TITLE

A refined culture system of oocytes from early antral follicles promotes oocyte maturation and embryo development in cattle

AUTHORS

Rodrigo Garcia Barros, Valentina Lodde, Federica Franciosi, and Alberto Maria Luciano*

*corresponding

ABSTRACT

The present study aimed to improve the in vitro culture of bovine oocytes collected from early antral follicles (EAFs) to support the progressive acquisition of meiotic and developmental competence. The rationale that drove the development of such a culture system was to maintain as much as possible the physiological conditions that support the oocyte growth and differentiation in vivo. To this extent, oocytes were cultured for 5 days, which parallels the transition from early to medium antral follicles (MAFs) in the bovine, and supports promoting a 3D-like structure were provided. Additionally, the main hormones (follicle-stimulating hormone, estradiol, progesterone, and testosterone) were added in concentrations similar to the ones previously observed in bovine EAFs. The meiotic arrest was imposed using cilostamide. The cultured cumulus–oocyte complexes (COCs) reached a mean diameter of 113.4 ± 0.75 µm and showed a progressive condensation of the chromatin enclosed in the germinal vesicle (GV), together with a gradual decrease in the global transcriptional activity, measured by 5-ethynyl uridine incorporation. The described morpho-functional changes were accompanied by an increased ability to mature and develop to the blastocyst stage in vitro, although not matching the rates obtained by MAF-retrieved oocytes. The described system improves the current state of in vitro culture of growing oocytes in the bovine species, and it can be used to increase the number of gametes usable for in vitro embryo production in animals of high genetic merit or with specific desirable traits.

INTRODUCTION

Assisted reproductive technologies (ARTs) have become essential to managing reproduction in animal breeding, restoring species on the brink of extinction, and treating infertility in humans. However, the small supply of mature, fertilizable oocytes is one of the main limitations to the success rate of assisted reproduction (Lonergan & Fair 2008, Galli 2017). Even though the mammalian ovary is potentially the source of thousands of oocytes enclosed into follicles at various stages of development, only a few of them reach a stage ensuring the generation of viable embryos and can be successfully used for in vitro embryo production (IVEP). In the cow, only fully grown oocytes that are enclosed in medium and large antral follicles (MAFs and LAFs, respectively) are routinely used in IVEP (Fair 2003). The remaining and most abundant part constitutes oocytes enclosed in primordial follicles – representing the ovarian reserve – and, to a lesser extent, growing oocytes in primary, secondary, and small antral follicles (EAFs). The oocytes contained in these follicle populations are not endowed with the molecular, biochemical, and cellular machinery that allows meiosis resumption and the formation of an egg suitable for fertilization and embryo development (Fair 2003).

In vitro growth of primordial follicles has the potential to produce mature oocytes capable of generating live offspring (Eppig & O'Brien 1996). However, this technique is still experimental and requires the development of specific multistep culture systems (McLaughlin & Telfer 2010), which are, to date, far from being efficient and reproducible (Telfer 2019). By contrast, oocytes enclosed

in EAFs, a population estimated between 100 and 1000 follicles per ovary in pubertal cows, may represent a more readily exploitable source of female gametes (Erickson 1966, Lussier et al. 1987, Silva-Santos et al. 2011, Modina et al. 2014).

In bovine, growing oocytes isolated from EAFs (0.5–2 mm) show a mostly uncondensed and dispersed chromatin in the germinal vesicle (GV, the oocyte nucleus), defined as GV0 configuration (Lodde et al. 2007). At this stage, oocytes are transcriptionally active and functionally coupled with the surrounding cumulus cells through gap junctions (GJs) (Lodde et al. 2007, 2008, Alam et al. 2018). However, they are incapable of resuming meiosis spontaneously and supporting embryo development. With the transition to MAFs (2–8 mm), the bovine oocyte undergoes significant changes, associated with the completion of the growth phase, such as the shift from GV0 to higher degrees of chromatin compaction, namely GV1, GV2, and GV3 configurations (Luciano et al. 2014). This transition is accompanied by progressive transcriptional silencing (Lodde et al. 2008), changes in the epigenetic signatures (Lodde et al. 2009, Labrecque et al. 2015, Lodde et al. 2017), and cytoplasmic organelle redistribution (Lodde et al. 2008). Also, the somatic compartment is affected by GV configuration transition, as demonstrated by the changes in the hormonal composition of the follicular fluid (Henderson et al. 1982, Kruip & Dieleman 1985) and major changes in the expression profile of cumulus cells (Dieci et al. 2016). Importantly, all these changes progressively confer a complete meiotic and embryonic developmental competence (Lodde et al. 2007, Luciano et al. 2011).

The definition of efficient in vitro culture protocols of EAF-derived oocytes may increase the ovarian reserve's exploitation, thus providing more oocytes suitable for ARTs. Furthermore, it would provide an invaluable experimental model to study the determinant of oogenesis. In cattle, several studies have been conducted on oocytes isolated from EAF, reporting significant variations in the maturation and blastocyst rates (5–79% and 4–12%, respectively) (Harada et al. 1997, Yamamoto et al. 1999, Hirao et al. 2004, Alm et al. 2006, Luciano et al. 2011, Taketsuru et al. 2012, Endo et al. 2013, Makita & Miyano 2014, Alam et al. 2018). Some of the culture conditions were somewhat consistent between different laboratories, such as the diameter of the follicle of origin, the initial oocyte diameter, the use of TCM199, the supplementation with hypoxanthine, and the CO2 partial pressure. Conversely, the presence of a collagen substrate, the length of culture, and the hormonal supplementation were identified as main variables between protocols, with the result that no consensus on the best practice for in vitro culture of growing oocytes have been achieved to date (Araujo et al. 2014, Simon et al. 2020).

Indeed, the transition from the growing phase to the fully grown state represents a critical step in oocyte development, and the consideration of the physiological requirements might prove essential in order to overcome the current limits of in vitro culture of growing oocytes (McLaughlin & Telfer 2010, Telfer et al. 2019). For instance, the physical environment should preserve the architecture of the COC complex, ensuring the bidirectional communications between the oocyte and the cumulus cells, which are essential to the progressive achievement of meiotic and embryonic developmental competence (Wigglesworth et al. 2013). Also, the hormonal milieu should be carefully considered to mimic the environment of a healthy growing follicle.

With this in mind, in vitro oocyte culture (IVCO) was refined to increase the meiotic and developmental competence. Compared to the previous IVCO (Luciano et al. 2011), the duration of the culture was prolonged to 5 days, a matrix-coated culture surface was introduced, the viscosity of the medium was increased, the hormonal supplementation was refined, and zinc sulfate was

supplemented. Particular attention was paid to maintaining adequate cyclic nucleotides levels by combining low FSH levels and the phosphodiesterase-3 (PDE3) inhibitor cilostamide to promote functional gap junction-mediated communications between oocyte and cumulus cells (Franciosi et al. 2014, Luciano et al. 2011). The efficiency of this culture system, named long IVCO (L-IVCO), was evaluated by assessing oocyte growth, chromatin remodeling, transcriptional activity, cumulus expansion after in vitro maturation (IVM), and meiotic and developmental competence.

MATERIALS AND METHODS

All chemicals and reagents used in this study were purchased from Merck–Sigma–Aldrich, Italy, except for those specifically mentioned.

Ovary collection

Bovine ovaries were recovered at a local abattoir (IT 2270M CE; Inalca S.p.A., Ospedaletto Lodigiano, LO, Italy) from 4 to 8 years old Holstein cows subjected to routine veterinary inspection and according to the specific health requirements. The ovaries were transported to the laboratory within 3 h in sterile saline (NaCl, 9 g/L), supplemented with penicillin (pen) 100 U/mL and streptomycin (strep) 0.1 mg/mL (pen/strep), at 26–28°C. The ovaries were washed twice in pen-/strep-supplemented saline and only those with more than 10 MAFs (2–8 mm) visible on the ovarian surface were processed to isolate COCs and used as a positive control in the IVEP experiments as previously described (Barros et al. 2020, Luciano et al. 2021). The COCs isolation procedures were conducted at 26–28°C. Unless otherwise specified, all the successive procedures were performed at 35–38°C on a warm plate.

Cumulus–oocyte complexes isolation and in vitro culture

Growing oocytes were retrieved from EAFs, submitted to L-IVCO, or used as control. A graphical representation of the experimental groups is provided in Fig. 1. Fully grown COCs were retrieved by aspiration of the follicular content, while growing oocytes were obtained by dissecting the follicles under the stereomicroscope. The two techniques are briefly explained below.

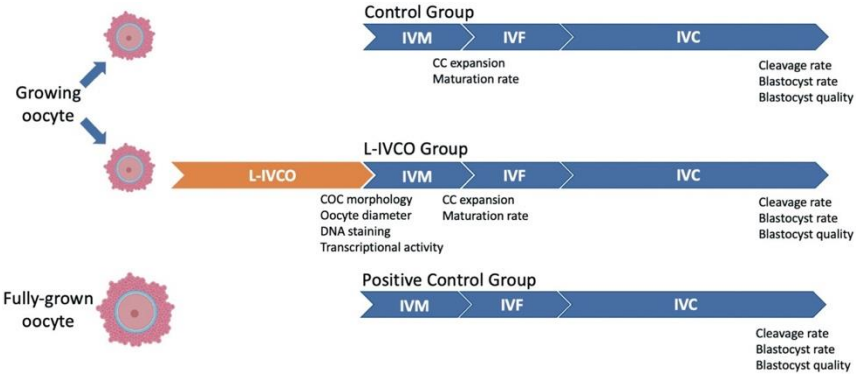


Figure 1: *Graphical representation of the experimental plan. L-IVCO group: growing oocytes retrieved from EAFs and cultured using the L-IVCO system; Control group: growing oocytes retrieved from EAFs and not undergoing L-IVCO culture; Positive control group: fully grown oocytes retrieved from MAFs. The culture treatment is indicated in the arrows: long in vitro oocyte culture (L-IVCO, orange arrow), in vitro maturation (IVM, blue arrow), in vitro fertilization (IVF, blue arrow), and in vitro embryo culture (IVC, blue arrow). The biological endpoints are reported below the arrows.*

Aspiration was conducted with a 19-G needle mounted on an aspiration pump (COOK-IVF, Brisbane QLD, Australia) with a vacuum pressure of −28 mm/Hg. COCs were washed in HEPES-based

manipulation medium (HM199) composed of TCM199 (M2520) with 25 mM HEPES and supplemented with 0.164 mM pen, 0.048 mM strep, 1790 units/L heparin, and 0.4% of fraction V BSA and examined under a stereomicroscope. Only compact COCs medium brown in color, with five or more complete layers of cumulus cells and a finely granulated homogenous ooplasm, were used.

After MAF aspiration, small pieces of ovarian cortex of 1.5–2.0 mm thick and approximately 3 cm2 were sliced parallel to the major axis of the organ and examined under a dissecting microscope. Follicles of 0.5–2 mm in diameter (EAF) were identified and isolated from the surrounding stroma and their wall was cut open using a 26 G needle to release the enclosed COC. Isolated COCs were washed twice in HM199 and immediately transferred to HM199 supplemented with 5 µM of the selective PDE3 inhibitor cilostamide (HM199-Cilo) to maintain the meiotic arrest. The oocytes' diameter, excluding the zona pellucida, was measured using an inverted microscope (Olympus IX50, magnification 10×) equipped with a CCD camera (3CCD Color Video Camera JVC, Japan, Model KYF55B). Only compact COCs with oocytes 100–110 µm in diameter, medium brown in color, with more than five layers of cumulus cells, homogeneous, and finely granulated ooplasm were selected for culture. The collection time of COCs from EAF did not last over 2–3 h. About 30–50 COCs were collected during each session.

The selected COCs were washed out of HM199-Cilo before culture in the L-IVCO medium, which was TCM199 supplemented with 2 mM GlutaMAX™ (ThermoFisher Scientific), 0.4% fatty acid-free BSA, 0.2 mM sodium pyruvate, 25 mM sodium bicarbonate, 0.1 mM cysteamine, 75 µg/mL of kanamycin and 4% polyvinylpyrrolidone (PVP; 360k molecular weight), 0.15 µg/mL zinc sulfate, 10−4 IU/mL of recombinant human FSH (r-hFSH, Gonal-F, Merck-Serono, Roma, Italia), 10 ng/mL E2, 50 ng/mL T, 50 ng/mL P4, and 5 µM cilostamide. COCs were placed individually in a well of a Biocoat™ collagen I-coated 96-well plate (Becton Dickinson Italia, Milan, Italy) containing 200 µL of L-IVCO medium. In order to establish the optimal concentration of E2, a range from 10 to 1000 ng/mL was assessed, as reported in Supplementary Fig. 2 and Supplementary Table 1 (see section on supplementary materials given at the end of this article).

The COCs were incubated for 5 days at 38.5°C under 5% CO2 in humidified air. Half of the volume was replaced with freshly prepared L-IVCO medium on day 2 and day 4.

Endpoints for the evaluation of oocyte development

Morphology of the COCs, oocyte diameter, and DNA staining during L-IVCO

To monitor the changes in morphology during the L-IVCO culture, each COC was photographed with an inverted microscope (Nikon Diaphot; Nikon Corp.) equipped with a digital camera (Nikon digital sight, DS5M) at the beginning of culture (d0) and on days 2, 4, and 5 (end of the L-IVCO). Changes in the size and shape of the cumulus, the formation/dimensions of antrum-like structures, signs of expansions, the presence of cellular debris, and the loss of cumulus cells were recorded. Based on the parameters observed at the end of the L-IVCO culture, COCs were categorized as class 1, class 2, class 3, and class 4.

The oocytes' diameter was determined after cumulus cell removal by gentle pipetting. Bright-field images of denuded oocytes were taken with a digital camera, and the oocyte diameter, excluding the zona pellucida, was measured using NIH ImageJ 1.58 software (Schneider et al. 2012). Successively, oocytes were fixed in paraformaldehyde (PFA) 4% in PBS for 1 h at room temperature (RT) and washed three times in PBS containing 0.1% polyvinyl alcohol (PVA), after which all

samples were mounted in an antifade medium (Vecta Shield; Vector Laboratories, Inc.) supplemented with DAPI dilactate of 1 µg/mL. Samples were analyzed with a conventional epifluorescence microscope (Eclipse E 600; Nikon Corp) to assess chromatin configuration and in bright field to observe if an intact nuclear envelope was present. Oocytes at the GV stage were classified according to the degree of chromatin condensation as previously described (Lodde et al. 2007): GV0, with a diffuse filamentous pattern of chromatin in the whole nuclear area; GV1, with a few foci of chromatin condensation; GV2, with chromatin further condensed into distinct clumps or strands; and GV3, with chromatin condensed in a single clump. Oocytes that resumed meiosis were classified as MI. Oocytes where the GV was not present and the chromatin was not visible or loosely dispersed in the cytoplasm or densely aggregated were classified as degenerated.

Global transcriptional activity during L-IVCO

Transcription was assessed in growing oocytes cultured as COCs from day 0 to day 5. Global transcriptional activity was evaluated using the Click-iT® RNA Imaging Kit (Invitrogen, Thermo Fisher Scientific), as previously described (Lodde et al. 2020) and following the manufacturer's guide.

Briefly, COCs were incubated with 2 mM of EU diluted in L-IVCO medium for 1 h at 38.5°C under 5% CO2, to allow incorporation. Cumulus cells were then mechanically removed, and oocytes were briefly washed in warm PBS/PVA, fixed in 4% PFA in PBS for 30 min RT, and washed again in PBS/PVA. Samples were permeabilized with 0.5% Triton-X 100 in PBS for 15 min RT, briefly washed in PBS/ PVA, and incubated in Click-iT® reaction cocktail for 30 min RT to enable the detection of incorporated EU. After further washing once in Click-iT® reaction rinse buffer in PBS/PVA, oocytes were mounted and stained in mounting Vectashield antifade mounting medium, with DAPI, using double-sticky tape between the slide and the cover glass. Samples were analyzed and imaged under an epifluorescence microscope as previously described (Lodde et al. 2020), maintaining identical acquisition settings for all the samples. Chromatin configuration was also evaluated in the same samples and classified according to the degree of chromatin compaction, as described above. The level of global transcriptional activity was estimated by assessing the mean fluorescent intensity value emitted by the incorporated EU using NIH ImageJ 1.58 software (Schneider et al. 2012). The mean fluorescent intensity emitted by the nuclear area was corrected by subtracting the mean background intensity of the oocyte's cytoplasm (Lodde et al. 2020).

Cumulus expansion and meiotic competence after in vitro maturation

After L-IVCO, COCs were washed in HM199 and cultured in groups of 20–25 for 24 h under standardIVM conditions. IVM medium was M199 (M3769) supplemented with 0.68 mM l-glutamine, 25 mM NaHCO3, 0.4% fatty acid-free BSA, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 µg/mL of kanamycin, and 0.1 IU/mL of r-hFSH (Luciano et al. 2011). As control, a group of freshly isolated COCS from EAFs (no L-IVCO) were in vitro matured. After IVM, the cumulus expansion was visually classified according to a previously reported scoring system (Barros et al. 2019), as: Grade 0 – no expansion, characterized by no morphological changes; Grade 1 – sign of expansion with partially denuded oocytes; Grade 2 – partial expansion, characterized by fair expansion and some clusters of not expanded cumulus cells; and Grade 3 – complete or nearly complete expansion. After scoring, COCs were denuded by gently pipetting and oocytes were fixed in PFA 4% in PBS for 1 h RT, washed three times in PBS/PVA, and mounted in DAPI-supplemented Vecta Shield. Samples were analyzed under an epifluorescence microscope and in bright field (Eclipse E 600) and classified as GV, MI, MII, and degenerated as previously described (Luciano et al. 2011).

Developmental competence after in vitro maturation, fertilization, and embryo culture
After IVM, oocytes were subjected to in vitro fertilization (IVF) and embryo culture (IVC) as previously described (Luciano et al. 2005). Briefly, the content of a straw of cryopreserved bull spermatozoa (C.I.Z., S. Miniato Pisa, Italy) was thawed, and sperm were separated with a 45–90% Percoll gradient. Sperm were counted and diluted to a final concentration of 0.75 × 106 spermatozoa/mL in fertilization medium, which was a modified Tyrode solution (Tyrode albumin lactate pyruvate, TALP) supplemented with 0.6% (w/v) fatty acid-free BSA, 10 µg/mL heparin, 20 µM penicillamine, 1 µM epinephrine, and 100 µM hypotaurine. COCs were cultured in 300 µL of IVF medium for 18 h in four-well dishes at 38.5°C under 5% CO2 in humidified air.

After fertilization, residual cells and spermatozoa were removed by vortexing for 2 min in 500 µL of synthetic oviduct fluid (SOF, (Tervit et al. 1972)) buffered with 10 mM of HEPES and 5 mM of NaHCO3 (SOF wash), rinsed twice, and then, transferred in groups of 15–30 in SOF embryo culture medium (Franciosi et al. 2010). The SOF embryo culture medium was buffered with 25 mM of NaHCO3 and supplemented with MEM essential and nonessential amino acids, 0.72 mM sodium pyruvate, 2.74 mM myo-inositol, 0.34 mM sodium citrate, and 5% calf serum (CS). Incubation was performed at 38.5°C with a humified gas mixture composed of 5% CO2, 5% O2, and 90% N2. After 186 h post-fertilization, the blastocyst rate was assessed under a stereomicroscope, and blastocysts were morphologically classified as not expanded, expanded, and hatched (Jakobsen et al. 2006). Briefly, blastocysts in which the blastocoel has just begun to form, and the cell types that were not distinguishable were classified as not expanded. Blastocysts were classified as expanded if the blastocoel was fully formed and the trophectoderm and inner cell mass were clearly distinguishable but still contained within a thinned zona pellucida. Hatched blastocysts were those outside the zona pellucida. The embryos were then fixed in 60% methanol in PBS, and the cell nuclei were counted under a fluorescence microscope after staining with 0.5 mg/mL propidium iodide.

In each experiment, COCs isolated from MAF were subjected to IVM, IVF, and IVC and used as positive control group.

Statistical analysis
All experiments were repeated at least three times. Data were analyzed by Graph Pad Prism 9.4 and are expressed as mean ±s.e.m. Distribution of the morphological COCs classes and of the oocytes presenting GV0 or GVc across 5 days of culture were analyzed by two-tailed Fisher's exact test. Preliminary normality tests were run to assess whether the data distribution was normal for oocyte diameter, chromatin configuration, expansion degree, meiotic competence, and blastocyst cell number. Data were analyzed with t-test if normally distributed; alternatively, Mann–Whitney test was applied. Data on transcriptional activity were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test. Data on cleavage and blastocyst rates were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Probabilities with values less than 0.05 were considered statistically significant.

RESULTS
Effect of L-IVCO on COC morphology
An initial characterization was carried out on 74 COCs isolated from EAFs and cultured for 5 days, by observing the morphology of the cumulus cells on days 0, 2, 4, and 5. According to the presence and proliferation of cumulus cells and the formation of antrum-like structures at day 5 (d5), COCs

were classified into four classes (Fig. 2). Class 1 showed compact cumulus of bigger size compared to the time of retrieval (d0), indicating proliferation. Signs of cumulus expansion or cell degeneration were not observed, while small antrum-like structures started to form around d4. Class 2 COCs were similar to class 1 but developed antrum-like structures that were larger at d5. Interestingly, both class 1 and 2 increased in volume and maintained a 3D structure, forming a dome-like structure previously observed in mouse and rat (Gore-Langton & Daniel 1990, Cain et al. 1995, Smitz et al. 1996, Cortvrindt et al. 1998) and bovine (Hirao et al. 2004).

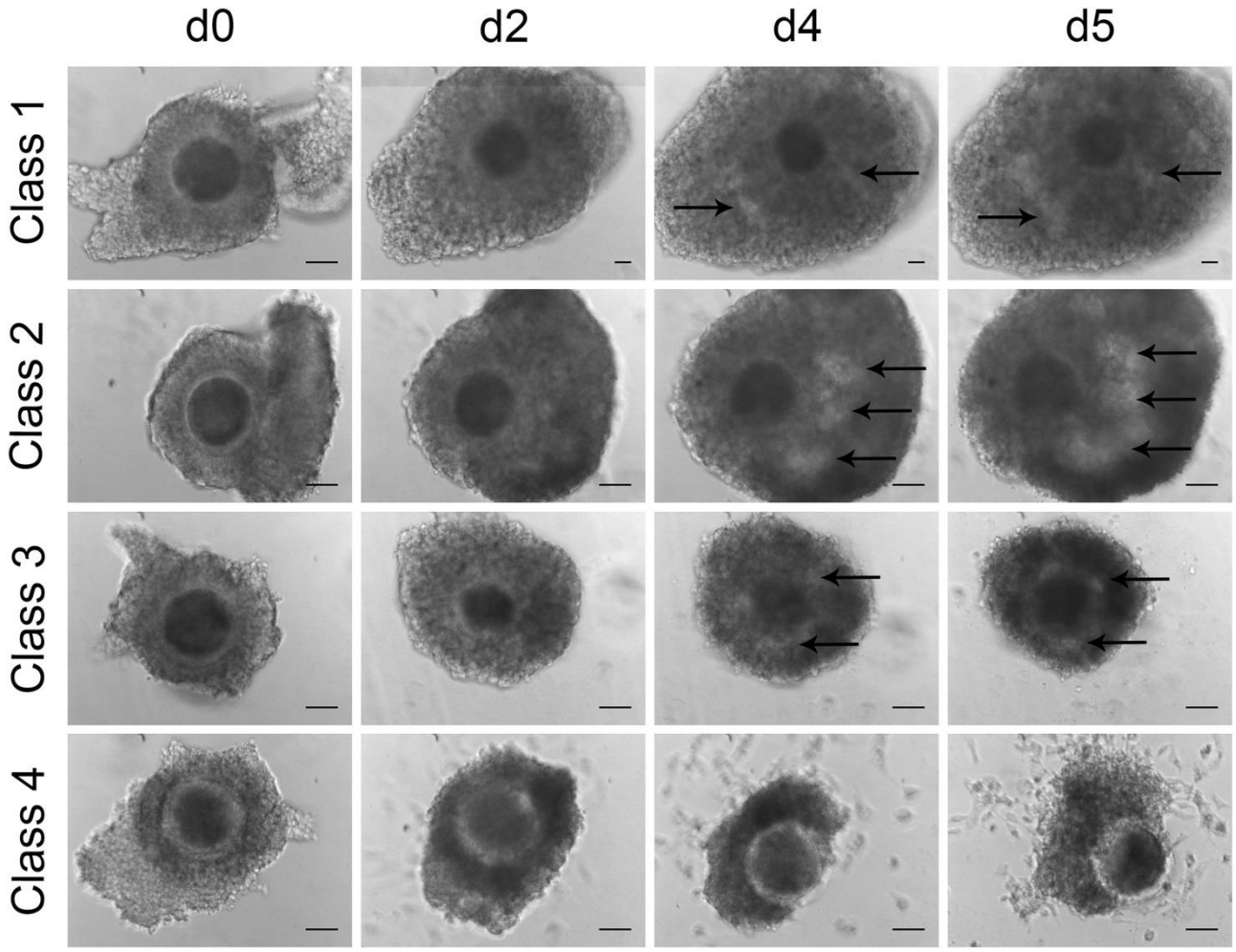


Figure 2: *Representative images of COCs morphology at the time of collection and after 2, 4, and 5 days of L-IVCO. After 5 days of L-IVCO, COCs were classified into 4 classes: Class 1, showing a compact cumulus with no sign of expansion and cell degeneration and reorganization of small antrum-like structures (arrows); Class 2, showing a compact cumulus with no sign of expansion and cell degeneration and with the reorganization of large antrum-like structures (arrows); Class 3, showing a cumulus of smaller size, with some disaggregated cells in the outer layer and limited formation of slim antrum-like structures (arrow); Class 4, showing significant loss of cumulus cells on more than 50% of the oocyte surface with signs of cell degeneration and abundant cellular debris. Scale bar 50 µm. Pictures are at 20× magnification, except d2, d4, and d5 of class 1 (first row), which are at 10× to better visualize the entire COC.*

In class 3, despite an initial increase in the size of the cumulus mass (d2), an overall decrease in the surrounding cumulus vestment, together with an increase in cellular debris, was observed. Furthermore, antrum-like formations were limited to tiny structures. Finally, class 4 COCs faced a

progressive loss of cumulus cells, reaching at least more than 50% of the oocyte surface by d5, along with signs of cell degeneration and abundant cellular debris. Class 4 was overall less represented than the other three classes (two-tailed, Fisher's exact test P≤ 0.0268, Table 1).

Table 1

COCs morphology and meiotic arrest at the end of the L-IVCO. At the end of the L-IVCO, COCs were classified into 4 classes: Class 1, showing a compact cumulus cell mass with no sign of cumulus expansion or cell degeneration and reorganization of small antrum-like structures in the cumulus mass (arrows); Class 2, showing a compact cumulus cell mass with no sign of cumulus expansion and cell degeneration and with the reorganization of large antrum-like structures in the cumulus mass (arrows); Class 3 where COCs were showing several layers of cumulus cell with no sign of cumulus expansion and some disaggregated cells in the outer layer of cumulus cells and limited formation of slim antrum-like structures in the cumulus mass (arrow); Class 4, showing significant loss of cumulus cells on more than 50% of the oocyte surface with signs of cell degeneration and abundant cellular debris. Data are presented as n (%).

COC morphology	Total COCs	GV	MI	Deg
Class 1	18 (24.3)	15 (83.3)	3 (16.6)	0 (0)
Class 2	19 (25.7)	14 (73.8)	4 (21.0)	1 (5.2)
Class 3	30 (40.5)	24 (80.0)	2 (6.7)	4 (13.3)
Class 4	7 (9.4)*	5 (71.5)	0 (0)	2 (28.5)

**Differences in the distribution of COCs morphologies in the cultured population (first column) P < 0.05, two-tailed Fisher's exact test). No differences were observed in GV arrested oocytes among classes.*

In general, no differences have been observed in the percentage of oocytes arrested at the GV stage between the classes (Table 1). However, when submitted to IVM, class 4 COCs did not resume meiosis or degenerate in more than 80% of the cases (Supplementary Fig. 1), supporting the hypothesis that they represent a non-viable class, as suggested by the morphological observations. Therefore, class 4 COCs were discarded and not used in further experiments.

Effect of L-IVCO on oocyte diameter and chromatin configuration

To assess the effects of L-IVCO on oocyte growth, the cumulus cells were removed, and the oocyte diameter was measured in 50 oocytes at the time of isolation from the EAFs and 67 oocytes at the end of L-IVCO. As reported in Fig. 3A, the mean diameter of oocytes significantly increased from 108.9 ± 0.6 to 113.4 ± 0.7 µm (P < 0.0001). However, they did not reach the diameter of fully grown GV oocytes, which approaches 120 µm (Fair et al. 1995, Hyttel et al. 1997). Furthermore, given that chromatin configuration has been recognized as a marker of oocyte differentiation and progressive

acquisition of competence (Lodde et al. 2007), the degree of differentiation associated with the growth was investigated by observing the chromatin configuration at d0 and d5 (Fig. 3B and C).

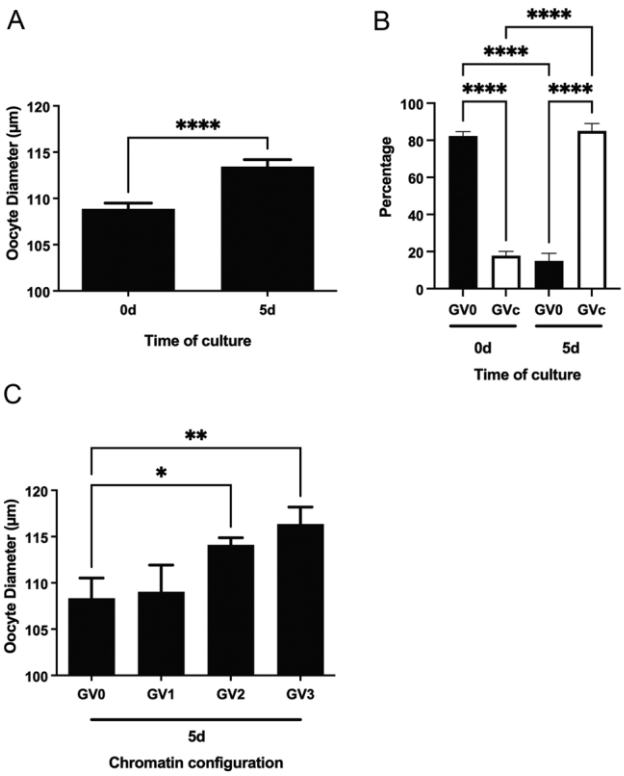


Figure 3: Effect of L-IVCO on oocyte growth and chromatin condensation. (A) Overall changes in diameter of growing oocytes between the time of collection (d0; $n = 54$) and after 5 days of L-IVCO (d5; $n = 58$). (B) The L-IVCO induces chromatin transition from uncondensed (GV0) to condensed stages (GVc = GV1+GV2+GV3). (C) The progressive condensation of the chromatin is accompanied by an increase in the size of the oocyte after 5 days of culture (d5). Data from five independent experiments ($n = 5$) were verified for normality and analyzed by Mann-Whitney test (A) and ANOVA followed by Tukey's multiple comparisons test (B, C). Values are means ± s.e.m.* and **** indicate significant differences between groups (*$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$).

While GV0 was the predominant configuration at the time of collection, by d5, more compacted GV configurations (GVc, as the sum of GV1-2-3) significantly increased (80%, Fig. 3B). Furthermore, oocytes displaying higher degrees of chromatin compaction (GV2-3) at d5 were on average bigger than 110 µm, while the oocytes that failed to rearrange their chromatin also had smaller diameters (<110 µm, Fig. 3C).

The remaining oocytes were either MI (0.0 ± 0.0 and 12.31 ± 4.93, at d0 and d5, respectively, $P > 0.05$) or degenerated (4.04 ± 2.49 and 9.28 ± 4.76, at d0 and d5, respectively, $P > 0.05$).

Effect of L-IVCO on transcriptional activity

The effect of L-IVCO on the global transcription of growing oocytes was investigated by visualizing the EU incorporation into nascent RNA (Fig. 4A). GV0 oocytes showed intense transcriptional activity at the time of collection, while it progressively decreased at d3 and d4, despite the oocytes still having a diffuse pattern of chromatin. Conversely, the transcriptional activity dropped

dramatically, starting from d1 in the oocytes with GV1-2-3 chromatin configuration. Overall, the decrease in the global transcriptional activity seems to anticipate the chromatin condensation during L-IVCO (Fig. 4B). Consistent with the results presented in Fig. 3, a progressive decline of GV0 stage oocytes was matched by an increase in GVc stage (Fig. 4C).

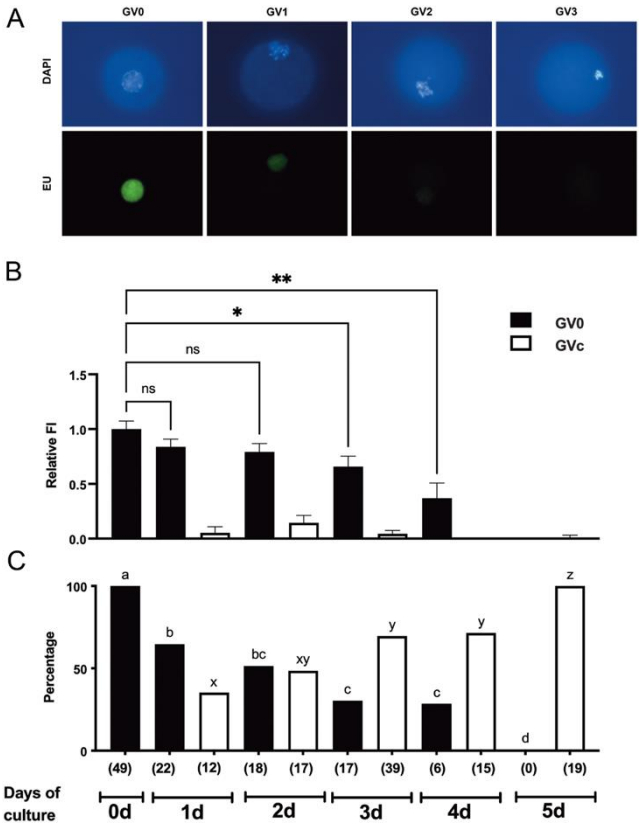
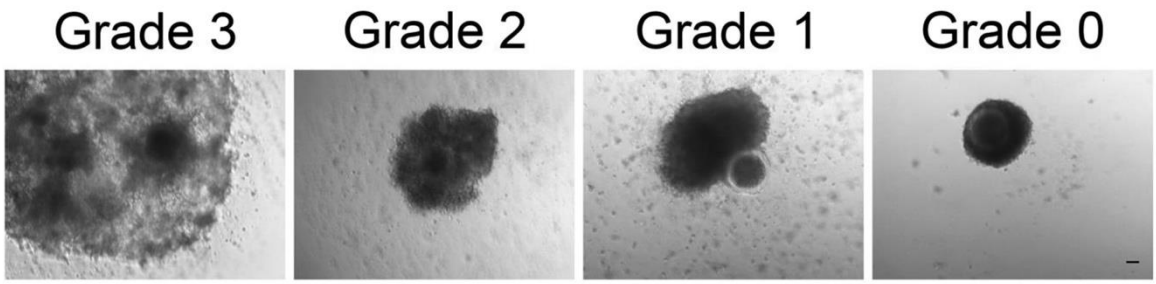


Figure 4: *Influence of L-IVCO treatment on the transcriptional activity and chromatin condensation of oocytes during 5 days of culture. (A) Representative images of chromatin configuration (DAPI) and transcriptional activity detection (EU). (B) Graph showing the intensity of the transcriptional activity of GV0 and GVc oocytes (i.e. as the total of the condensation stages GV1, 2 and 3), at time of collection (d0), after 1 day (d1), 2 days (d2), 3 days (d3), 4 days (d4), and 5 days of culture (d5) and the comparison of intensities of GV0 oocytes at the different time points. (C) Graph showing the proportion of oocytes presenting GV0 or GVc across 5 days of culture. Data from three independent experiments (n = 3) were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test and values are means ± s.e.m. Distribution of the oocytes presenting GV0 or GVc across 5 days of culture was analyzed by two-tailed Fisher's exact test. * and ** indicate significant differences between groups (* P < 0.05; ** P < 0.01).*

Effects of L-IVCO on cumulus expansion and oocyte meiotic competence

After L-IVCO, COCs were submitted to standard IVM protocol, and the degree of cumulus expansion and the capacity to complete meiosis I were assessed. COCs isolated from EAFs and submitted directly to IVM were used as control. As reported in Fig. 5, approximately 30% of the L-IVCO COCs reached the highest expansion score (grade 3), a significantly higher rate compared to the control (approximately 11%). Notably, almost 40% of the control COCs did not show any signs of expansion.



	Grade 3 (%)	Grade 2 (%)	Grade 1 (%)	Grade 0 (%)
Control + IVM	11.34 ± 3.66[b] (9)	49.45 ± 1.00[a] (41)	0.0 ± 0.0[b] (0)	39.21 ± 3.88[a] (33)
L-IVCO +IVM	29.60 ± 4.32[a] (18)	27.94 ± 8.48[b] (17)	29.25 ± 8.47[a] (19)	13.21 ± 4.18[b] (9)

Figure 5: *Data showing the expansion degree of cumulus cells in COCs isolated from EAFs and submitted directly to IVM (Control) or COCs isolated from EAFs and submitted to L-IVCO followed by IVM (L-IVCO). After IVM, the expansion degree (Grade) of the cumulus cells was classified according to a scoring system: Grade 0 – no expansion, characterized by no morphological changes; Grade 1 – sign of expansion with partially denuded oocytes; Grade 2 - partial expansion, characterized by fair expansion and some clusters of not expanded cumulus cells; and Grade 3 – complete or nearly complete expansion. Data from seven independent experiments were analyzed by paired t-test and values are means ± s.e.m. Different letters indicate significant differences in the columns (P < 0.05). Scale bar represents 40 µm.*

L-IVCO induced a significant increase in the percentage of oocytes that matured after IVM (Fig. 6), paralleled by a significant decrease in the percentage of oocytes arrested in GV, indicating that L-IVCO promotes the acquisition of oocyte meiotic competence. The remaining oocytes were degenerated (4.7 ± 3.1 and 14.2 ± 4.6, in control and L-IVCO, respectively, P > 0.05).

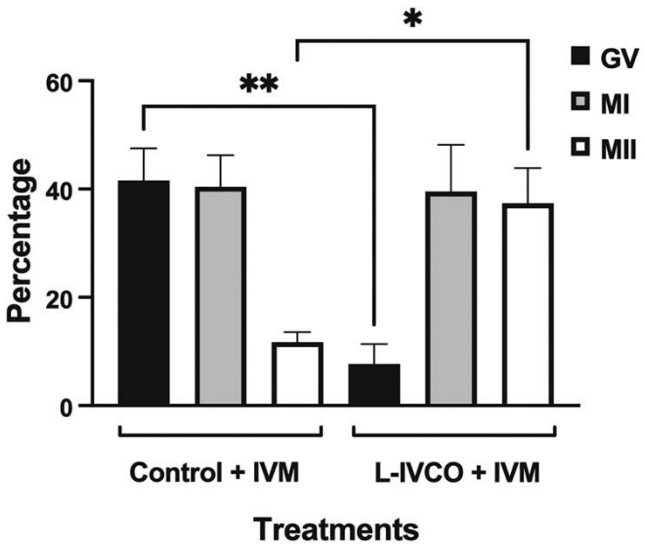


Figure 6*: Effect of L-IVCO treatment on the oocyte meiotic competence acquisition after IVM. Graphic is showing the percentage of oocytes in the GV, MI, and MII stages of growing oocytes when submitted directly to IVM (control + IVM; n = 60) or cultured in L-IVCO for 5 days prior to IVM (L-IVCO + IVM; n = 55). Data from seven independent experiments (n = 7) were analyzed by unpaired t-test and values are means ± s.e.m. * and ** indicate significant differences in comparison between homologous stages (* P < 0.05; ** P < 0.01).*

Effect of L-IVCO treatment on embryonic developmental competence acquisition

To assess the effect of L-IVCO on the acquisition of embryonic developmental competence, in vitro grown COCs (L-IVCO) were in vitro matured and fertilized, and the embryos were cultured for 8 days. COCs collected from EAFs and not submitted to L-IVCO were used as control, while fully grown COCs, collected from MAFs, represented the positive control.

As shown in Fig. 7A and B, growing COCs achieved the blastocyst stage only if L-IVCO was cultured previously, while growing control oocytes failed to support embryo development after the first few cell cleavages. Notably, the L-IVCO group did not develop to the blastocyst stage at the same rate as the fully grown oocytes; however, when blastocysts quality parameters were compared, no significant differences were observed neither in the percentages of expanded and hatched blastocysts (Fig. 7C) nor in the blastocyst cell number (Fig. 7D) between the two experimental groups.

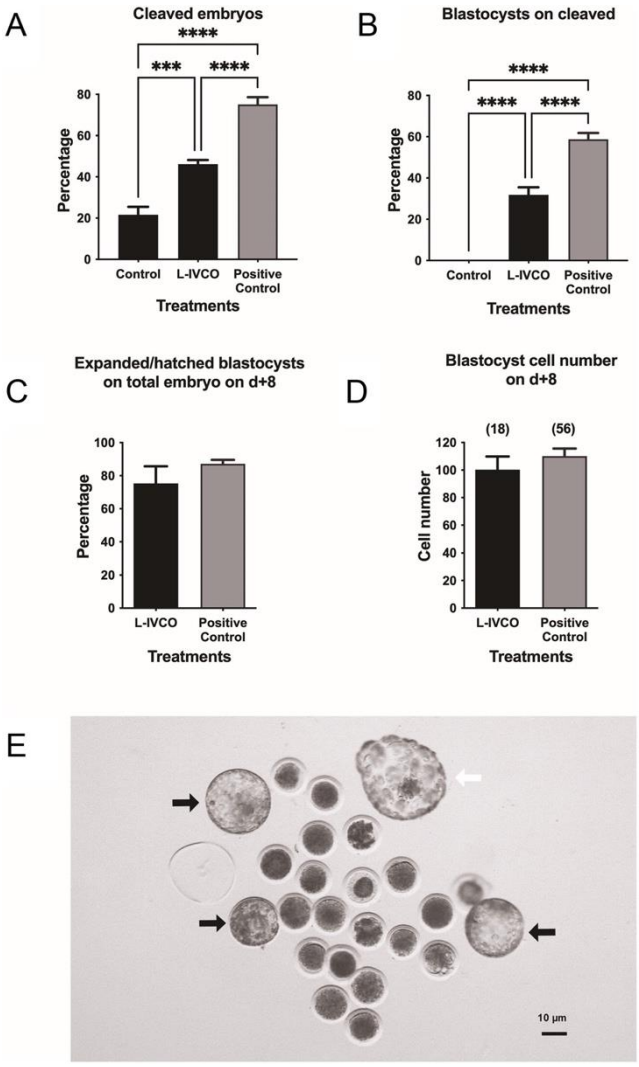


Figure 7*: Effect of L-IVCO on cleavage rate (A), blastocyst rates on cleaved embryos (B) and blastocyst quality, as the percentage of expanded and hatched blastocyst on total embryos (C) and blastocyst cell number (D). A representative image of hatched (white arrow) and expanded (black arrows) blastocysts after L-IVCO and IVP (E). Growing oocytes were cultured for 5 days in L-IVCO (L-IVCO, n = 125) before being submitted to IVP. A group of growing oocytes (control, n = 127) and a group of fully grown oocytes (positive control, n = 291) were submitted directly to IVP as negative and positive controls, respectively. Data on cleavage and blastocyst rates were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. The percentage of expanded and hatched blastocyst on total embryos (C) and blastocyst cell number (D) were analyzed by Mann–Whitney test. All values are means ± s.e.m. Data from five independent experiments (n = 5). *,**, and **** indicate significant differences between groups (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).*

DISCUSSION

The present study shows that growing bovine oocytes collected from EAFs can be cultured in vitro using a completely defined 3D-like system on a flat substratum that enables the oocytes to grow, differentiate, and progressively acquire meiotic and developmental competence over 5 days. This culture system, named L-IVCO, was developed to recreate the physiological conditions of the bovine growing follicle as much as possible. For instance, particular attention was paid to the hormonal milieu, whereby E2, T, P4, and FSH concentrations were retrieved from published studies on bovine folliculogenesis (Henderson et al. 1982, Dieleman et al. 1983, Ireland & Roche

1983, Kruip & Dieleman 1985, Kaneko et al. 1991, Silvan et al. 1993, Beg et al. 2002, Modina et al. 2007, Monniaux et al. 2008, Soares et al. 2017, Sakaguchi et al. 2019) and experimentally assessed when contrasting reports were found. This was the case of E2, whose follicular concentrations in the growing follicle are in the range of 10 ng/mL (Henderson et al. 1982, Kruip & Dieleman 1985). However, previous studies reported the use of E2 concentrations in culture ranging from 10 to 1000 ng/mL (Hirao et al. 2004, Taketsuru et al. 2012, Endo et al. 2013, Makita & Miyano 2014, Alam et al. 2018). As reported in Supplementary Fig. 2 and Supplementary Table 1, increasing E2 levels in culture did not further benefit the oocyte growth, meiotic competence, and cumulus expansion. Therefore, it was established to use the minimum effective concentration.

Together with E2, T, which acts as an estrogen precursor, was included for its role in supporting oocyte and follicle growth (Walters et al. 2008, Endo et al. 2013, Alam et al. 2018), promoting antrum formation (Endo et al. 2013), maintaining intercellular transzonal connections between oocyte and granulosa cells, and being overall beneficial for meiotic competence acquisition (Makita & Miyano 2015). P4 was selected for the antiapoptotic function in granulosa cells (Luciano et al. 1994). Finally, FSH supplementation was demonstrated to be beneficial during the growing phase, sustaining the functionality of GJs and promoting the regular chromatin transition and oocyte growth (Luciano et al. 2011). Besides hormones, cilostamide was added to sustain the intraoocyte levels of cyclic nucleotides, thus preventing meiotic resumption (Luciano et al. 2011, Franciosi et al. 2014).

Additional refinements were adopted to maintain a 3D organization of the cumulus cells, previously reported to be a successful condition for mammalian oocytes (Eppig et al. 1996). In the L-IVCO, a 3D-like environment was provided by adding in the medium PVP, a synthetic polymer used in medicine for its solubility in water and biocompatibility (Hirao et al. 2004). PVP increased the viscosity, and together with a matrix-coated culture surface, supported 3D growth (Hirao et al. 2004).

Finally, the culture period was adjusted to span the time required for an early antral follicle to grow from about 0.5 mm to more than 2 mm (Lussier et al. 1987). In cows, this range corresponds to the transition from a transcriptionally active growing oocyte of 100–110 µm of diameter to a fully grown oocyte of about 120 µm (Fair et al. 1995) and it takes approximately 5 days.

Growing oocytes collected from EAFs have a diffuse pattern of chromatin configuration termed GV0, while more compact arrangements are observed in MAFs-derived oocytes, called GV1, GV2, and GV3 (Luciano et al. 2014). Indeed, the large-scale configuration of chromatin within the GV has been indicated as a marker of oocyte growth and competence in several mammals with increasing compaction being typical of gametes at more advanced stages of differentiation (reviewed in Luciano & Lodde 2013). Hence, the degree of chromatin compaction has been used to track the growth and differentiation process during L-IVCO.

While the GV0 configuration was prevalent at the time of collection, as expected, by the end of the L-IVCO, GV0 oocytes were almost completely replaced by more condensed chromatin stages (collectively named GV condensed (GVc), 80%), suggesting differentiation and competence acquisition.

From a functional standpoint, chromatin condensation has been associated with the progressive silencing of transcription, with GV0 oocytes being actively synthesizing RNAs, and GV1, GV2, and

GV3 stages being either marginally de novo transcribing or not transcribing at all (Lodde et al. 2007, 2008, Luciano et al. 2011, Lodde et al. 2020). Compared to the previous IVCO where transcriptional silencing was observed over the span of 24–48 h of culture ((Luciano et al. 2011) and unpublished data), the newly developed L-IVCO sustained the transcriptional activity for longer, likely due to zinc supplementation (Lodde et al. 2020), a trace element that is typically absent from culture media unless introduced with the serum (Tarahomi et al. 2019).

Given that the oocyte stores mRNAs during the growth phase and uses them for protein synthesis during the later stages of oogenesis and early embryogenesis (Bachvarova 1985), it is believed that an oocyte that is still actively transcribing has not accumulated all the transcripts necessary for the following developmental steps and is, therefore 'not-competent'. Therefore, it was predicted that the process of progressive chromatin condensation would be accompanied by transcriptional silencing. Indeed, a progressive fading of detectable transcriptional activity over 4 days of culture was observed, even though the results are seen to suggest that transcriptional silencing preceded chromatin compaction in vitro. Furthermore, the finding that the transcriptional activity at d4 in the residual GV0 oocytes was already very low, along with the observation that transcriptionally active oocytes were few or none at d5 may be considered an indication that the culture timing should not be further extended since in vitro culture of transcriptionally inactive oocytes negatively impacted the developmental competence in mice (De La Fuente & Eppig 2001). Seemingly, more efforts are needed to further refine the growing oocytes in vitro culture to improve the transcriptional activity.
When in vitro matured and fertilized, approximately 20% of the L-IVCO oocytes produced blastocysts, as opposed to the inability of naïve EAF-derived oocytes to support embryo development beyond the cleavage. Furthermore, this result represents a considerable improvement when compared to previous reports (Hirao et al. 2004, Luciano et al. 2011, Alam et al. 2018). Although encouraging, the developmental ability does not match the one observed in fully grown oocytes, collected from MAFs, in line with the generally accepted concept that some aspects of in vivo oocyte growth cannot be completely recapitulated in vitro (De La Fuente & Eppig 2001). On this line, it should be noted that also the diameter of L-IVCO oocytes does not reach that of MAFs-retrieved oocytes, probably indicating that additional adjustments shall be considered to further sustain the oocyte growth and competence acquisition.

However, tempting speculation on the data of embryo development is that meiotic competence is achieved by 40% of the L-IVCO oocytes. If the not matured oocytes are removed, the remaining ones seem to achieve the same performances as fully grown oocytes in terms of cleavage and blastocyst development, with the great majority that fertilizes and cleaves, and one out of two forming blastocysts. However, this hypothesis could only be experimentally verified if mature oocytes were identified without removing the cumulus cells, but if confirmed, it would suggest that the acquisition of the meiotic competence represents the major bottleneck in the transition from growing to fully grown oocytes. Ultimately, it must be acknowledged that the full acquisition of developmental acquisition to term can only be proven by in vitro transfer to recipients.

It remains to be determined why not all the oocytes that reached the GV1, GV2, and GV3 stages in vitro underwent meiotic maturation. As already mentioned, some intraovarian control mechanisms seem to be disrupted by the in vitro culture of isolated COCs (De La Fuente & Eppig 2001). Probably, other molecular and biochemical features besides chromatin compaction should be considered when testing the efficiency of in vitro growth protocols, such as the appearance of M-phase characteristics and modifications of cytoplasmic components (Dieci et al. 2013). Indeed,

cytoplasmic maturity might be an issue for the efficient block of the polyspermy or the ability to remodel the sperm chromatin (Gioia et al. 2005).

Further studies are therefore needed to understand if failure to fertilize properly might account for the lower cleavage rates of L-IVCO COCs compared to in vivo fully grown oocytes.

Furthermore, since oocyte – somatic cell interactions are pivotal for proper oogenesis and folliculogenesis and the two cell types constantly exchange message molecules via different signaling routes, the somatic compartment must also be considered (Albertini et al. 2001, Eppig 2001). With this in mind, the extent of differentiation of L-IVCO treated cumulus cells was tested by assessing the ability to undergo expansion when matured in in vitro. Notably, only 30% of the tested COCs expanded, and despite being significantly more competent than the not-cultured counterpart, cumulus cells seem to experience a belated differentiation compared to the gamete. Whether this lack of response is caused by 'companion cell' intrinsic factors, or by an insufficient provision of enabling factors by the oocyte has not been investigated yet (Vanderhyden et al. 1990).

By conducting morphological observations during culture, it was observed that in the somatic compartment of some COCs, signs of antrum formation appeared and eventually regressed, with different dynamics, despite the indistinguishable morphology at the time of collection. Overall, these findings suggest that a further improvement of the culture conditions might come from a deeper characterization of the somatic cells. By better characterizing the differentiation program of the somatic cells, it may be possible to support their cultural needs and ideally identify non-invasive biomarkers for more accurate COC selection.

Overall, the approach described herein poses the basis to design culture strategies to support the specific oocyte needs at the growing – fully grown – transition and expand the source of oocytes usable for fertility preservation programs in animals of high genetic merit or with specific desirable traits. L-IVCO can provide a valuable tool to dissect the cellular and molecular processes that control the final oocyte development.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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AUTHOR CONTRIBUTION STATEMENT

AML, VL, and RGB conceived the study. RGB, AML, and VL performed the experiments. RGB and VL performed and analyzed the experiments on the assessment of the transcriptional activity. All authors participated in data analysis. The manuscript was drafted by RGB and revised by AML, VL and FF. All authors read and approved the final manuscript.

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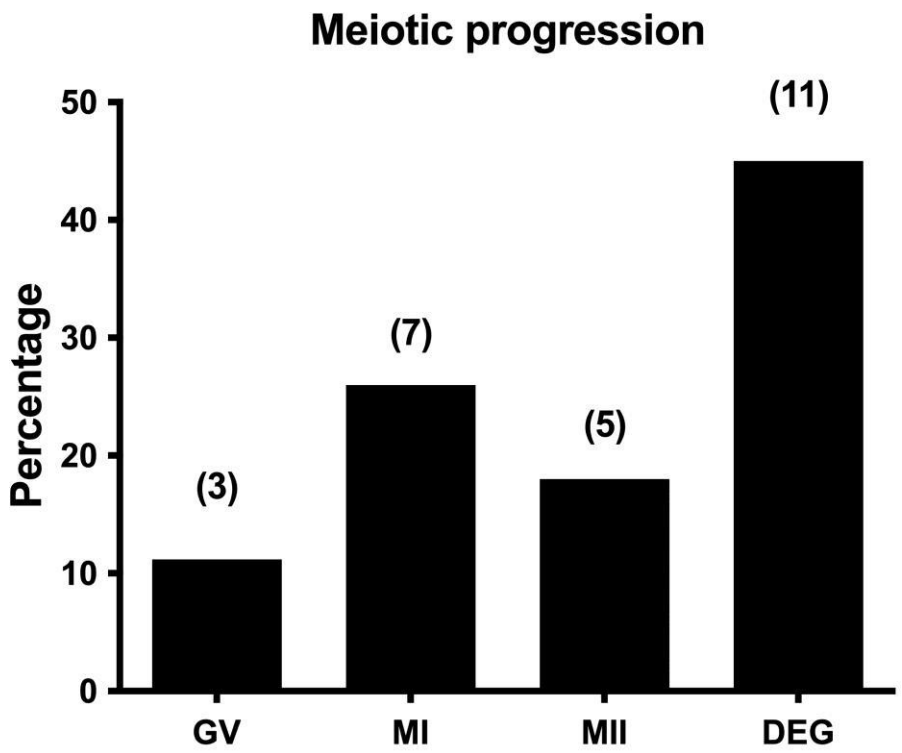
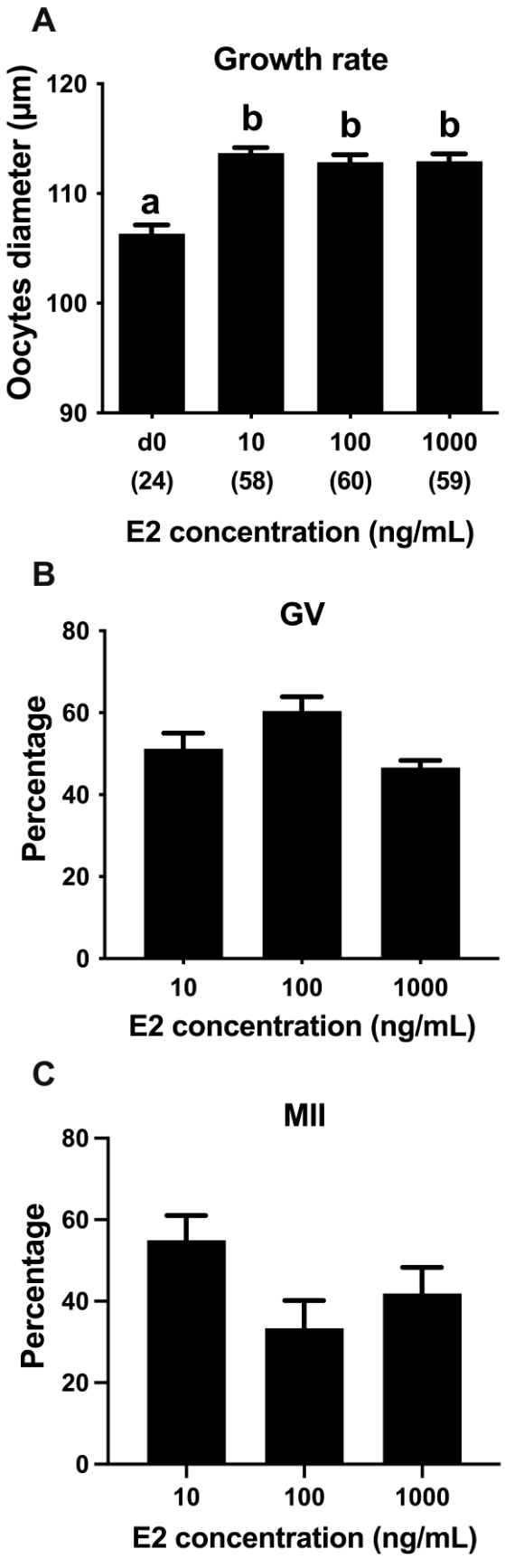
SUPPLEMENTARY MATERIALS

Supplementary Figure 1: Meiotic progression of Class 4
Graph showing the meiotic progression of oocytes Class 4 after IVM. Only 18% of oocytes could reach the MII stage at the end of the culture, while 82% degenerated or remained arrested at GV or MI stage. Class 4 oocytes were excluded from subsequent not used in downstream experiments based on this data. Cumulative data from 7 independent experiments.



Supplementary Figure 2: Effect of different E2 concentrations during L-IVCO
(A) Effect of different E2 concentrations on oocyte growth measured as diameter at the time of collection (0d, n=24) and after 5 days of L-IVCO with different concentrations of E2: 10 ng/mL (n=58), 100 ng/mL (n=60) and 1000 ng/mL (n=59). Data were analyzed by 1- way ANOVA followed by Kruskal-Wallis test and values are means ± SEM. Different letters indicate significant differences between groups ($P<0.0001$; N=3).
(B) Graph showing the percentage of oocytes arrested at the GV stage after 5 days of L-IVCO at different concentrations of E2 (10, 100 and 1000 ng/mL; n= 31, 33 and 34 respectively). Data were analyzed by 1-way ANOVA followed by Kruskal-Wallis test and values are means ± SEM (N=3).
(C) Effect of different E2 concentrations (10, 100 and 1000 ng/mL; n= 45, 39 and 46 respectively) on the percentage of oocytes reaching MII after IVM. No significant differences in MII rate were observed between treatments with different concentrations of E2, although the percentage of MII was numerically higher in 10 ng/mL than 100 and 1000 ng/mL of E2. Data were analyzed by 1-way ANOVA followed by Kruskal-Wallis and values are means ± SEM ($P=0.1431$; N=4).


A
Growth rate
Oocytes diameter (μm)
120
110
100
90
a
b
b
b
d0
(24)
10
(58)
100
(60)
1000
(59)
E2 concentration (ng/mL)
B
GV
Percentage
80
60
40
20
0
10
100
1000
E2 concentration (ng/mL)
C
MII
Percentage
80
60
40
20
0
10
100
1000
E2 concentration (ng/mL)

Supplementary Table 1. Effect of different E2 concentrations on cumulus expansion after IVM
Effect of different concentrations of E2 (ng/mL) during L-IVCO on expansion degree of cumulus cells after IVM. After IVM, the expansion degree of cumulus cells was visually classified according to a subjective scoring system: grade 3 - complete or nearly complete expansion; grade 2 - partial expansion, characterized by fair expansion and some clusters of not expanded cumulus cells; grade 1 - sign of expansion with partially denuded oocytes; grade 0 - no expansion, characterized by no morphological changes. Data from four independent experiments were analyzed by 1-way ANOVA followed by Tukey's multiple comparison test. Values are means ± SEM.

E2 (ng/mL)	Grade 3	Grade 2	Grade 1	Grade 0
10	29.9 ± 10.6 (14)	26.8 ± 9.6 (12)	26.2 ± 13.8 (10)	16.9 ± 5.5 ab (7)
100	18.2 ± 6.5 (8)	27.9 ± 9.4 (12)	33.8 ± 11.5 (13)	19.9 ± 4.8 a (8)
1000	12.3 ± 5.2 (5)	38.8 ± 9.8 (15)	43.3 ± 11.7 (16)	6.4 ± 4.0 b (2)