rather than an axiomatic setting, with somatic biomarkers recorded alongside nuclear milestones to resolve protocol-specific interactions[80,81].

In sum, the present work situates human GV-oocyte maturation within its somatic context and shows that the state of the cumulus layer is not epiphenomenal but proximal to the oocyte’s probability of re-entering meiosis. Under co-culture, cumulus cells manifest a lower-ROS / higher-AMPK phenotype and cohesive organization that together delineate a buffered, energy-

competent niche; under denudation, this protective architecture is lost and maturation is less likely to proceed. These observations reinforce a field-wide pivot from treating the oocyte as an isolated unit to engineering the microenvironment that supports it[82]. Future optimization of human IVM will therefore rely not only on hormonal triggers but on recreating and monitoring the somatic ecology—structural, metabolic, and redox—that permits maturation to unfold on time with minimal collateral stress.

This doctoral thesis investigated the influence of cumulus cell co-culture on nuclear maturation and the cellular microenvironment of human germinal-vesicle (GV) oocytes. The study aimed to clarify whether maintaining the natural cumulus–oocyte complex (COC) could improve meiotic resumption and modulate oxidative and energy-related signaling in the surrounding cumulus cells during in-vitro maturation (IVM).

Primary cumulus cells and GV-stage oocytes were collected from follicular aspirates of patients undergoing ovarian tissue cryopreservation. The oocytes were cultured either together with their companion cumulus cells (co-culture) or after denudation. The proportion of oocytes that resumed meiosis, indicated by germinal vesicle breakdown (GVBD), was significantly higher in the co-culture group compared with denuded oocytes. Parallel immunocytochemical analyses revealed that cumulus cells cultured together with oocytes showed a lower level of reactive oxygen species (ROS) and higher fluorescence intensity of AMP-activated protein kinase (AMPK) than cumulus cells cultured alone. Morphologically, co-cultured cumulus cells maintained a more cohesive, expansion-like structure, consistent with an improved functional state.

These results demonstrate that preserving the integrity of the cumulus–oocyte complex establishes a more balanced oxidative–metabolic environment that facilitates meiotic progression. The findings align with current international developments in human IVM that emphasize microenvironmental optimization—such as biphasic CAPA-IVM systems and low-oxygen culture conditions—to mimic the physiological follicular niche. By linking the oocyte's maturation status to the redox and energy state of its supporting cells, this work contributes mechanistic insight into the somatic–germ cell interactions governing human oogenesis in vitro.

The study's main limitation lies in its small sample size and the focus on early nuclear maturation endpoints. Future investigations integrating cumulus co-culture with modern IVM protocols, combined with functional analysis of the AMPK–redox pathway, will help determine

whether the observed microenvironmental benefits translate into improved fertilization and embryo development outcomes.

In summary, the present research identifies the cumulus–oocyte unit as an active metabolic and antioxidative niche that promotes oocyte maturation. Understanding and preserving this cellular interaction will be essential for developing safer and more physiologic in-vitro maturation strategies in human assisted reproduction.

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7. Appendix

Appendix A. Baseline clinical and endocrine characteristics of patients

Baseline clinical and endocrine characteristics of patients whose follicular material was used for the in vitro experiments are summarized in Table A1. All laboratory parameters were obtained from routine clinical testing performed prior to or at the time of ovarian puncture. These data are provided for descriptive purposes only and were not used for patient stratification or group allocation, as experimental comparisons were performed at the oocyte and cumulus-cell level.

Due to the retrospective use of available clinical data, not all parameters were available for every patient. No formal statistical comparisons were conducted for baseline variables.

Table A1. Baseline clinical characteristics of patients

Parameter	**Value**
Number of patients	22
Age (years)	38 ± 7
AMH (ng/mL)	1.21 (0.6–2.4)
FSH (IU/L)	6.7 ± 2.1
LH (IU/L)	5.9 ± 2.4
Estradiol, E2 (ng/L)	185 (40–620)
Progesterone (µg/L)	0.6 (0.2–1.4)

Values are presented as mean ± standard deviation or median (range), as appropriate.

8. Preliminary publications

1. **Ban, Z.; Todorov, P.; Rahimi, G.; Skala, C.; Isachenko, V.** Reciprocal Interactions Between Human GV-Oocytes and Cumulus Cells: Effects on GVBD, ROS Production, and AMPK Expression. *Medicina* **2025**, **61**, 2107. Published 26 November 2025. https://doi.org/10.3390/medicina61122107