Effects of lactate, super-GDF9 and low oxygen tension during biphasic in vitro maturation on the bioenergetic profiles of mouse cumulus-oocyte-complex

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CONFLICT OF INTEREST

Authors report no conflict of interest.

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ABSTRACT

In vitro maturation (IVM) is an alternative assisted reproductive technology (ART) with reduced hormone related side-effects and treatment burden compared to conventional IVF. Capacitation (CAPA)-IVM is a biphasic IVM system with improved clinical outcomes compared to standard monophasic IVM. Yet, CAPA-IVM efficiency compared to conventional IVF is still suboptimal in terms of producing utilizable blastocysts. Previously we have shown that CAPA-IVM leads to a precocious increase in cumulus cell (CC) glycolytic activity during cytoplasmic maturation. In the current study, considering the fundamental importance of CCs for oocyte maturation and cumulus-oocyte complex (COC) microenvironment, we further analyzed the bioenergetic profiles of maturing CAPA-IVM COCs. Through a multi-step approach, we (i) explored mitochondrial function of the in vivo and CAPA-IVM matured COCs through real-time metabolic analysis with Seahorse analyzer; and to improve COC metabolism (ii) supplemented the culture media with lactate and/or super-GDF9 (an engineered form of growth differentiation factor 9) and (iii) reduced culture oxygen tension. Our results indicated that the pre-IVM step is delicate and prone to culture related disruptions. Lactate and/or super-GDF9 supplementations failed to eliminate pre-IVM induced stress on COC glucose metabolism and mitochondrial respiration. However, when performing pre-IVM culture under 5% oxygen tension, CAPA-IVM COCs showed a similar bioenergetic profiles compared to in vivo matured counterparts. This is the first study providing real-time metabolic analysis of the COCs from a biphasic IVM system. The currently used analytical approach provides the quantitative measures and the rational basis to further improve IVM culture requirements.

INTRODUCTION

In vitro fertilization (IVF) involves the stimulation of ovaries with exogenous gonadotropins for several days, followed by the triggering of ovulation (e.g. with human chorionic gonadotropin (hCG) administration). In vitro maturation (IVM), on the other hand, is an alternative assisted reproductive technology (ART) where immature cumulus-oocyte-complexes (COCs) are collected from small/mid- antral follicles and matured in vitro. Patient preparation for IVM requires minimal (2-3 days) or no gonadotropin stimulation, and no hCG priming is necessary [1,2]. Therefore, it is a mild and patient friendly approach with reduced risks of hormone-related side effects and lower treatment costs per cycle.

Earlier research has shown that standard IVM systems are leading to spontaneous oocyte nuclear maturation, upon removal of the COC from the inhibitory follicular environment by a lack of intrinsic factors regulated by somatic cell compartment and loss of cumulus-oocyte connections [3]. This precipitous resumption of meiosis is however not accompanied with cytoplasmic maturation (or capacitation). In order to remediate, bi-phasic IVM systems, with a pre-IVM step, allowing oocyte capacitation prior to triggering nuclear maturation have been developed [4–8]. CAPA-IVM, being one successful example of a bi-phasic IVM system, has improved the competence of the oocytes derived from small antral follicles and led to increased oocyte maturation and clinical pregnancy rates [9–13] through synchronized nuclear and cytoplasmic maturation [11,14]. Even though CAPA-IVM is superior to the standard IVM systems, a current challenge to reduce the high attrition rate from mature oocytes to utilizable blastocysts, which are still lower in number compared to conventional IVF [13]. One potential solution for reducing the efficiency gap between CAPA-IVM and conventional IVF could be through optimizing the culture conditions (such as media formulation) to mimic the in vivo environment, thus mitigating the negative effects inflicted on the oocytes by in vitro culture [3].

The follicular microenvironment depends on several energy substrates including glucose, lactate, and pyruvate, in varying concentrations as well as oxygen [15]. For obtaining a competent oocyte, proper utilization of follicular fluid (FF) nutrients and oxygen are important as much as the substrate availability [16]. During in vivo maturation, cumulus cells (CCs) take up the glucose from FF and perform glycolysis to provide the oocytes with pyruvate [17,18], which is then metabolized by oocytes through mitochondrial oxidative phosphorylation (OXPHOS) for ATP generation [17,19,20]. FF also supplies sufficient levels of oxygen to the COCs residing in an avascular environment [21,22] for energy transformation through pyruvate oxidation [23]. In fact, it was shown that the oocyte is the main receiver of the FF oxygen, as only up to 5% of this essential gas is absorbed by CCs within the COCs [24]. Like glucose, lactate in the medium is metabolized by the CCs to produce pyruvate which can support the oocyte during maturation [25]. Furthermore, mouse oocytes can metabolize culture media pyruvate, but not lactate-derived pyruvate, to convert into energy in

mitochondria [26]. Nevertheless, oxidation of pyruvate is crucial for oocytes regardless of the source it has been derived from [27].

Alterations in metabolic activity are known culture-induced negative effects, and IVM-inflicted perturbations in COC metabolism have been observed in several species [28–32]. Recently, we have shown an impaired precocious increase in glycolytic activity in CCs during the pre-IVM phase of the mouse CAPA-IVM when compared to their in vivo matured counterparts [33], which may contribute to the lower in vitro oocyte competency. Consequently, limiting pre-IVM glycolysis and/or focusing on improving CC function might possibly enhance CAPA-IVM culture outcomes. The former could be potentially achieved through supplementing lactate to CAPA-IVM basal media, which was missing in the culture medium used in the former study. Lactate is the end-product of lactic acid fermentation, with a known role in inhibiting the activity of the glycolytic enzyme phosphofructokinase (PFK) in several tissues [34]. In early-stage murine embryos, lactate also reduces glycolytic activity indirectly through its conversion into pyruvate [35]. On the other hand, CC function could be improved through supplementing IVM media with oocyte-secreted factors (OSFs). OSFs enhance oocyte quality through orchestrating several CC metabolic functions; including glycolysis, amino acid uptake and cholesterol biosynthesis (reviewed in [36,37]). CAPA-IVM media supplementation with cumulin, a heterodimer of bone morphogenic protein-15 (BMP15) and growth differentiation factor-9 (GDF9), regulates the expression levels of ovulatory cascade genes to a level more resembling that of in vivo matured CCs [38]. Similarly, the engineered OSF super-GDF9, which has over 1000-fold greater bioactivity than wild-type GDF9 [39], visibly improved COC mucification and extracellular matrix (ECM) elasticity in CAPA-IVM which was more comparable to the ECM morphology of in vivo matured COCs [38].

Regulating oxygen tension of the culture environment could be another possible approach to improve COC metabolism. While there is a long-standing consensus on the requirement of low oxygen tension during in vitro embryo culture, the optimal oxygen concentration for IVM is still controversial. The low oxygen (2%-9%) in the in vivo COC environment [40,41] is overridden in the in vitro settings where oxygen concentrations are at atmospheric levels. Oocyte mitochondria use substrates produced by CC glycolysis to perform OXPHOS that will provide the necessary energy throughout oocyte maturation, fertilization, and preimplantation development (reviewed in [37]). Supraphysiological oxygen levels could trigger mitochondrial activity and lead to imbalanced ATP generation [42], as well as the accumulation of reactive oxygen species (ROS). Regulating oxygen tension during IVM to levels comparable to those in vivo would also offer a way of by-passing the CC metabolism and targeting the oocyte OXPHOS directly. Thus, reducing oxygen concentrations during IVM could be beneficial for sustaining COCs innate bioenergetic profile.

Hence in this study, we initially studied the mitochondrial function of in vivo and CAPA-IVM matured COCs, in order to gain a better understanding of the possible adverse effects of

defective CC glycolysis on the ATP production of the oocytes. Then, we hypothesized that supplementing CAPA-IVM media with lactate and super-GDF9, either separately or in combination, could restore the defective CAPA-IVM CC glucose metabolism and basal respiration through limiting glycolysis and/or improving CC function. Finally, we analyzed the effect of low oxygen tension during pre-IVM culture on oocyte competence and mitochondrial function.

MATERIALS AND METHODS

Animal model

Animals used in this study were the F1 mice of C57BL/6j x CBA/ca hybrid (Charles River Laboratories, France). All mice were housed and bred with the approval of Vrije Universiteit Brussel's local ethical committee (approval no: 21-216-1) and in accordance with the Belgian Legislation for animal care.

COC collection

Ovaries were collected from 19- to 21-day-old prepubertal female mice in Leibovitz L15 (Sigma-Aldrich, Belgium) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin (all from Thermo Fisher Scientific, Belgium). This study included three types of in vivo samples: (i) baseline-COCs (baseline), retrieved in mice that received no hormonal stimulation; (ii) in vivo GV-COCs, collected 48 h after stimulation with 2.5 IU Folligon (Intervet, Netherlands); and (iii) in vivo MII-COCs, collected after the stimulation with 2.5 IU Folligon for 48 h followed by 2.5 IU Chorulon (Intervet) for 14 h. Mice from the second and the third groups are considered as in vivo super-ovulated (SO) mice. Different from in vivo samples, CAPA-IVM COCs were collected from unstimulated mice that did not receive any hormonal stimulation. Collection medium was supplemented with 0.2 mM IBMX (3-isobutyl-1-methylxanthine; Sigma-Aldrich) to prevent meiotic resumption prior to culture. COCs for CAPA-IVM culture and in vivo baseline and GV-COCs were collected from the harvested ovaries, through puncturing small antral follicles with insulin needles. In vivo MII-COCs were collected from the fallopian tubes.

CAPA-IVM culture

Basal culture medium for CAPA-IVM was prepared as described previously [14]. Briefly, alpha-MEM (Thermo Fisher Scientific) was supplemented with 2.5 % FBS, 5 ng/ml sodium selenite, 5 μg/mL apo-transferrin, 5 ng/ml insulin, 10 nM 17-beta estradiol (E2) (all from Sigma-Aldrich) and 2.5 mIU/ml FSH (Merck, Belgium). Basal media was supplemented with 25 nM CNP-22 (Phoenix Europe, Germany) for pre-IVM culture and 50 ng/mL recombinant mouse epiregulin (EREG; Bio-techne, United Kingdom) for IVM culture. Individual COCs were cultured for 48 h in pre-IVM media, followed by 18 h in IVM media, in 96-well round bottom ultra-low attachment plates (Corning) at 37 ^{O}C, 5% CO_2 and 100% humidity.

Evaluation of MII oocyte rate and oocyte competence

At the end of the IVM culture, COCs were processed for evaluation of several different end points. For one set of experiments, COCs were denuded mechanically to assess the maturation rate and measure MII oocyte diameters. Maturation rate was calculated by dividing the number of oocytes with a visible polar body to the total number of denuded oocytes. For another set of experiments, oocyte competence was assessed through performing IVF and embryo culture (details were explained in [38]). In IVF experiments, cleavage rate on day 2 (D2) and blastocysts rates on D5 were assessed as end points. Cleavage rate was calculated over the total number of inseminated COCs, whereas D5 blastocyst rate was calculated over the total number of cleaved embryos on D2.

Enzymatic assays: Sample preparation and assays

For the enzymatic assays, in vitro matured GV and MII COCs were collected at the end of pre-IVM and IVM cultures, respectively. Mechanically denuded oocytes and their corresponding cumulus cells were collected in pools of five (one biological replicate), snap frozen and stored at -80 ^{O}C. Cell lysis was performed as described before [33] using an in-house prepared lysis buffer and through applying three freeze-thaw cycles. Protein content was measured using a modified Bradford assay [43]. Samples were aliquoted and stored at -80 ^{O}C until performing the assays. Phosphofructokinase (PFK) (E.C.: 2.7.1.11) and lactate dehydrogenase (LDH) (E.C.: 1.1.1.27) activities and concentrations of pyruvate and lactate were measured in samples via enzymatic assays (all from Sigma-Aldrich). All assays have been optimized for the current sample types as explained in the previous studies [33,44] and specific protocol settings for each assay can be found in [33]. All assays were performed on a SpectraMax i3 (Molecular Devices) using appropriate 96-well plates.

Seahorse analyzer

The mitochondrial function of in vitro (with CAPA-IVM) and in vivo (baseline and SO) grown and matured COCs were studied using the Seahorse metabolic flux analyzer (XFe96) and the Mito Stress Test (Agilent Technologies). The Mito Stress Test allows for the study of mitochondrial function in real-time, through serial injection of several inhibitors of the electron transport chain and recording the oxygen consumption rate (OCR) of the cells. Basal cellular respiration, as well as mitochondrial respiration, maximal respiration, proton leak, ATP-coupled respiration, non-mitochondrial oxygen consumption, spare respiratory capacity and coupling efficiency are calculated using Seahorse Analytics (seahorseanalytics.agilent.com). Definitions of the reported endpoints are provided in Table 1.

The XFe96 sensor cartridge was hydrated overnight with sterile water in a CO_2 free incubator at 37°C. Following the first hydration step, water was replaced with calibrant solution (preincubated overnight at 37°C in a CO_2-free incubator) and the plate placed back into the CO_2 free incubator setting (at 37°C) for 1 hour. Meanwhile, Seahorse plates were coated with

Cell-Tak (Corning) following the basic coating protocol of the manufacturer. COCs were collected at the end of pre-IVM and IVM and washed three times in droplets of assay medium (prepared by supplementing XF DMEM with 1 mM pyruvate, 2 mM glutamine and 5 mM glucose [45]) before transfer into the Seahorse plate. Similarly, baseline, in vivo GV- and MII-COCs were collected following the same preparation protocol as described above. 5-15 COCs (one biological replicate) were transferred per assay well together with 20 μl XF assay medium (final assay volume per well is 180 μl), and the plate was placed into the CO_2 free incubator at 37°C to de-gas for 1 hour. Final concentrations (per well) for Mito Stress Test Kit reagents' oligomycin, FCCP (carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone) and Rot/AA (rotenone/antimycin A) were 1 μM, 2.5 μM and 2.5 μM, respectively [45]. Upon completing the runs with the Seahorse, the contents of each well were lysed using 75 μl extraction buffer (2% sodium dodecyl sulfate, 60 nM Tris, 100 mM DTT, protease and phosphatase inhibitor cocktails) [46]. Supernatants were collected after incubating the samples at 37°C for 30 mins followed by centrifuging at 4°C at 10,000 rpm for 15 mins. Protein concentration per sample (μg/μl) was calculated using Qubit Protein Assay (Thermo Fisher) and sample protein concentration was used to normalize the data. Three separate Mito Stress Test runs were completed, each with COCs from individual experiments.

CellROX and JC-1 staining, imaging and image analysis

CellROX Deep Red (Thermo Fisher) was used to measure the intracellular ROS induced oxidative stress. Mitochondrial membrane potential was calculated using the MitoProbe JC-1 assay kit (Thermo Fisher). All media and oil were pre-equilibrated at 37°C, 5% CO_2 and 100% humidity prior to use. Immunofluorescence labeling was carried out in 25 μl drops of staining solution (5 μM CellRox and 2 μM JC-1 in M16 medium) under oil. GV oocytes were mechanically denuded, transferred to the staining dish (one condition per droplet) and incubated for 30 mins at 37°C, 5% CO_2 and 100% humidity. Following three washing steps in M16 medium, oocytes were imaged in glass bottom confocal dishes (MatTek) in 2 μl dops of M16, under oil, on a Zeiss LSM 800 confocal microscope using live imaging chamber (37°C, 5% CO_2, and humidity).

Three images with a z-step of 10 μm were taken on a 40x magnification. Accumulated ROS signal was detected at 653 nm (red). JC-1 aggregates, indicating active mitochondria membrane potential was detected at 565 nm (orange), while JC-1 monomers reflective of electrochemical potential loss was measured at 493 nm (green). Fluorescence signal was measured for each channel, on all z-stacks, within the region of interest (in the ooplasm) as the mean intensity value using the image analysis wizard in ZenBlue (Zeiss software). The intensity values from three stacks were averaged as an individual value for each sample. Averaged intensity values were normalized against average intensity obtained in CAPA-IVM control. Overall mitochondrial membrane potential per GV oocyte was calculated as the aggregate to monomer ratio (orange/green) detected in the ooplasm [47].

Experimental Design

This study consisted of three parts, as illustrated in Figure 1. Designs of each experiment, as well as assessed end points, are explained below.

Experiment I: Mitochondrial function in the in vivo matured vs CAPA-IVM COCs

As a follow-up to the previously published results [33], mitochondrial function of the in vivo and in vitro matured COCs was assessed using the Seahorse metabolic flux analyzer and Cell-ROX staining. Furthermore, mitochondrial membrane potential was assessed through JC-1 staining. In vivo matured COCs and CAPA-IVM COCs were obtained as described above following hormonal stimulations and after standard CAPA-IVM culture. In order to track changes taking place during oocyte capacitation (following Folligon injection in vivo, during pre-IVM in vitro) a baseline in vivo condition was also included.

Experiment II: Individual and combined effects of lactate and super-GDF9 supplementation on CAPA-IVM COC metabolism

The individual effect of lactate on CAPA-IVM COC metabolism was studied trough supplementing the basal culture media with Calcium L-lactate hydrate (Sigma-Aldrich). An initial dose finding study was performed, during which the effect of two doses of lactate (1 mM and 2 mM) - added at the pre-IVM step- was evaluated on the MII rate and oocyte size. Dose selection for this initial dose finding study was based on literature[48]. No disruption in the system was detected with either of the doses (Supplementary Figure 1 A and B). Given the acidity of the environment affects oocyte maturation, and lactate concentration decreases in the reproductive tract during maturation [15], the effect of lactate in our CAPA-IVM system was tested through addition either to pre-IVM only (2 mM) or to both culture steps where lactate concentration was decreased during IVM (pre-IVM 2 mM and IVM 1 mM). End points were assessment of the MII oocyte rate, oocyte competence and performing enzymatic assays.

The individual effect of super-GDF9 on CAPA-IVM COC metabolism was studied by supplementing basal culture media with super-GDF9 (50 ng/ml in pre-IVM and 25 ng/ml in IVM). We have previously demonstrated that given doses of OSF supplementation of our CAPA-IVM system during both culture steps led to enhanced mucification and elasticity of the extracellular matrix in COCs, a feature that was in-line with cumulus cell gene expression results [38]. Thus, we applied the same culture protocol in the current study. We used super-GDF9 in the current study given its higher potency and purer chemical composition compared to cumulin [39]. Preparation of the engineered super-GDF9 is explained in detail in [39] and [38]. Given that oocyte competence and MII rates following super-GDF9 supplementation have already been assessed and reported in a previous study and concluded to be comparable [38], cultures from this condition were utilized to perform enzymatic assays.

Following the identification of the individual effects of lactate and super-GDF9, their combined effect was tested against individual supplementations. For combined testing, the pre-IVM culture step was supplemented with 2 mM lactate and 50 ng/ml super-GDF9 followed by the IVM culture step with 1 mM lactate and 25 ng/ml super-GDF9. Cultures from this experimental condition were performed to assess MII oocyte rate, MII oocyte size, oocyte competence, mitochondrial function, ROS accumulation and mitochondrial membrane potential.

Experiment III: Effect of oxygen tension

As a follow-up set of experiments, the effect of culture environment oxygen concentration was assessed. Considering the collective results obtained from experiments I and II, we focused on adjusting the oxygen tension during the pre-IVM step. For the control group, standard CAPA-IVM culture settings, as described above, were followed. For the experimental group (low oxygen CAPA-IVM), the COCs were cultured at 37 ^{O}C, 5% CO_2, 6% O_2 and 100% humidity during pre-IVM and 37 ^{O}C, 5% CO_2, atmospheric O_2 and 100% humidity during IVM. SO COCs were also included for assessment of mitochondrial function with the Seahorse Mito Stress Test. MII oocyte rate and oocyte competence, as well as mitochondrial function, ROS levels and mitochondrial membrane potential were assessed and compared between groups.

Statistical analysis

Maturation, D2 cleavage and blastocyst rates were arcsine transformed prior to statistical analysis with one-way ANOVA followed by Tukey's post hoc test. For the enzymatic assays and Mito Stress Test, all relevant groups were compared through ordinary one-way ANOVA using Sidak's correction on log_2 transformed data. For the Mito Stress Test, average OCR was calculated per group and per outcome, with individual values collected from three to four repeat tests. For CellROX and JC-1 staining, values collected from each experimental condition were normalized to the average value of the CAPA-IVM control GV oocytes, and the statistical difference was calculated either with one-way ANOVA (Experiments 1&2; unpaired; Sidak's correction) or with paired t-test (Experiment III). For all tests, significance was considered when $p<0.05$ and confidence interval was 95%. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, California, USA, www.graphpad.com).

RESULTS

Respiration patterns of immature SO and CAPA-IVM COCs are dramatically different

Mitochondrial respiration data highlight that cytoplasmic (Figure 2A) and nuclear (Figure 2B) maturation follow distinct patterns, as well as in vivo and in vitro maturation. Compared to baseline, mitochondrial respiration decreases in SO GV-COCs following folligon injection. On the other hand, mitochondrial respiration of CAPA-IVM GV-COCs is comparable to the baseline COCs, showing a different pattern to their SO counterparts. This difference between

in vivo and in vitro GV-COCs is also evident, as basal respiration rates of the two groups are significantly different (Figure 2C; p: 0.0077). Both mitochondrial and basal respiration increase during in vivo maturation (GV to MII transition) whereas in CAPA-IVM both decrease (Figure 2A, B and C; neither of the changes are statistically significant).

No differences were observed in ATP-production coupled respiration between the groups, although an increasing trend was observed for CAPA-IVM GV-COCs compared to SO (Figure 2D; p: 0.070). Proton leak was found to be significantly higher in CAPA-IVM GV-COCs compared to SO GV-COCs (Figure 2E; p: 0.0068). As explained in Table 1, basal respiration is the net sum of processes capable of oxygen consuming that are used to produce ATP and proton leak [49]. Coupled respiration is the coupled part of respiratory oxygen flux that pumps the fraction of protons across the inner mito-membrane which is utilized by the phosphorylation system to produce ATP from ADP and Pi. We detected higher coupling efficiency in SO GV-COCs compared to their in vitro counterparts (Figure 2G; p: 0.0317), supporting the higher proportion of ATP production in this group compared to proton leak, despite comparable ATP-coupled respiration between the groups, indicating higher basal respiration is mainly due to increased proton leak. As overall OCR are different between the groups, comparison between absolute spare respiratory capacity values would have been misleading, given it is a mathematical formula calculated by subtracting basal respiration from maximal respiration. Instead, we compared the spare respiratory capacity percentages, which did not show any difference between groups (Figure 2H).

CellROX staining of GV oocytes showed increased ROS content in oocytes following cytoplasmic maturation -both in vivo and in vitro- compared to baseline (Figure 2J; p (baseline vs SO): 0.0006, p (baseline vs CAPA-IVM): <0.0001). On the other hand, CAPA-IVM GV oocytes had significantly higher ROS levels compared to SO GVs, indicating the further ROS accumulating effect of the in vitro environment (Figure 2J; p: 0.036). While the mitochondrial membrane potentials of both SO and CAPA-IVM GV oocytes were significantly higher compared to baseline oocytes (both p values: <0.0001), no differences were detected between the two groups (Figure 2K).

Individual supplementation of lactate and super-GDF9 have limited effect on CAPA-IVM COC metabolism

We first compared the effect of lactate addition to pre-IVM only, and also to both steps of CAPA-IVM culture, to identify the best treatment protocol. Maturation rates and oocyte competency were comparable between the control and lactate supplemented group regardless of the CAPA-IVM step the COCs were cultured with lactate (Supplementary Figure 1 C-E).

Next, we assessed the glycolytic activity of both CCs and oocytes with enzymatic assays following lactate supplementation. Specific enzymatic assays (PFK and LDH activities, pyruvate and lactate concentrations) were selected based on the upregulated glycolysis observed in the previous study [33]. Lactate supplementation during the pre-IVM step of CAPA-IVM culture did not cause any changes in CCs for any of the studied enzymes or metabolites (Figure 3A, C, E, G). Even though the mean pyruvate concentrations within GV-CCs were almost halved after culturing with lactate compared to control, this change did not reach significance (Figure 3A; p:0.63). Both PFK and LDH activities as well as lactate concentrations were not different between the two conditions (Figure 3C, E, G). Within MII-CCs, pyruvate levels between the conditions were comparable to the GV stage (Figure 3A), while there was an increasing (yet not significant) pattern for PFK activity, LDH activity and lactate concentrations (Figure 3C, E, G) as the exposure time to lactate increased. The highest mean PFK and LDH activities, as well as lactate concentrations, were observed in the MII-CCs obtained following media lactate supplementation during both steps of CAPA-IVM (Figure 3C, E, G). Even though pyruvate concentrations of control CCs were halved during GV-to-MII transition, this difference did not reach significance (Figure 3A). Similarly, media lactate supplementation during pre-IVM or both steps did not affect CC pyruvate levels since they were comparable between GVs and both lactate MIIs. LDH activity (Figure 3E; p: 0.0051) and lactate concentration (Figure 3G; p: 0.0098) of MII-CCs increased significantly compared to GV-CCs when lactate was added throughout the CAPA-IVM culture period, instead of only in the pre-IVM step. A similar pattern was also observed for PFK activity, but it did not reach significance (Figure 3C).

Pyruvate levels of CCs were significantly higher compared to oocytes from control GV- and MII-COC, as well as lactate supplemented GV-COCs (Supplementary Figure 2A). On the other hand, PFK activity of CCs compared to oocytes were significantly higher in all of the analyzed groups (Supplementary Figure 2B). LDH activity of CCs and oocytes were comparable only in lactate supplemented GV-CCs (Supplementary Figure 2C). Significant differences between MII stage CC and oocyte lactate concentrations were observed when culture media was supplemented with lactate either during pre-IVM or both steps of CAPA-IVM (Supplementary Figure 2D).

GDF9 supplementation of pre-IVM media did not affect pyruvate and lactate concentrations of GV-CCs (Figure 3B and H). On the other hand, GDF9 supplementation significantly increased LDH activity in GV-CCs compared to the control group (Figure 3F; p <0.0001). While PFK activity in GDF9 supplemented GV-CCs almost doubled, this increase did not reach significance (Figure 3D). Pyruvate concentrations and PFK activities of MII-CCs were comparable regardless of the media supplementation (Figure 3B and D). While a slight increase in mean LDH activity and lactate concentration were observed in MII-CCs after GDF9 supplementation, this did not reach significance (Figure 3F and H). During GV-to-MII transition a significant decrease in pyruvate was detected, both in the control group and GDF9

supplemented group MII-CCs compared to GV-CCs (Figure 3B; p values: 0.0038 and 0.0003, respectively). On the contrary, LDH activity was significantly higher in the control group MII-CCs compared to GV-CCs (Figure 3F; p: 0.0327).

Pyruvate levels of CCs from both GV-COC conditions were significantly higher than in oocytes (Supplementary Figure 3A). Both PFK and LDH activities of CCs compared to oocytes were significantly higher in all of the analyzed groups (Supplementary Figure 3 B and C). CC lactate concentrations were higher than those in oocytes of GV-COCs from the control group, and GDF9 supplemented MII-COCs (Supplementary Figure 3D).

Combined lactate+super-GDF9 supplementation does not restore the boost in pre-IVM respiration

Oocyte maturation rates and IVF results, reflecting oocyte competence, of the groups are provided in Table 2. No differences were detected regarding the mature oocyte rate, D2 cleavage rate or D5 blastocyst rate, between any of the groups. When measuring the diameters of MII oocytes, we found that oocytes from the lactate only supplemented (mean diameter: 71.7±1.6 μm, n: 68) and GDF9 only supplemented (mean diameter: 71.6±1.9 μm, n: 52) groups were larger in size compared to the control group (mean diameter: 70.7±2.1 μm, n: 66; respective p values are 0.0064 and 0.0357), while they were comparable to the lactate+GDF9 combined group.

Mitochondrial respiration after pre-IVM (cytoplasmic maturation) was comparable in all CAPA-IVM groups with or without supplementation (Figure 4A). Although the experimental groups that received GDF9 only and lactate+GDF9 exhibited increased mitochondrial respiration profiles (Figure 4A), no significant differences were detected in either basal respiration (Figure 4C) or in maximal respiration (Figure 4D) between GV-COCs. Similarly, no differences were detected in ATP-coupled respiration (Figure 4E), proton leak (Figure 4F), non-mitochondrial oxygen consumption (Figure 4G) or coupling efficiency (Figure 4H) between any of the GV-COC groups.

Mitochondrial respiration profiles of CAPA-IVM MII-COCs were more distinctive (Figure 4B). Both the control and lactate only groups had lower mitochondrial respiration when compared to the GDF9 only and lactate+GDF9 supplemented groups (Figure 4B). While mitochondrial respiration profiles for the control and lactate only groups were lower compared to their GV states, for the GDF9 only and lactate+GDF9 supplemented groups, their mitochondrial respiration profiles were comparable to their immature counterparts.

Within MII-COCs, when compared to the lactate only group, the GDF9 only and lactate+GDF9 mix supplemented groups exhibited significantly higher basal respiration (Figure 4C; p values are 0.0005 and 0.0002; respectively), maximal respiration (Figure 4D; p values are 0.0002 and

0.0005; respectively), ATP-production coupled respiration (Figure 4E; p values are 0.021 and 0.0026; respectively), proton leak (Figure 4F; p values are 0.0001 and 0.0004; respectively) and non-mitochondrial oxygen consumption (Figure 4G; p values are 0.0164 and 0.0125; respectively). The GDF9 supplemented group also had significantly higher maximal respiration (Figure 4D; p: 0.047), proton leak (Figure 4F; p: 0.0253) and non-mitochondrial oxygen consumption (Figure 4G; p: 0.0003) when compared to the control MII-COCs. Furthermore, the lactate+GDF9 supplemented group had a significantly higher basal respiration rate (Figure 4C; p: 0.0233) and non-mitochondrial oxygen consumption rate (Figure 4G; p: 0.0002) when compared to control group.

When comparing changes during the pre-IVM to IVM transition, both basal respiration and maximal respiration were significantly lower in lactate only supplemented MII-COCs compared to the same group GV-COCs (Figure 4C and D; p: 0.0014 and 0.0054, respectively). While an overall decrease was observed in MII-COC proton leak, the lactate-only supplemented condition exhibited the most pronounced decrease in proton leak (Figure 4F; p: <0.0001) followed by the control group (p: 0.016), while no such significance was observed for the other groups. Although coupling efficiency tended to increase during final nuclear maturation, indicating global basal respiration dedicated for ATP production increased during nuclear maturation in all CAPA-IVM groups, only MII-COCs that were cultured with the lactate+GDF9 mix during CAPA-IVM had a significantly higher values when compared to their respective GV-COCs (Figure 4H; p: 0.0340). Non-mitochondrial oxygen consumption significantly decreased during the GV-to-MII transition in the control group (Figure 4G; p: 0.008); whereas for the other groups, non-mitochondrial oxygen consumption of GV- vs MII-COCs was comparable.

Finally, no differences were detected in ROS accumulation and mitochondrial membrane potential between CAPA-IVM oocytes, whether the culture media was supplemented with lactate and/or GDF9 or not (Figure 5).

Low oxygen tension during pre-IVM restores basal respiration profile of CAPA-IVM COCs

Oocyte maturation rates and IVF results, indicating oocyte competence, of the groups are provided in Table 2. No differences were detected regarding mature oocytes rate, MII oocyte diameter, D2 cleavage rate or D5 blastocyst rate between groups (Table 2).

Mitochondrial respiration profiles showed that when COCs were cultured in low oxygen during pre-IVM, their profile resembled SO GV-COCs more than CAPA-IVM control GV-COCs cultured in atmospheric oxygen (Figure 6A). On the other hand, mitochondrial respiration of the same group of COCs were lower compared to both SO and CAPA-IVM control groups at MII stage (Figure 6B). No differences were detected in basal respiration (Figure 6C), ATP-production coupled respiration (Figure 6D) and spare respiratory capacity % (Figure 6H)

between groups. Proton leak was significantly higher in control CAPA-IVM GV-COCs compared to SO GV-COCs (Figure 6E; p: 0.087). Supporting the higher proton leak with regular CAPA-IVM GV-COCs, their coupling efficiency was significantly lower compared to SO GV-COCs (Figure 6G; p: 0.048). The only difference detecting during the GV-to-MII transition was in coupling efficiency, which significantly increased in the control CAPA-IVM group as the COCs matured (Figure 6G; p: 0.0358).

No differences were detected in ROS accumulation and mitochondrial membrane potential between CAPA-IVM oocytes whether they were cultured in atmospheric oxygen or low oxygen during the pre-IVM culture period (Supplementary Figure 4).

DISCUSSION

IVM is an alternative ART protocol that is mainly offered to women suffering from polycystic ovarian syndrome (PCOS) [2]. Although the benefits of IVM include a lower risk of developing ovarian hyperstimulation syndrome (OHSS) and a reduced treatment burden for patients, its clinical applications are not well established yet due to lower efficiency rates compared to conventional IVF [13]. Through recent advancements partially revealing the complexity of oocyte maturation, and concomitantly, introduction of bi-phasic IVM systems, IVM oocytes' developmental competence has been successfully enhanced. CAPA-IVM is one such bi-phasic IVM protocol, which uses C-type natriuretic peptide (CNP) during the pre-IVM step to regulate cAMP concentrations to block oocyte meiosis resumption, followed by an IVM step with epidermal growth factor network peptides and FSH to induce nuclear maturation [9,14]. CAPA-IVM has proven its superiority over standard IVM protocols in humans, through clinical studies showing improved maturation and pregnancy rates [9,11–13]. Despite this success, the high attrition rates from mature oocytes to good quality embryos is still keeping the efficiency of CAPA-IVM at lower levels compared to conventional IVF. Hence, establishing culture strategies addressing this issue is the current fundamental challenge.

Recently we have shown perturbances in CC glucose metabolism of CAPA-IVM COCs that are inflicted during pre-IVM culture, and lead to precociously increased glycolytic activity compared to their in vivo counterparts [33]. Considering the metabolic needs and the microenvironment of the maturing small-/mid-antral COCs, we hypothesized that further optimization of CAPA-IVM could be possible through focusing on adjusting culture media nutrient concentrations and/or oxygen tension [33], which are both important factors in regulating CC and oocyte metabolism. Cooperation between both compartments of the COC is important not only for their individual performance, but also to provide the required energy for supporting oocyte maturation, fertilization and preimplantation embryo development [50–52]. Several studies have attributed the major glycolytic role to the CCs and mitochondrial ATP production to the oocytes within the COCs [17,18,20,24,33]. Even though oocytes from the previous study did not exhibit any altered metabolic pattern that could be associated with

lower competency, that analysis was limited to spectrophotometric measurements of glycolysis and tricarboxylic acid cycle enzymes and metabolites, without providing any direct insights into oocyte OXPHOS and mitochondrial function [33]. Therefore, in the current study, we initially evaluated the COC mitochondrial function and components of cellular oxygen consumption in real-time with the Seahorse extracellular flux analyzer, to reveal the oocyte's metabolic performance and demands during maturation. The decision to analyze COCs as a whole complex rather than focusing on the individual cell types was made based on proven essential bi-directional communication between CCs and oocytes [29,37]. Nevertheless, as 95% of the oxygen is directed to cumulus-enclosed-oocytes [24], real-time OCR of COCs can be used as a straightforward indicator of functionality of oocyte mitochondria.

Analyzing real-time OCR, we found that following Folligon injection, mitochondrial respiration of the SO GV-COCs decreased compared to baseline COCs. On the contrary, mitochondrial respiration did not change compared to baseline levels when COCs were placed in pre-IVM culture. This dramatic difference between SO GV-COCs and CAPA-IVM GV-COCs was observed also in the basal respiration rates, showing that OCR was significantly higher in in vitro cultured GV-COCs. Taken together with the previous results indicating a boost in CC glycolysis [33], current results show that it is the oocyte's increased OXPHOS that stimulates CC glycolysis during pre-IVM, since pyruvate oxidation by oocytes cannot be completed without substrates provided by CCs [27,53].

Basal respiration is the net sum of processes capable of consuming oxygen which are ATP-production coupled respiration and proton leak [49]. We found that in CAPA-IVM GV-COCs, proton leak is significantly higher compared to SO GV-COCs. Proton leak reflects the level of protons migrating to the mitochondrial matrix without resulting in ATP production [54]. Furthermore, CellROX staining of GV oocytes showed that cytoplasmic maturation increases the abundance of ROS. Although ROS are in general perceived as damaging by-products of OXPHOS, they are also important regulatory signaling molecules responsible for growth and development [55], a finding supported through our current results as well. However, given the significantly higher CellROX signal in the CAPA-IVM GV oocytes compared to SO oocytes, it is plausible to say that the pre-IVM culture is inflicting oxidative stress.

We did not observe any significant differences between respiration profiles of MII-COCs matured in vivo vs in vitro, possibly indicating that even though perturbances were inflicted in pre-IVM, the bioenergetic profile of COCs was able to resume normo-patterns during IVM. Even though, GV-to-MII transition was marked by a decrease in mitochondrial respiration and basal OCR, supporting the findings by Scantland et al. [56], the percentage of basal respiration dedicated for ATP-production increased, probably in an attempt by the oocyte to prevent ATP deficit. The only significant change during GV-to-MII transition was detected in non-mitochondrial oxygen consumption, which was significantly lower in CAPA-IVM MII-COCs than GV-COCs. Non-mitochondrial oxygen consumption is presumed to increase when there

is an increase of cytoplasmic ROS [57]. Similarly, its decrease can possibly be explained as removal of the ROS from the environment. As proton leak between both groups of MII-COCs are comparable, indicating an absence of mitochondrial potential exploitation, this detected decrease in non-mitochondrial oxygen consumption could be another indication for restoration of normal bioenergetic patterns.

Lactate supplementation to culture media did not induce any significant changes in CC glycolysis. When media was supplemented with lactate during both steps of CAPA-IVM, lactate concentrations and LDH activity increased in CCs, without changing pyruvate concentration or PFK activity. While this is indicative of lactate being taken up from the environment by the CCs and metabolized, comparable mitochondrial respiration in COCs supports the findings of Dumollard et al. indicating that lactate-derived pyruvate cannot be used by oocytes to produce ATP [26]. GDF9 only supplementation to culture media slightly increased the activity of glycolytic enzyme PFK in CCs, although not significantly. On the other hand, while GDF9 supplementation led to a significant increase in LDH activity, we did not observe any difference in CC pyruvate and lactate levels. Given that mitochondrial respiration increased in GDF9 supplemented CAPA-IVM COCs, even though we did not observe a significant increase in CC PFK activity, it is clear that more substrates were delivered to the oocyte for ATP production by the CC compartment, as oocyte and CC metabolisms are interdependent[37,58,59].

Real-time metabolic analysis of COCs after individual or combined supplementation of lactate and GDF9 showed that none of the tested supplement combinations were able to restore the in vivo COC's respiration profile (all were either comparable to or higher than the control, which was already dramatically higher compared to SO GV-COC). When COCs were supplemented with lactate only throughout CAPA-IVM culture, they exhibited the lowest levels of mitochondrial and basal respiration compared to other MII-COCs. While these levels were comparable to the control group, they were significantly lower than the COCs supplemented with GDF9 only and lactate+GDF9 mix. The same group also exhibited the maximum decrease in proton leak during GV-to-MII transition, as well as decreased basal respiration. Yet, the ratio of ATP-coupled respiration to proton leak was comparable within all CAPA-IVM MII-COCs, indicating that while this decrease might not have any biological relevance it could result in decreased ATP concentrations within oocytes. Furthermore, results indicated that GDF9 is the principal substance inflicting changes in oocyte's oxygen consumption, since GDF9 only and lactate+GDF9 mix supplemented COCs exhibited comparable OCR for all analyzed components, both at GV and MII stages. COCs supplemented with GDF9 only and lactate+GDF9 mix exhibited comparable levels of ATP-production coupled respiration to control COCs. The most significant difference between the control group and the GDF9 supplemented groups was detected at non-mitochondrial OCR, yet there was not a significant difference in ROS production and mitochondrial function between MII-COCs. In leukocytes, the increase in non-mitochondrial OCR is attributed to the increase of

inflammatory enzymes [60]. COC expansion and ovulation during oocyte maturation have been compared to an immune-like response, with similar characteristics to inflammation [61]. While IVM CCs generally have aberrant expression of genes from the aforementioned processes [62], we recently showed that OSF addition during CAPA-IVM culture significantly regulates expression of genes involved in CC expansion to a level comparable to in vivo matured COCs [38]. Proper CC function is a prerequisite for ovulation and fertilization [63], and OSFs play a crucial role for CCs to attain their role [64]. Given that CAPA-IVM COCs exhibit better mucification in the presence of GDF9, this could be a possible reason for significantly higher non-mitochondrial OCR. Still, the significant increase in overall oxygen consumption following GDF9 supplementation requires further studies for clarification.

Lactate is the end-product of lactic acid fermentation, with a known role of inhibiting the activity of the glycolysis enzyme PFK in several tissues [34]. Even though lactate-only supplementation to CAPA-IVM media limited COC oxygen consumption during IVM, it failed to act as a glycolysis-limiting nutrient as expected during the pre-IVM step. Consequently, we concluded that regardless of the culture media supplementation, it is the pre-IVM culture environment itself that is boosting metabolic activity in COCs. The direct relationship between culture environment oxygen concentration and the ATP content of IVM oocytes has been shown by Hashimoto et al. [42]. Oxygen concentration of the reproductive tract varies between 2%-9% [40,41] which is significantly lower than atmospheric oxygen concentrations. However, the optimal oxygen microenvironment to use during in vitro culture is still one of the most debated topics of IVM optimization strategies, as studies have reported contradictive results. Banwell et al. indicated that changing oxygen concentrations during mouse IVM does not affect oocyte maturation, fertilization, or embryo development rates [65]. On the other hand, Preis et al. showed improved oocyte competence following IVM at 5% oxygen [66]. In bovine IVM, 5% oxygen tension reduced the number of mature oocytes, but those that matured exhibited better embryonic developmental capacity compared to the oocytes matured under 20% oxygen [42]. In the current study, given no differences were observed in COC metabolic activity following media nutrient supplementation, we cultured the COCs in a low oxygen environment during pre-IVM to physically limit their access to oxygen and tested oocyte competency and COC metabolism.

The most striking improvement in COC metabolism was obtained when COCs were cultured at low oxygen tension during pre-IVM. Following real-time metabolic analysis of COCs, we found that GV-COCs cultured under 5% oxygen during pre-IVM had decreased mitochondrial respiration, which was more similar to SO-GVs than GV-COCs grown under 20% oxygen. While not significant, basal respiration of the 5% oxygen group was also lower than for the 20% oxygen group. Proton leak in the 5% oxygen group was comparable to the SO-GV group, while GV-COCs cultured under 20% oxygen had significantly higher proton leak compared to SO-GVs. Similar to the proton leak, coupling efficiency of GV-COCs cultured at 5% oxygen was comparable to SO GV-COCs. Surprisingly, we did not detect any difference in GV oocyte ROS

levels and mitochondrial membrane potential after altering oxygen tension. On the other hand, both mitochondrial respiration and basal OCR of MII-COCs that were cultured under 5% oxygen during pre-IVM were slightly lower compared to both SO and 20% oxygen COCs.

In embryos, higher metabolic activity is related to lower developmental potential due to molecular and cellular damage inflicted by ROS accumulation [67–69]. Elevated oxygen concentrations increase ROS generation [70], which leads to severe damage in DNA. In fact, the highest DNA damage rates are found in the most metabolically active embryos [71]. Upon sensing external stimuli, such as oxygen tension, cells normally respond to maintain homeostasis through regulating the expression of several genes [72]. However, transcriptional machinery is regulated differentially in oocytes compared to somatic cells, as mRNA transcription ceases by the time the oocyte is ready for nuclear maturation [51,73]. Following the resumption of meiosis, maternally stored mRNAs are translated to support the zygote until activation of their genome [74]. Therefore, in addition to higher ROS accumulation due to hyper-active metabolism, oocytes might not be equipped to maintain homeostasis through stimulating transcriptomic changes upon encountering hyperoxia during CAPA-IVM, ultimately leading to lower embryo quality.

Both GV and MII oocytes exhibit a hooded mitochondrial ultrastructure, with fewer cristae, which is an atypical phenotype with a reduced capacity for OXPHOS, compared to other cell types [56,75–77]. In contrast, the energy demands of an oocyte during cytoplasmic maturation is massive given the essential biological transition including cytoskeleton remodeling, organelle reorganization and nuclear reprograming [78]. Essentially, a cell's energy demand is the driving force for ATP production, which is challenged under stress conditions. The fact that we did not observe any significant differences in spare respiratory capacity % in both GV- and MII-COCs indicates preserved mitochondrial flexibility, despite stress-inducing pre-IVM conditions. Moreover, considering the assessed endpoints for oocyte competence between COCs cultured under 5% vs 20% O_2 during pre-IVM did not reflect any changes, the current slightly lower mitochondrial respiration profile of the MII-COCs cultured under 5% oxygen during pre-IVM is not alarming. Recently, the adenosine salvage pathway has been suggested as an alternative and complementary ATP production pathway to prevent energy deficit during oocyte maturation [56,79]. This two-step reaction that produces ATP through accumulated cAMP could be the reason for comparable oocyte competence between the two groups. However, given that mitochondria are inherited maternally, oocytes with dysfunctional mitochondria might still lead to deficiencies post maturation, and cause lower rates of good quality embryos in IVM systems. Thus, it is necessary to focus on improving basal respiration rates and balancing ROS levels of MII-COCs during the IVM step following pre-IVM culture at 5% oxygen.

Our study did not highlight any improvement in oocyte competence, with the current assessment criteria, following media supplementation of lactate and/or GDF9. Our current

unstimulated pre-pubertal mouse model is successful in imitating the low competence human oocytes obtained from 2-8 mm follicles after minimal FSH stimulation. Our long-term aim is to assess the success of the advances in our mouse CAPA-IVM protocol by embryo implantation potential, live birth rates and newborn health. On the other hand, one limitation of the model is the absence of any infertility etiology that interferes with the detection of short-term improvements in oocyte competence [38]. Testing our current findings in a PCOS mouse model would be very interesting, given patients with PCOS have a distorted metabolic profile compared to normo-ovulatory patients which causes increased oxidative stress in oocytes [80]. A ROS regulatory role of CAPA-IVM has already been proposed through observed changes in CC gene expression [12]. In this study, even though reduced oxygen tension was successful in improving the bioenergetic profile, it did not change the intensity of accumulated ROS in GV oocytes. Hence, antioxidant supplementation could also be utilized to improve ROS accumulation during CAPA-IVM under low oxygen concentrations.

To the best of our knowledge, this is the first study reporting the respiratory profiles of mouse COCs with real-time metabolic analysis, both during bi-phasic IVM and during in vivo maturation. Results show that pre-IVM culture is boosting GV oocytes' OXPHOS and inflicting oxidative stress, and neither individual nor combined supplementations of lactate and GDF9 are effective in limiting pre-IVM CC glycolysis. However, the reduction of oxygen tension during pre-IVM, revealed a culture strategy to regulate OCR and metabolism of CAPA-IVM COCs, paving the way for future investigations in the human CAPA-IVM system.

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FIGURES

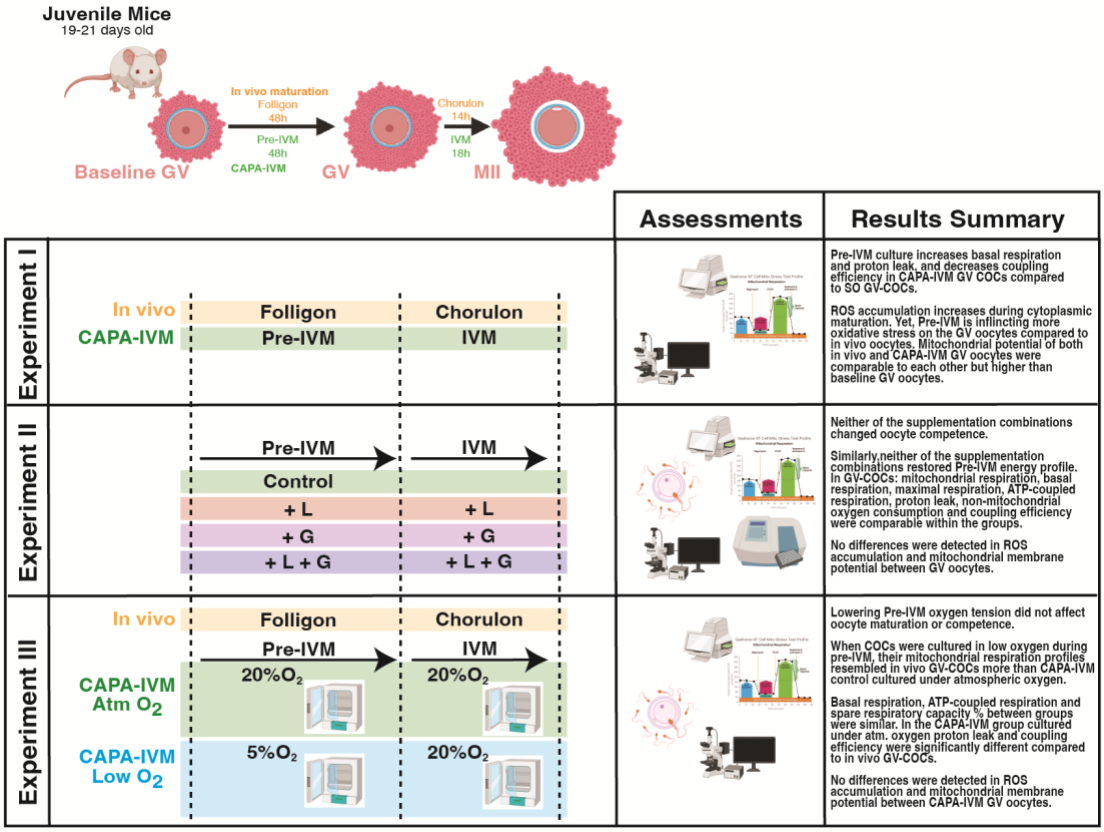


Figure 1: Illustration of experimental design and brief summary of results. In Experiment I, mitochondrial function (Seahorse Analyzer, confocal imaging) of in vivo and CAPA-IVM matured COCs was studied. Experiment II focused on individual (enzymatic assays, IVF, Seahorse Analyzer) and combined effects (IVF, Seahorse Analyzer, confocal imaging) of lactate and super-GDF9 supplementation to CAPA-IVM culture media. In Experiment III, effect of low oxygen tension during pre-IVM on oocyte competence (IVF) and COC metabolism (Seahorse Analyzer, confocal imaging) was assessed. L: Lactate. G: Super-GDF9. Atm: Atmospheric. Figure was created using BioRender.

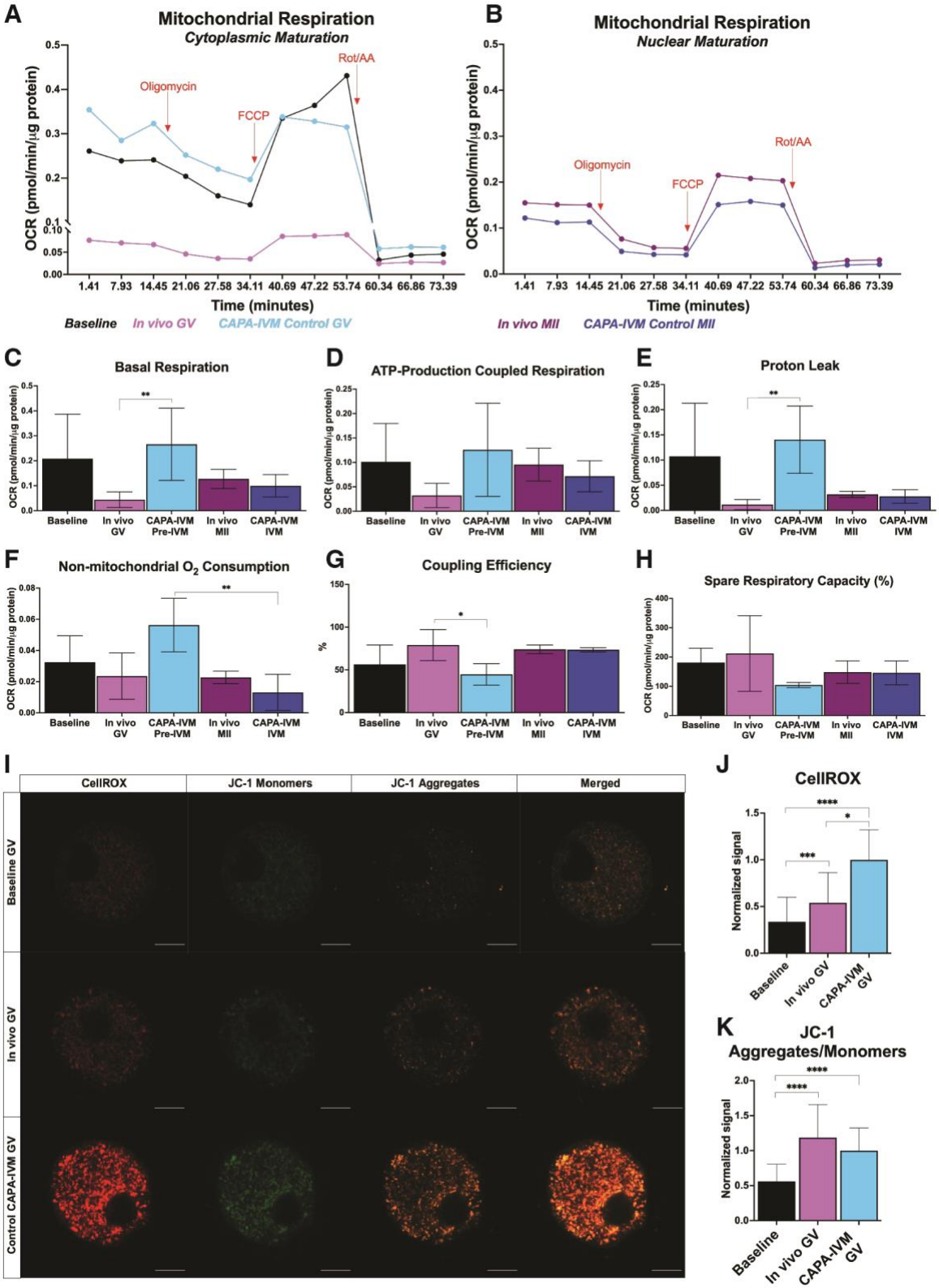


Figure 2: Mitochondrial function, ROS accumulation and mitochondrial membrane potential were compared between in vivo vs CAPA-IVM matured COCs. **(A-H)** show results obtained through real-time metabolic analysis with Mito Stress Test (Seahorse Analyzer). **(I-K)** Panels illustrates CellROX (red), JC-1 monomer (green) and JC-1 aggregate (orange) signals collected through imaging GV oocytes. Scale bar is 20 µm. *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001. Error bars indicate standard deviation. OCR: Oxygen consumption rate.

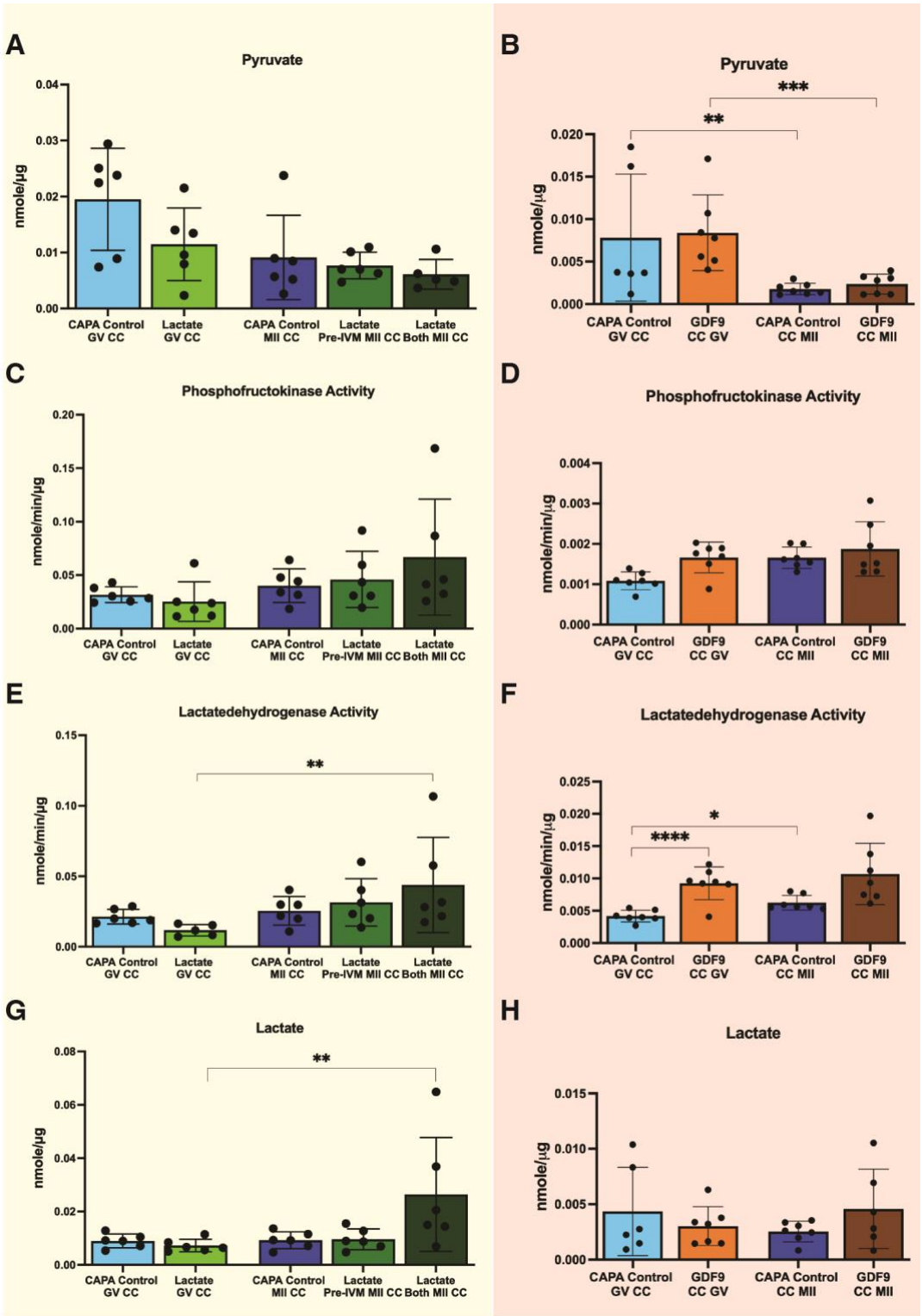


Figure 3: Individual effects of lactate (**A, C, E, G;** yellow background) and super-GDF9 (**B, D, F, H;** red background) supplementation during CAPA-IVM culture were assessed through enzymatic assays in cumulus cells. Each dot represents one biological replicate. *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001. Error bars indicate standard deviation.

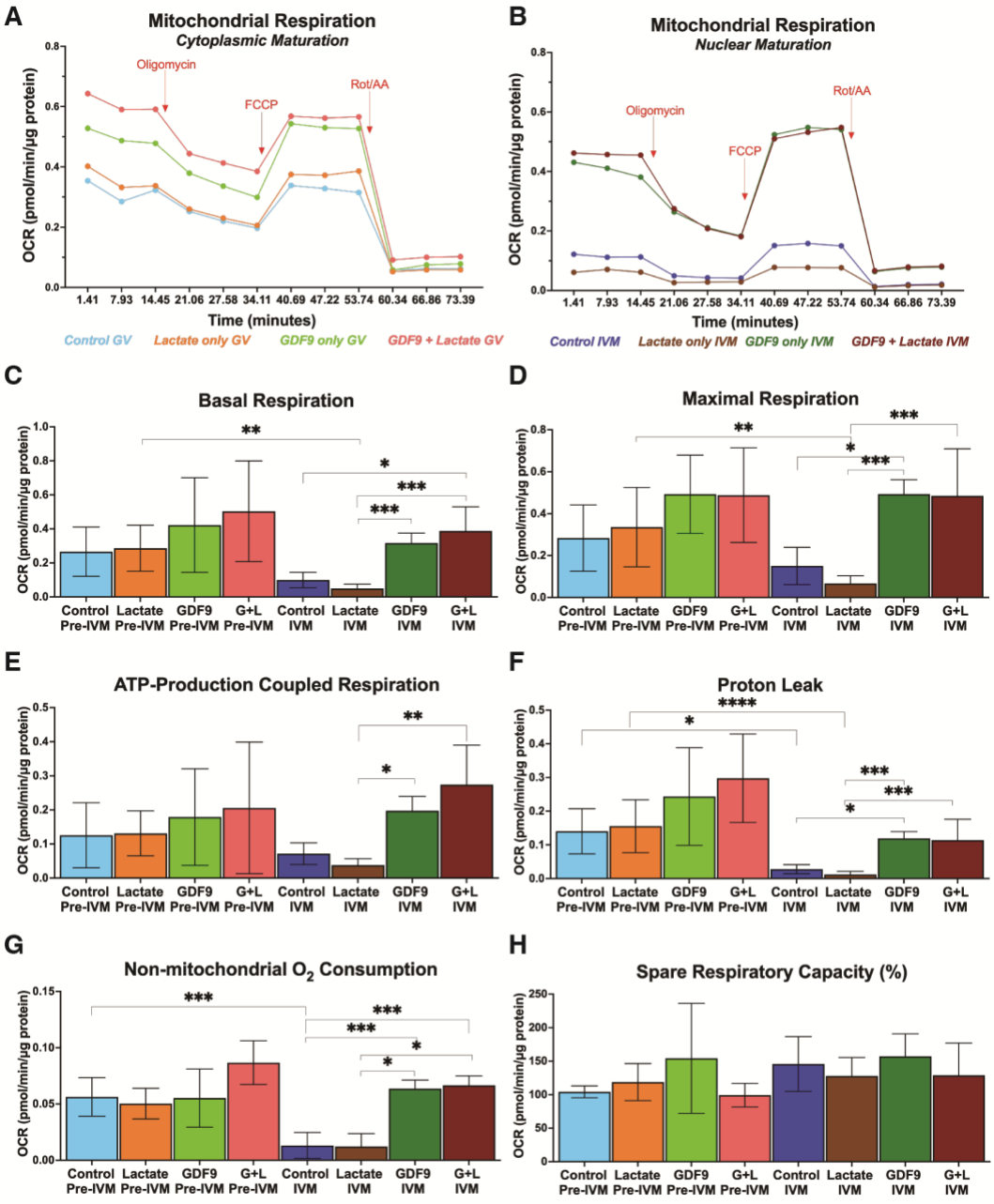


Figure 4: Mitochondrial function was compared within CAPA-IVM cultured GV- and MII-COCs. through real-time metabolic analysis with Mito Stress Test (Seahorse Analyzer). *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001. Error bars indicate standard deviation. L: Lactate. G: Super-GDF9. OCR: Oxygen consumption rate.

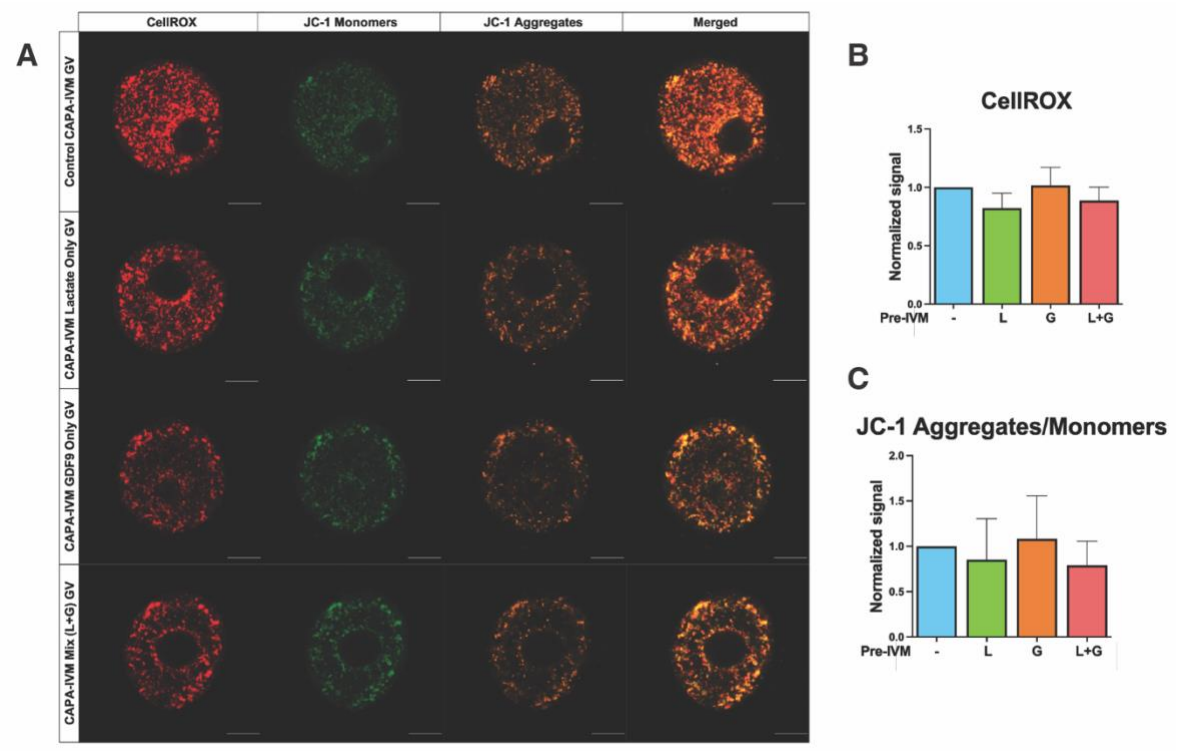


Figure 5: ROS accumulation and mitochondrial membrane potential were compared within CAPA-IVM cultured GV oocytes. **(A)** Panel illustrates CellROX (red), JC-1 monomer (green) and JC-1 aggregate (orange) signals collected through imaging GV oocytes. Scale bar is 20 μm. **(B)** shows average CellROX signal and **(C)** shows mitochondrial membrane potential, both data collected from three separate experiments. L: Lactate. G: Super-GDF9.

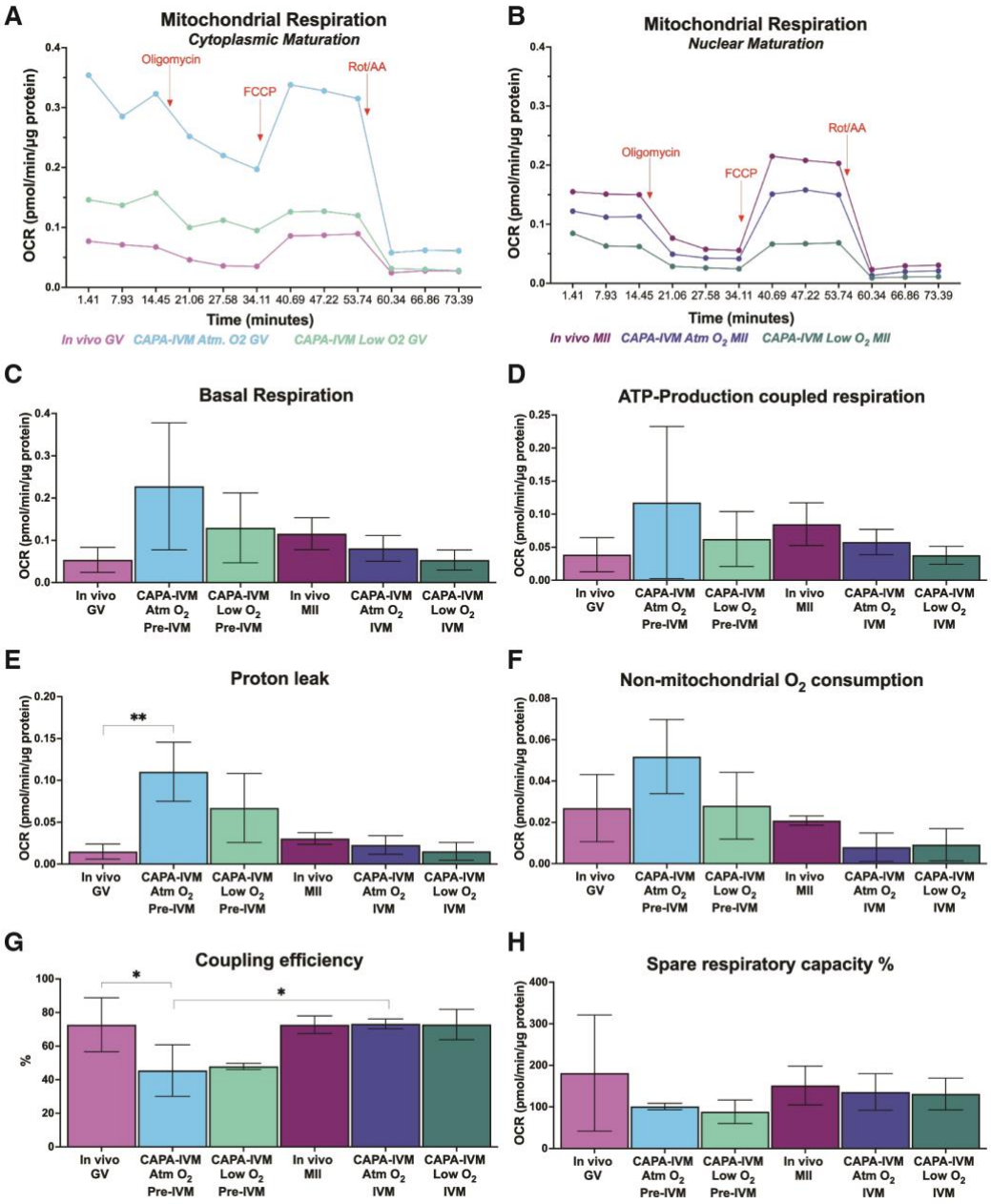


Figure 6: Mitochondrial function was compared between SO COCs, and CAPA-IVM COCs cultured under atmospheric oxygen vs low oxygen during pre-IVM, through real-time metabolic analysis with Mito Stress Test (Seahorse Analyzer). *: $p<0.05$, **: $p<0.01$. Error bars indicate standard deviation. Atm: Atmospheric. OCR: Oxygen consumption rate.

TABLES

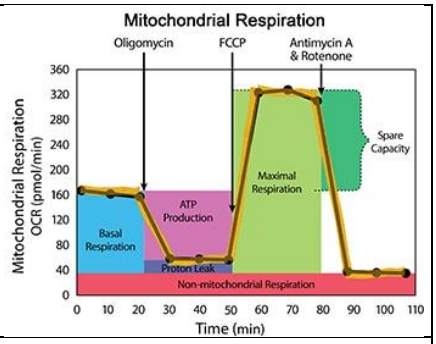
Mitochondrial respiration	Cellular mitochondrial respiration profile is indicated using Mito Stress Test. Following injections of three inhibitors, mitochondrial profile of the given cell type is determined. 
Basal respiration	Respiration required to meet energy demand of the cell. It is the sum of respirations used to perform ATP synthesis and associated with proton leak.
Proton leak	Indicates protons migrated to the mitochondrial matrix without resulting in energy production. Oligomycin addition inhibits the activity of ATP synthase and shows the rate of oxygen consumed by proton leak.
ATP-production coupled respiration	Indicates respiration dedicated for mitochondrial ATP synthesis.
Coupling efficiency	Identifies the percentage of OCR that is coupled with ATP synthesis.
Maximal respiration	Cell's efficiency to respond increased energy demand under stress. FCCP disrupts mitochondrial membrane potential and drives oxygen consumption to the maximum.
Spare respiratory capacity	Through FCCP stimulated max OCR spare respiratory capacity can be measured and it indicates cell's flexibility to respond increased energy demand under stress.
Non-mitochondrial respiration	Rot/AA shuts down mitochondrial respiration and enables measurement of oxygen consumed by non-mitochondrial processes. This could increase as a response to ROS or inflammatory processes.

Table 1: Explanation of Mito Stress Test assay parameters [49].

Experiment II				
	MII oocyte (%)	**MII oocyte size (μm)**	**Cleavage (%)**	**Blastocyst/Cleaved %**
Control	77.7 ± 3.5	70.7 ± 2.1[a]	53 ± 19.1	78 ± 5
Lactate only	80.3 ± 7.1	71.7 ± 1.6[b]	49.7 ± 16.9	80.7 ± 18.1
GDF9 only	73 ± 6	71.6 ± 1.9[bc]	59.3 ± 9.5	62 ± 27.8
Lactate + GDF9	75.7 ± 8.1	71.3 ± 1.6[abc]	55.3 ± 30	70.3 ± 36.1
Experiment III				
	MII oocyte (%)	**MII oocyte size (μm)**	**Cleavage (%)**	**Blastocyst/Cleaved %**
Control Atm O_2	77.7 ± 3.5	70.7 ± 2.1	57 ± 16.4	79.7 ± 2.9
Control Low O_2	78.3 ± 6.8	70.9 ± 1.9	38 ± 19.9	73.3 ± 30

Table 2: Effect of lactate and/or super-GDF9 addition (Experiment II) and pre-IVM oxygen tension (Experiment III) on CAPA-IVM oocyte maturation and competence.