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# Embryotrophic effect of a short-term embryo coculture with bovine luteal cells

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## Abstract

The coculture with somatic cells is an alternative to improve suboptimal *in vitro* culture (IVC) conditions and promote embryo development. Several cell types have been used for this purpose, but there is no information about using luteal cells in short-term coculture with embryos. Consequently, this study aimed to assess the effect of a short-term coculture of early bovine embryos-luteal cells on the *in vitro* development and embryo quality. Presumptive embryos were cultured from day 0 to day 2 in medium alone (control) or cocultured with bovine luteal cells (BLC-1). Then, embryos from both groups were cultured in medium alone from day 2 to day

8. The development rates on day 8 were compared between groups. The level of reactive oxygen species (ROS) and proliferation rates were evaluated in day 2 embryos and late apoptosis and proliferation rates were determined in day 7 blastocysts. Our results showed that the coculture with bovine luteal cells increased the blastocyst rate compared to the control (50.4% vs. 29.8%; $P < 0.01$), but there were no differences in the cleavage rates on day 2. The rate of stage 6 blastocysts was higher in the coculture (37.3% vs. 23.8% control; $P < 0.01$), without differences in the expansion and hatching rates compared to the control. The ROS level in day 2 embryos was higher in the coculture than the control (82 vs. 57.1; $P < 0.05$), and the cell proliferation rate was higher in the coculture (48% vs. 13% control; $P < 0.01$), without differences in the mean number of cells between groups. In day 7 blastocysts, the apoptosis rate decreased in the coculture with bovine luteal cells from day 0 to day 2 (4.1% vs. 10.9% control; $P < 0.01$), whereas the cell proliferation rate and the mean number of cells did not differ between groups. This is the first report of a short-term coculture of *in vitro* produced embryos and bovine luteal cells. Our model could be an alternative to increase the efficiency of the *in vitro* production of embryos in cattle.

Keywords: coculture; bovine luteal cells; embryotrophic effect; bovine embryo quality

## 1. Introduction

The number of *in vitro* produced embryos per year in the commercial bovine embryo transfer (ET) industry has grown, increasing the demand for the

improvement of the *in vitro* fertilization (IVF) technology [1]. However, the efficiency of *in vitro* embryo production (IVP) remains low, probably because the *in vitro* environment cannot mimic the *in vivo* environment resulting in embryos with an altered quality. After IVP, the blastocyst yield is mainly affected by the intrinsic quality of the oocytes, whereas blastocyst quality is determined by the culture environment after fertilization [2]. Besides, it is accepted that embryos produced *in vitro* are inferior in quality to those produced *in vivo*. Several studies support this based on morphological data, cryotolerance, transcript expression profiles and pregnancy rates after ET (reviewed by [3]). Therefore, it is necessary to evaluate the quality of IVP embryos through methods that will allow the selection of the most suitable environment of development for the production of more competent embryos. Measurement of reactive oxygen species (ROS), apoptosis, and proliferation cell levels can be used as indicators of embryo quality [4-7]. The Ki-67 proliferation immunostain has not been used yet as an invasive biomarker of quality in IVP bovine embryos.

It is widely agreed that *in vitro* culture (IVC), whether based on single or sequential media, does not mimic the changing environment encountered by embryos descending the oviduct and entering the uterus [1]. Thus, coculture with somatic cells can be an alternative to improve suboptimal *in vitro* culture conditions and promote embryo development. This can be accomplished through two main mechanisms. First, somatic cells could remove deleterious components from the culture medium, protect against oxidative stress and modulate the medium

66 physicochemical conditions. Second, somatic cells may also secrete embryotrophic
67 factors [8].

68 The corpus luteum is a transient endocrine gland within the adult ovary which is
69 formed from the follicle wall after ovulation [9]. It is formed by at least four different
70 types of cells: large luteal cells, small luteal cells, capillary endothelial cells and
71 fibroblast [10]. Its formation and limited lifespan in the mammalian ovary is critical
72 for fertility as the corpus luteum produces progesterone ($P_4$). It is the essential
73 steroid hormone that permits embryo implantation and sustains the pregnancy [11].
74 Progesterone produced by luteal cells exerts an embryotrophic effect during early
75 pregnancy through endometrial changes [12-15] but also through a direct effect on
76 the embryo [16, 17]. Nevertheless, only two studies have reported the use of luteal
77 cells in coculture with early embryos. In the first one, bovine luteal cells were
78 cocultured with bovine blastocyst or trophoblastic vesicles produced *in vivo* to
79 investigate the production of $P_4$ [18]. The second one is a more recent study that
80 proposed a long-term embryo-luteal cells coculture to evaluate steroidogenic and
81 prostanoid interactions [19]. There is no information about using short-term
82 cocultures. Also, the quality of the embryos was not studied in detail in the previous
83 studies.

84 Consequently, the aim of this study was to assess the effect of a short-term
85 coculture system of early bovine embryos-luteal cells on the *in vitro* development
86 and embryo quality.

87 2. Materials and methods

88 2.1. Sample collection

89 For the IVP the bovine ovaries were collected from a local slaughterhouse,
90 transported to the laboratory at 28 to 35ºC within 2 h of slaughter and washed with
91 phosphate-buffered saline (PBS) immediately after arrival. For the primary culture
92 cell, the ovaries were kept at 4°C until use.

93 2. 2. Experimental design

94 The day 0 was defined as the beginning of IVC immediately after 5 h of IVF.
95 Presumptive embryos were cultured during 48 h (from day 0 to day 2) in medium
96 alone (control) or cocultured with bovine luteal cells. Then, embryos from both
97 groups were cultured in medium alone from day 2 to day 8. The development rates
98 on day 2 and 8 were compared between the groups. The ROS level and
99 proliferation rates were evaluated in day 2 embryos, and late apoptosis and
100 proliferation rates were determined in day 7 blastocysts.

101 2. 3. Primary culture of corpora lutea

102 The ovaries were classified [20], and only those with corpus luteum at the early-
103 luteal stage were dissected. The luteal tissue was mechanically separated from its
104 fibrous capsule, minced into small pieces, washed with PBS, and incubated with 1
105 IU/mL collagenase type IV (17104019; Gibco®) in Dulbecco's Modified Eagle's
106 Medium/Nutrient Mixture F-12 Ham (DMEM/F-12, D8900; Sigma-Aldrich®) for 30
107 min at 37°C in a shaking water bath. The cell suspension was filtered through a cell
108 strainer (CSS010100; BIOFIL®) to remove undigested tissue fragments and
109 centrifuged for 10 min at 200 X g. The pellet was resuspended in DMEM/F-12

supplemented (s-DMEM/F-12) with 100 IU/mL penicillin and 100 µg/mL streptomycin (15140122; Gibco®), 2 nM L-glutamine (G8540; Sigma-Aldrich), and 10% fetal bovine serum of biotechnological quality (FBS; Internegocios), and centrifuged twice for 5 min at 200 X g. Cell number and viability were estimated in a hemocytometer with the use of the trypan blue exclusion method. The cells were seeded in a T-75 culture flask with s-DMEM/F-12 and incubated in a humidified atmosphere of 5% $CO_2$ in air at 39°C. The medium was renewed every 48 h until the cell confluence.

### 2. 4. Bovine luteal cells purification

The primary cell culture was trypsinized, cells were resuspended in 2 mL of s-DMEM/F-12, placed on the top of a discontinuous Percoll gradient [21], and centrifuged at 200 X g for 30 min at room temperature (RT). The discontinuous density gradient was formed by laying 2 mL each of the following phases, 40%, 37.5%, 35%, 30%, 20% and 10% Percoll (P4937; Sigma-Aldrich) in a 15 mL centrifuge tube. Bovine luteal cells of passage 1 (BLC-1) were recovered from the 40-37.5% and 35-37.5% interphases and centrifuged twice for 5 min at 200 X g. Immunocytochemistry of 3β-Hydroxysteroid dehydrogenase enzyme was performed to confirm the luteal nature of the passage 1, according to the methodology previously published [22]. The purity was higher than 98%. Finally, BLC-1 were frozen in DMEM/F-12 supplemented with 20% FBS and 7.5% dimethyl sulfoxide (DMSO, D8418; Sigma-Aldrich), and stored at -80°C until use.

### 2. 5. Preparation of monolayers of bovine luteal cells for embryo coculture

Five days before IVF, the bovine luteal cells were warmed, resuspended in Synthetic Oviductal Fluid (SOF) [23, 24] supplemented with 5% FBS, seeded in a concentration of 25000 cells/mL in 50 µL-droplets under mineral oil and incubated in a humidified atmosphere of 5% $CO_2$ in air at 39°C. Two days before IVF, the medium of the droplets was renewed, and the day of IVF, before adding the embryos, the medium was replaced by SOF supplemented with 2.5% FBS. The medium from three droplets was pooled, frozen and stored at -20°C to measure the concentration of $P_4$ at the beginning of embryo coculture. Samples from three independent replicates were measured.

### 2. 6. Measurement of progesterone

The concentrations of P4 were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (EA 74; Oxford Biomedical Research®). According to specifications, samples and standard solution were added to a microplate. The diluted enzyme conjugate was added and the mixture was shaken and incubated for 1 h at RT. Then, the plate was washed and the bound enzyme conjugate was detected by the addition of tetramethylbenzidine. The absorbance was read after 30 min using a microplate reader (Biochrom EZ Read 400) at 650 nm, supported by Software Galapagos.

### 2. 7. Oocyte collection and *in vitro* maturation

Cumulus-oocyte-complexes (COC) were aspirated from ovarian follicles of 2 to 6 mm using an 18 ga-needle mounted on a 10 mL-syringe. The COC were selected under a stereomicroscope and washed three times in medium 199 (M-199,

11150059; Gibco®) supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin and 10% FBS. Only COC with homogeneous ooplasm and multilayered cumulus cells were used for the *in vitro* maturation (IVM). The maturation medium was M-199 supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin, 10% FBS, 0.3 mM sodium pyruvate (P2256; Sigma-Aldrich), and 1.5 IU/mL menotropin (hMG; Menopur®, Ferring). Groups of 10 COC were placed in 100 µL-droplets and were incubated for 22 h under mineral oil in 5% $CO_2$ in humidified air at 39ºC.

2. 8. *In vitro* fertilization

Frozen semen from an Holando-Argentino bull (CRB, Buenos Aires, Argentina) was thawed in a water bath at 37ºC for 30 s and centrifuged twice at 490 X g for 5 min in modified M-199 [25] supplemented with 5 mM caffeine (C0750; Sigma-Aldrich) and 20 µg/mL heparin (H3149; Sigma-Aldrich). Sperm concentration was adjusted to 15 x $10^6$/mL. The fertilization medium was modified M-199 with 10 mg/mL bovine serum albumin (BSA, A9418; Sigma Aldrich), 2.5 mM caffeine and 10 µg/mL heparin. The COC were washed with modified M-199 with 10 mg/mL BSA and subsequently exposed to the sperm suspension for 5 h in 100 µL-droplets (10 COC per droplet) at 39ºC in 5% $CO_2$ in humidified air.

2. 9. *In vitro* embryo culture

After IVF, the presumptive embryos were denuded of cumulus cells by vortexing for 3 min and cultured in groups of 25 to 30 in 25 µL-droplets of SOF with 2.5% FBS according to the experimental design. *In vitro* culture was performed at 39°C

under 5% $CO_2$ in humidified air. On day 2, embryos were transferred to new droplets of SOF and cultured until day 8.

2. 10. Assessment of embryo development

Cleavage was evaluated on day 2, and the total number of blastocysts and the stage of development (stage 5: early blastocyst, stage 6: regular blastocyst, stage 7: expanded blastocyst and stage 8: hatched blastocyst) were recorded on day 8.

2. 11. Measurement of reactive oxygen species in day 2 embryos

The intracellular ROS level was measured in a total of 76 day 2 embryos, based on the 2′,7′-Dichlorofluorescin diacetate (DCFH) assay as previously described [26, 27]. The embryos were incubated in the dark for 30 min at 39ºC in 0.1% polyvinyl alcohol (PVA) in PBS, supplemented with 153 µM DCFH (D6883; Sigma-Aldrich), washed three times in PBS-PVA and immediately analyzed in an epifluorescence microscopy (Leica DM LS) using excitation and emission wavelengths of 450 to 490 nm and 515 to 565 nm respectively. The images were acquired with a digital camera (Leica DC180) attached to a microscope (DM4000B and capture software LASZ; Leica Microsystems, Wetzlar, Germany). The arbitrary values of transmittance were measured to quantify the fluorescence emission intensity using Leica QWin V3 software. The means were compared between the experimental groups.

2. 12. Assessment of cell proliferation in embryos by immunofluorescence of Ki-67

A total of 119 day 2 embryos and 32 day 7 blastocysts were fixed in 4% paraformaldehyde (P6148; Sigma-Aldrich) for 30 min, washed twice in PBS-PVA, permeabilized with 0.2% Triton X-100 in PBS for 15 min, and incubated in 3% FBS and 0.1% Tween-20 in PBS (blocking solution, BS). Then, they were incubated with 1:100 mouse anti-Ki-67 monoclonal IgG antibody (Clone MIB-1, M7240, Dako) for 1 h (except the control) and then rinsed in BS and incubated with 1:100 FITC labeled goat anti-mouse IgG (sc-2078, Santa Cruz Biotechnology) for 1 h. The Embryos were stained with Hoechst 33342 (H1399; Invitrogen) in PBS-PVA for 10 min and immediately analyzed by epifluorescence microscopy (Leica DM LS) using excitation and emission wavelengths of 450 to 490 nm and 515 to 565 nm respectively. All incubations were performed at RT. The total cell number and the Ki-67 positive cells per embryo were recorded for each experimental group to determine the proliferation cell rate.

2. 13. Assessment of DNA fragmentation in blastocysts by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

A total of 52 day 7 blastocysts were fixed in 4% paraformaldehyde for 30 min at RT, washed twice in PBS-PVA and permeabilized with 0.5% Triton X-100 in 0.1% sodium citrate (W302600; Sigma-Aldrich) for 1 h at RT. Positive and negative controls were incubated with 100 IU/mL DNAse (D5025; Sigma-Aldrich) for 1 h at 37ºC. The TUNEL reaction was performed according to the kit manufacturer protocol (In Situ Cell Death Detection Kit, Roche). Hoechst 33342 was used as a contrast stain and samples were mounted on slides for their observation with an epifluorescence microscope (DM4000B and capture software LASZ; Leica

Microsystems, Wetzlar, Germany) using excitation and emission wavelengths of 450 to 490 nm and 515 to 565 nm respectively. The total cell number and the TUNEL positive cells per blastocyst were recorded for each experimental group to determine the late apoptosis rate.

### 2. 14. Statistical analysis

Data were analyzed using InfoStat software and *p*-values < 0.05 were considered to be statistically significant. Rates of cleavage and blastocysts were analyzed by Chi-square tests. Levels of ROS (fluorescence emission intensity of DCFH) and rates of proliferation and apoptosis per embryo were analyzed by Mann–Whitney U test.

## 3. Results

The steroidogenic activity of the cells used for embryo coculture was confirmed by the detection of $P_4$ from the culture medium. The concentration of $P_4$ ± SEM at the beginning of the coculture period was 65.5 ± 3.9 pg/mL.

Coculture with bovine luteal cells increased the blastocyst rate compared to the control group ($P < 0.01$; Table 1). However, there were no differences in the cleavage rates on day 2 (Table 1). On the other hand, the rate of stage 6 blastocysts was higher in the coculture ($P < 0.01$), without differences in the expansion and hatching rates compared to the control (P = NS; Table 1).

The ROS level in day 2 embryos was higher in the coculture than the control ($P < 0.05$; Fig. 1). Besides, the cell proliferation rate was higher in the coculture (P <

240 0.01; Fig. 2), without differences in the mean number of cells ± SD between groups
241 (8.3 ± 4.3 vs. 8.2 ± 4.4; P = NS).

242 In day 7 blastocysts, the apoptosis rate decreased in the embryos cocultured with
243 bovine luteal cells from day 0 to day 2 (P < 0.01; Fig. 3), whereas the cell
244 proliferation rate (Fig. 4) and the mean number of cells ± SD did not differ between
245 groups (76.7± 22 vs. 83.5 ± 26.2; P = NS).

246 4. Discussion

247 The coculture of embryos with different types of somatic cells has been beneficial
248 for *in vitro* embryo development, and several studies have been carried out in this
249 area during recent years (reviewed by [8]). In the present study, we have
250 demonstrated that bovine luteal cells improve the embryo development in a short-
251 term coculture system. Furthermore, it significantly decreases the late apoptosis
252 rate in blastocysts indicating an improvement in their quality.

253 The presence of several cell types in the corpus luteum brought about the need to
254 develop an effective method of purification of bovine luteal cells. According to
255 previous reports [28, 29], cell purification methods in the case of primary cultures
256 are carried out in a stage previous to seeding, after the stage of enzymatic
257 digestion. In our study, the purification was carried out in a second stage, after
258 trypsinization of the primary culture of corpora lutea, using a Percoll density
259 gradient centrifugation method. This simple yet innovative modification showed
260 more benefits in contrast with the method that carries the centrifugation before
261 seeding (data not shown). For the selection of Percoll concentrations used in this

study, we took into consideration a previous study in goats [21] due to the possible similarities of corpus luteum in ruminants. Thus, we were able to obtain luteal cells that were cryopreserved for the experiments of coculture. Based on a study that showed that the steroidogenic capacity of bovine luteal cells is not affected by cryopreservation [29], a single pool of cryopreserved luteal cells was used for all coculture replicates trying to reduce variation between them.

In the present study, the blastocyst rate was significantly higher in the coculture with bovine luteal cells than in control (50.4% vs. 29.8%, respectively). There is only one report in which bovine luteal cells were used in coculture with bovine embryos from IVF. Contrary to our results, the blastocyst rates were similar in the presence or absence of luteal cells (less than 30%). However, they reported differences in embryo quality. The coculture group significantly increased the amount of grade I and grade II embryos, but it was only based on a non-invasive morphological assessment [19].

As the arrest of embryo development occurs in the first mitotic divisions [30], we have proposed a short-term coculture system embryo-bovine luteal cells for the first 48 h of IVC. In our study, the cleavage rate after the coculture period did not show differences between experimental groups (~ 80%). However, the embryotrophic effect of the luteal cells was later evidenced. Furthermore, a study comparing the effect of a short and a long coculture system using bovine oviductal epithelial cells did not show differences between both periods [31]. The differences observed concerning the blastocyst rate between our result and Torres et al. [19] might be related to the stage in which the embryos were cocultured with luteal

cells. In contrast with our system, they used a long-term coculture without taking into account that the critical period associated with the arrested embryonic development at earlier stages. Besides, the experimental design of that research was not similar to the present study.

When evaluating the rates of the different stages of blastocysts, the coculture with bovine luteal cells did not modify the rate of expanded blastocysts or hatching. However, the rate of stage 6 blastocyst was higher in the coculture group than the control. This change in the development kinetics demonstrates an embryotrophic effect exerted by luteal cells.

We report for the first time the detection of the cell proliferation antigen Ki-67 in early bovine embryos from IVF. The day 2 embryos that were cocultured with bovine luteal cells showed a higher rate of cell proliferation (Ki-67 positive mark). This increase was reflected in a higher yield of blastocysts compared to the embryo culture in the absence of cells. In blastocysts, there were no differences between the experimental groups and, in both cases; high cell proliferation rates were observed (~ 90%). A study in murine blastocysts cocultured with mesenchymal stem cells and embryonic fibroblasts from the same species showed similar rates [32]. Likewise, a study was carried out in domestic felines in which the effect of epidermal growth factor (EGF) on the competence for development and embryo quality was evaluated by Ki-67 assessment [33]. In this case, variations associated with EGF supplementation were observed, but the cell proliferation rate was low (less than 20%) when compared to what was observed in our bovine embryo production system. Therefore, the assessment of Ki-67 antigen expression

in day 2 embryos could be used as an invasive marker of embryo quality, with a potentially predictive of embryonic competence.

Late apoptosis was evaluated in day 7 blastocysts using the TUNEL assay as an embryo quality parameter. Studies in bovine embryos and other mammalian species have associated lower levels of apoptosis with an improvement of embryo quality [4, 5, 34, 35, 36]. Although the period of coculture was limited to the first 48 h of IVC, our data indicated a significant decrease in the apoptosis rate in the coculture group compared to the control (4.1% vs. 10.9%). Therefore, our results suggest that the lower rate of apoptosis in blastocysts from coculture with BLC-1 involves an increase in its quality.

We hypothesized that the embryo coculture with luteal cells was associated with low levels of ROS. Therefore, the ROS level was evaluated after the period of coculture. Previous reports have associated higher levels of ROS with a negative impact on embryo development [37, 38]. However, we observed higher levels of ROS in the embryos with the highest competence of development to blastocyst stage. It is known that oxygen is essential for embryo development and that ROS are produced as a consequence of aerobic metabolism. During the preimplantation period, energy is needed for cell growth and differentiation [39]. So, the highest levels of ROS observed in embryos with greater cell proliferation on day 2 may be associated with an increased metabolic activity. On the other hand, enzymatic and non-enzymatic antioxidants produced by embryos and BLC-1 could compensate the increase in ROS, thus avoiding oxidative damage. Additional studies are

needed to investigate the redox status in the coculture and the possible late consequences of the increase in ROS levels.

Besides the general mechanisms of coculture, $P_4$ and prostanoids synthesized by bovine luteal cells [19] could be involved in the embryotrophic effects observed in our coculture system. The concentrations of $P_4$ detected in the culture medium of bovine luteal cells were similar to those reported by Batista et al. [29]. Lysophosphatidic acid (LPA) is a well-known mediator of cell signaling in reproductive tissues [40]. Studies in cattle showed that luteal cells have receptors for LPA [41, 42]. It was also observed that the *in vitro* supplementation of LPA after 48 h of IVC did not modify the percentages of bovine blastocysts. Nevertheless, it affected the transcription levels of the embryo quality markers [43]. An increase in the transcription of antiapoptotic genes (bcl2) and growth (igf2r), and a decrease in the proapoptotic genes (bax) were also observed [44]. These findings are related to what was observed in this work. Therefore, LPA could be one of the main mediators involved in the embryotrophic effect observed in our short-term coculture system.

This is the first report of a short-term coculture of IVP embryos and bovine luteal cells. The results indicate that the embryo coculture with BLC-1 could be an alternative to improve IVP. Considering the possible adverse long-term effects of the coculture, additional studies are needed before recommending our coculture system as an alternative to optimize IVP. Finally, our coculture system could be a valuable model for the study of the cellular interactions and the embryotrophic mechanisms involved.

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490

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493

494 Table 1

495 *In vitro* development of bovine embryos after IVC in control or coculture with bovine
496 luteal cells (BLC-1).

| Group | Oocytes n (%) | Cleaved n (%) | Day 8 Blastocysts n (%) | Blastocyst stage n (%) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Stage 5 | Stage 6 | Stage 7 | Stage 8 |
| **Control** | 141 | 118 (83.7) | 42 (29.8)[a] | 4 (9.5) | 10 (23.8)[a] | 18 (42.9) | 10 (23.8) |
| **Coculture BLC-1** | 133 | 111 (83.5) | 67 (50.4)[b] | 2 (3) | 25 (37.3)[b] | 29 (43.3) | 11 (16.4) |

497

498 Data are expressed as the means of three replicates. Values with different
499 superscripts in a column are significantly different ($P < 0.05$).

500 Captions figures

501 Figure 1

502 Level of ROS in day 2 embryos after IVC in control or coculture with bovine luteal
503 cells (BLC-1). 1) The circles indicate embryos with low levels of ROS and the
504 asterisks those with high levels. 2) Intensity of fluorescence of DCFH
505 (transmittance) expressed as the means ± SEM (control: 57.1 ± 5.7 vs. coculture:
506 82 ± 8). Scale bar = 200 µm

507 *indicates significant differences ($P < 0.05$).

508 Figure 2

509 Proliferation rate in day 2 embryos after IVC in control or coculture with bovine
510 luteal cells (BLC-1). 1) Hoechst 33342 nuclear staining and Ki-67

511 immunofluorescence in the same embryo from control (A and B) and coculture (B
512 and C). The triangles indicate proliferating cells with green nuclear mark. 2)
513 Proliferation rate per embryo expressed as the means ± SEM (control: 13 ± 2.28%
514 vs. coculture: 48 ± 4.36%). Scale bar = 100 µm.

515 *indicates significant differences (P < 0.05).

516 Figure 3

517 Apoptosis rate in day 7 blastocysts after IVC in control or coculture with bovine
518 luteal cells (BLC-1). 1) Hoechst 33342 nuclear staining and TUNEL positive cells
519 (green) in the same blastocyst from control (A and B) and coculture (C and D). 2)
520 Apoptosis rate per embryo expressed as the means ± SEM (control: 10.9 ± 2.65%
521 vs. coculture: 4.1 ± 1.37%). Scale bar = 100 µm.

522 Figure 4

523 Proliferation rate in day 7 blastocysts after IVC in control or coculture with bovine
524 luteal cells (BLC-1). 1) Hoechst 33342 nuclear staining and Ki-67
525 immunofluorescence in the same blastocyst. Proliferating cells shown green
526 nuclear mark. 2) Proliferation rate per embryo expressed as the means ± SEM
527 (control: 88 ± 2.3% vs. coculture: 90 ± 1.4%). Scale bar = 100 µm.

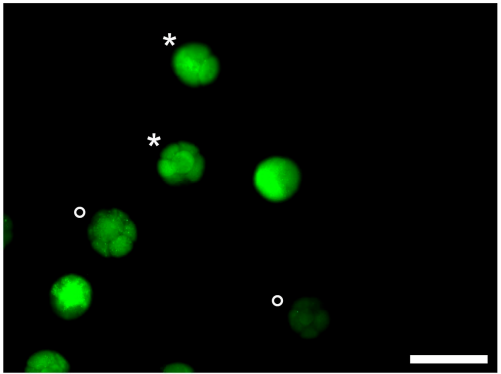
528 *indicates significant differences (P < 0.05).

529

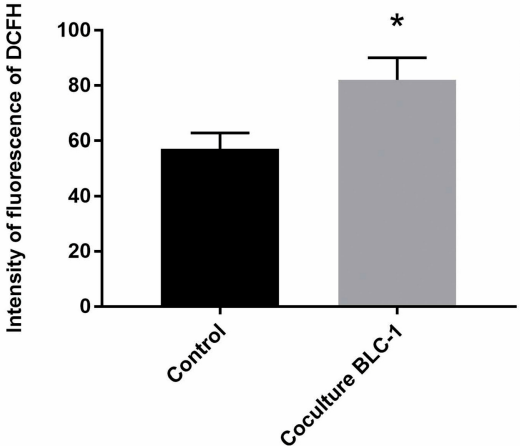
530

531

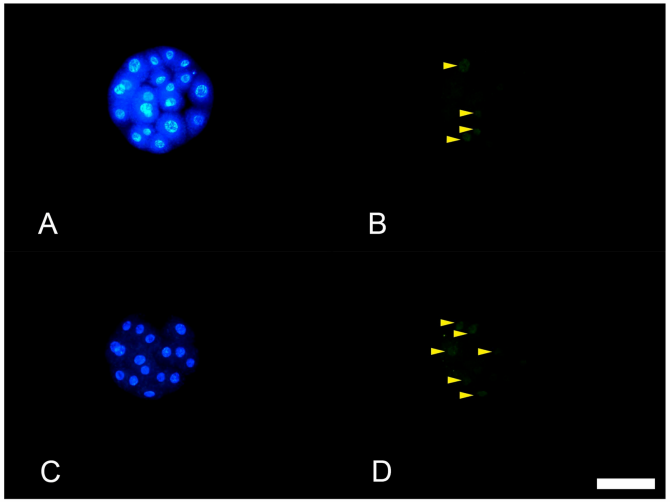
1)



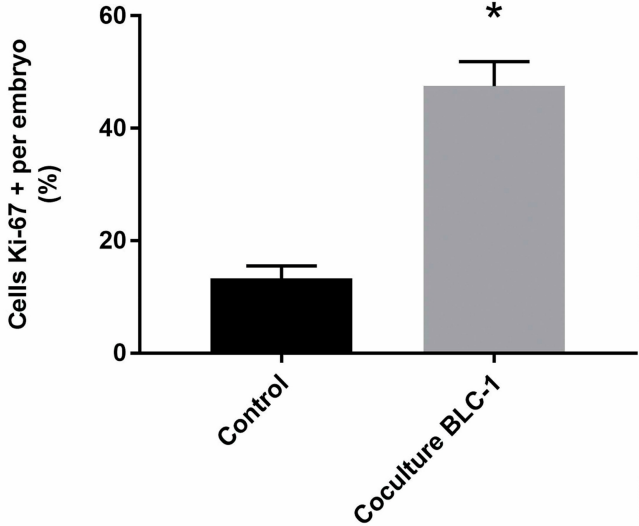
2)


Intensity of fluorescence of DCFH
100
80
60
40
20
0
*
Control
Coculture BLC-1

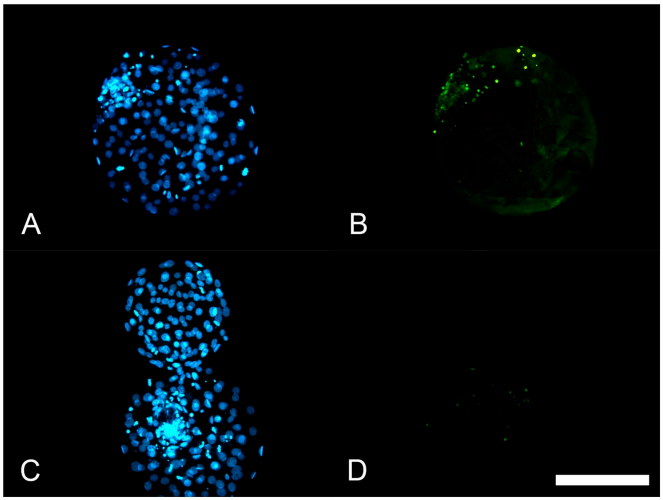
1)


HOECHST 33342
Ki-67
CONTROL
COCULTURE
A
B
C
D

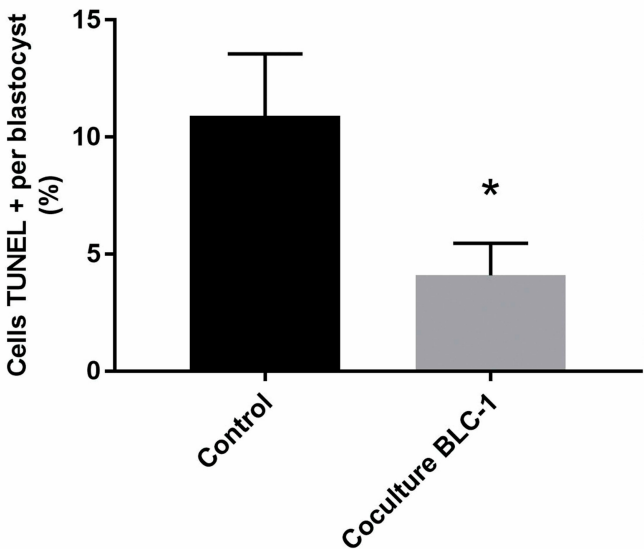
2)


Cells Ki-67 + per embryo (%)
60
40
20
0
*
Control
Coculture BLC-1

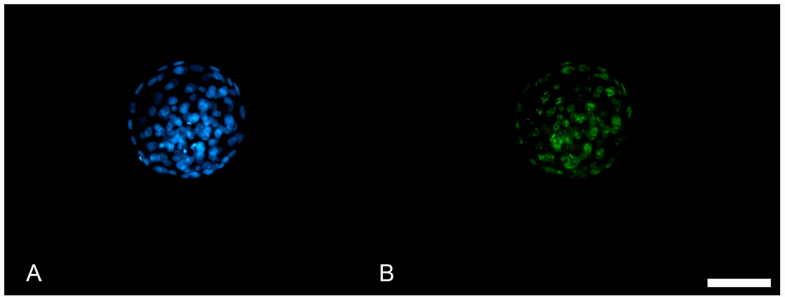
1)


HOECHST 33342
TUNEL
CONTROL
COCULTURE
A
B
C
D

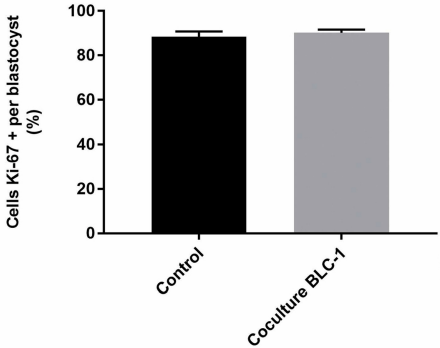
2)


Cells TUNEL + per blastocyst (%)
15
10
5
0
*
Control
Coculture BLC-1

1)


HOECHST 33342
Ki-67
A
B

2)


Cells Ki-67 + per blastocyst (%)
100
80
60
40
20
0
Control
Coculture BLC-1