Oocyte developmental capacity is influenced by intrinsic ovarian factors in a bovine model for individual embryo production

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ABSTRACT

The ovary and its hormones may have major effects on the *in vitro* developmental capacity of the oocytes it contains. We related intrinsic ovarian factors namely the presence of corpus luteum (CL) and/or dominant follicle (>8 mm) and the follicular count to cumulus expansion (CE), embryo development, and blastocyst quality in a bovine model. Cumulus-oocyte-complexes (COCs) were aspirated from follicles between 4 and 8 mm in diameter. *In vitro* embryo production was performed in a fully individual production system. The follicular fluid from which COCs were collected was pooled (per ovary) to evaluate the estrogen, progesterone, and insulin-like growth factor-1 (IGF-1) concentrations. Cumulus oocyte complexes collected from ovaries without a CL presented a greater CE than COCs derived from ovaries bearing CL. The absence of ovarian structures increased the blastocyst rate when compared to oocytes derived from ovaries with a CL, a dominant follicle, or both. Blastocysts derived from ovaries without a dominant follicle presented higher total cell numbers and a lower proportion of apoptosis than blastocysts derived from ovaries containing a dominant follicle. Cumulus oocyte complexes collected from ovaries with high follicular count resulted in higher cleavage than from ovaries with low follicular count, but the blastocyst rate was similar between groups. Ovaries bearing a CL had greater progesterone and IGF-1 follicular fluid concentrations in neighbouring follicles than ovaries without a CL. Selection for bovine ovaries without CL or dominant follicle can have positive effects on CE, embryo development, and blastocyst quality in an individual embryo production system set-up.

1. Introduction

Oocyte quality, defined as the capacity of oocytes to develop into an embryo with high implantation potential (Rienzi *et al.*, 2012) may be affected by diet (Adamiak *et al.*, 2005; Fouladi-Nashta *et al.*, 2007), metabolic stress (Leroy *et al.*, 2012; Gu *et al.*, 2015), disease (Roth *et al.*, 2013; Dickson *et al.*, 2020), and aging (Miao *et al.*, 2009). However, within healthy individuals, the estrous cycle stage and its hormonal tides can also influence the oocyte developmental capacity (Machatková *et al.*, 1996). Conflicting results on the potential impact of corpus luteum and follicles at diverse stages of development on oocyte developmental capacity are reported in the literature (Boediono *et al.*, 1995; Sugulle *et al.*, 2008; Hajarian *et al.*, 2016). The corpus luteum is a transient reproductive gland that mostly produces progesterone (P_4), a steroidal hormone required to establish and maintain pregnancy (Davis and Rueda, 2002). The corpus luteum might affect the milieu and growth of neighbouring follicles by increasing their intrafollicular P_4 concentration. Follicles developing in the adjacencies of the corpus luteum may have higher P_4 levels than those growing in the contralateral ovary of the same animal (Kor, 2014; El-Shahat *et al.*, 2019; Satué *et al.*, 2020). Furthermore, the blood flow (and nutrients supply) of ovaries bearing an active corpus luteum is higher than in ovaries with no corpus luteum (Ford and Chenault, 1981; Acosta *et al.*, 2003; Abdelnaby *et al.*, 2018). Yet, many inconsistencies on the impact of corpus luteum on embryo developmental capacity and quality exist within published literature (Sugulle *et al.*, 2008; Hajarian *et al.*, 2016; Argudo *et al.*, 2020).

In most mammals, follicular development happens in waves (Baird, 1987; Sirois and Fortune, 1988; Fortune, 1994), and for each wave in a mono-ovulatory species, one follicle grows into dominant while the others (subordinate follicles) regress (Hodgen, 1982; Gougeon, 1986; Savio, 1990). The fate of subordinate follicles are influenced by a plethora of factors, but the inhibitory effect of the dominant follicle remains pivotal (Matton et al., 1981; Wolfsdorf et

al., 1997). The dominant follicle suppress FSH secretion from the pituitary gland via estradiol (E_2) and inhibin production (Fortune, 1994), but other factors may exert their inhibitory action at the ovarian level. Subordinate follicles also secrete E_2 and inhibin, but in a lower extend. Thus, the presence of a dominant follicle may affect the intrafollicular concentration of several paracrine factors in adjacent follicles, affecting the developmental capacity of the oocytes they contain. Among them, the intrafollicular concentrations of factors such as insulin-like growth factors (IGF), which have a crucial role in follicular growth and oocyte development (Yoshimura *et al.*, 1996; Palma *et al.*, 1997), may also be affected either by the presence of a corpus luteum or dominant follicle (Einspanier et al., 1990; Lucy, 2000). In cows, it was reported that the early stages of corpus luteum and granulosa and theca interna cells of large antral follicles have the highest mRNA expression of IGF-1. Similarly, it was shown that the mRNA expression and immunolocalization of IGF-1 in granulosa cells depend on the stage of follicular development (Schams *et al.*, 2002). Interestingly, Yuan *et al.* (1998) stated increasing levels of IGF-1 mRNA from early to mid-dominance, and other authors found increasing concentrations of IGF-1 in the follicular fluid (FF) of large and preovulatory follicles (Einspanier *et al.*, 1993; Sudo *et al.*, 2007; Velazquez *et al.*, 2009). Furthermore, it has recently been suggested that oocyte developmental capacity could be affected by follicular counts (FC) (Mossa *et al.*, 2012; Favoreto *et al.*, 2019). Some studies have demonstrated that better fertility is correlated with high FC in humans (Te Velde and Pearson, 2002; Broekmans *et al.*, 2006) and cattle (Ireland *et al.*, 2008; Santos *et al.*, 2016). Nevertheless, contradictory results have been published on the lack of effect between FC and fertility in cows (Santos *et al.*, 2013; Silva-Santos *et al.*, 2014).

Autocrine and paracrine factors are secreted by group cultured cumulus-oocyte-complexes (COCs) and embryos, which can affect the same or neighboring COCs/embryos and result in stimulating their development (Paria and Dey, 1990; O'Neill, 2008). Nevertheless,

non-cleaved or underdeveloped embryos may also secrete inhibitory factors in the group culture, adversely affecting the development of neighboring blastocysts (Tao *et al.*, 2013; Wang *et al.*, 2021). Furthermore, it has been suggested that metabolic by-products such as ammonia or lactate or depletion of necessary substrates by dying embryos have more adverse effects on fast-developing embryos (Reed *et al.*, 2011). We recently optimized a fully individual bovine embryo production system to investigate conditions that may affect development and blastocyst quality in mono-ovulatory species (Azari-Dolatabad *et al.*, 2021). Thus, this study aimed to determine the effect of intrinsic ovarian factors e.g., corpus luteum, dominant follicle, and FC on *in vitro* cumulus expansion (CE), embryo development, and blastocyst quality in a serum-free individual culture system. We hypothesized that those intrinsic ovarian factors may affect oocyte quality and thus resulting embryo development and quality, as can be demonstrated in an individual culture system, which allows to follow the fate of a single oocyte and correlate this with ovarian factors. Furthermore, to associate the effect of corpus luteum and dominant follicle on COC development, we evaluated the P_4, E_2, and IGF-1 concentrations in follicles (4 to 8 mm) from ovaries with and without a corpus luteum or dominant follicle.

2. Materials and methods

2.1. Media and reagents

Tissue culture media (TCM)-199 medium, gentamycin, and phosphate-buffered saline (PBS) were purchased from Life Technologies Europe (Ghent, Belgium). All other chemicals not otherwise listed were obtained from Sigma-Aldrich (Overijse, Belgium). All media were filtered before use (0.22 μm; GE Healthcare-Whatman (Diegem, Belgium)).

2.2. Reproductive tract selection

Slaughterhouse-derived Belgian Blue cow reproductive tracts were collected and only non-pregnant uteri (and ovaries) without evidence of gross lesions were selected. The average

sacrifice age of Belgian Blue cows in Belgian conditions is around five years (Azari-Dolatabad et al., 2021). Endometrial cytobrush samples after cut-opening each uterine horn were collected and all samples contained less than 5% polymorphonuclear leucocytes (Pascottini et al., 2015). Selection criteria for paired ovaries included the presence of a corpus luteum >1.5 cm (in diameter) and no follicle >2.5 cm (in diameter) in each ovary. The corpus luteum was classified as an early or late luteal stage if it was reddish or if the color of the corpus luteum was orange/brown, respectively (De Wit et al., 2000). For each ovary, the diameter of the larger follicles was measured using a digital caliper and categorized as >8, >12, or >15 mm.

2.3. In vitro embryo production and follicular fluid collection

The experimental design is depicted in Figure 1. In 12 replicates, a total of 54 genital tracts (108 ovaries) were selected and the individual embryo production was performed as previously described (Azari-Dolatabad *et al.*, 2021). Briefly, the ovaries were cut from each reproductive tract and washed three times in warm physiological saline supplemented with kanamycin (25 mg/mL), sterilized with 90% ethanol, and dried with clean towels. Keeping track of the ovary of origin, COCs and FF were aspirated from 4-8 mm diameter follicles using an 18-gauge needle attached to a 10 mL syringe and pooled into microcentrifuge tubes (1.5 mL) and placed in an incubator (38.5 °C) for 30 min to allow to sediment. On the one hand, the COCs-free FF supernatants (250–400 μL) were carefully collected, transferred to another microcentrifuge tube (1.5 mL), and centrifuged at 2460 × *g* at 4 °C for 7 min. After centrifugation, supernatants were collected and stored at −20 °C until further analysis. Next, 500 μL of HEPES-TALP was added to the microcentrifuge tubes containing the COCs and transferred to a 35 × 10 mm petri dish (Thermo Fisher Scientific, Waltham, MA USA; one petri dish per ovary). This procedure was repeated 3 times to assure the collection of all the COCs. Only COCs with uniform cytoplasm and surrounded by at least three or more compact layers of cumulus cells were selected. *In vitro* maturation (IVM) was performed individually in petri

dishes (60 × 15 mm; Thermo Fisher Scientific, Waltham, MA USA) containing 17 droplets of 20 µL of maturation medium (TCM-199 with 20 ng/mL epidermal growth factor (EGF) and 50 µg/mL gentamycin) covered with 7.5 mL paraffin oil (SAGE oil for tissue culture, ART-4008-5P, Cooper Surgical Company) for 22 h at 38.5 °C in 5% CO_2 in humidified air. For *in vitro* fertilization (IVF), a discontinuous 45/90% Percoll® gradient (GE Healthcare Biosciences, Uppsala, Sweden) was prepared as previously described (Wydooghe et al., 2014) and frozen thawed spermatozoa from a bull of proven fertility was used. Fertilization was done individually in droplets of 20 µL of IVF-Tyrode's Albumin Lactate Pyruvate (IVF-TALP) medium covered with 7.5 mL paraffin oil. To do so, the sperm concentration was adjusted to 1x106 spermatozoa/mL using IVF–TALP medium enriched with bovine serum albumin (BSA; Sigma A8806, 6 mg/mL) and heparin (25 mg/mL). Individual COCs were then transferred to each droplet and incubated for 21 h at 38.5 °C in 5% CO2 in humidified air. After 21 h of fertilization, COCs from each dish were pooled and vortexed for 3 minutes. Cumulus-free presumed zygotes were washed 3 times in a synthetic oviductal fluid (SOF) medium and individually transferred to 20 µL SOF droplets supplemented with 0.4% BSA (Sigma A9647), insulin, transferrin, and selenium (5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL selenium), and covered with 7.5 mL paraffin oil. Afterward, presumed zygotes were incubated at 38.5 °C for 8 days in 5% CO_2, 5% O_2, and 90% N_2.

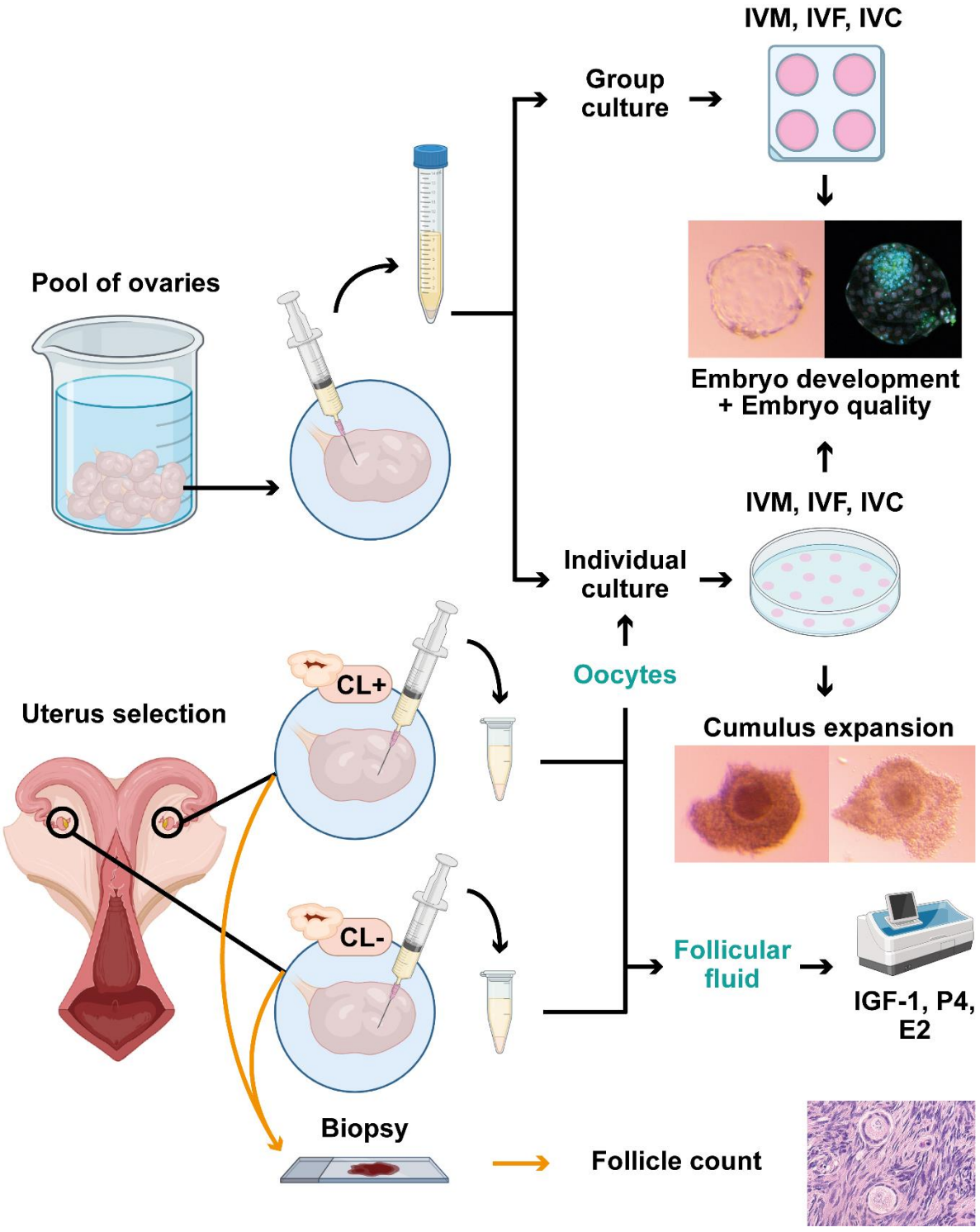

Figure 1. Schematic representation of the study design. *In vitro* maturation (IVM); *in vitro* fertilization (IVF); *in vitro* culture (IVC); with corpus luteum (CL+); without corpus luteum (CL-); insulin-like growth factor (IGF)-1; progesterone (P_4), and estradiol (E_2).

Alongside each replicate, individual and group controls were included. The individual control consisted of COCs selected from a random pool of ovaries with different structures (corpus luteum and dominant follicle) and cultured as described above. For the group control, COCs from a random pool of ovaries were matured and fertilized in groups of 60 in 500 µL of maturation and fertilization medium without oil coverage. *In vitro* culture was done in groups of 25 zygotes in droplets of 50 µL of SOF medium covered with 900 µL of paraffin oil. In all cases (group and individual culture systems), cleavage rate was determined at 45 h post insemination as the percentage of cleaved embryos out of presumed zygotes. Blastocyst rates were assessed on days 7 and 8 post insemination as the percentage of blastocysts out of presumed zygotes. The hatching rate was assessed as the percentage of hatching/hatched blastocysts out of the total number of embryos that reached the blastocyst stage at day 8 post insemination.

2.4. Hormonal evaluation of the follicular fluid

Follicular fluid concentrations P_4, E_2, and IGF-1 were measured in a subset of 30 samples (15 cows). Of the ovaries, 12, 10, and 8 had CL, DF (>8 mm), and both structures, respectively. Progesterone and E_2 concentrations were measured with an auto-chemistry analyzer (Cobas 6000 c 501, Roche Diagnostics, Indianapolis, IN) using the Progesterone III and the Estradiol III kits (Roche Diagnostics, Indianapolis, IN). Follicular fluid samples were diluted at 1:5 for P_4 analysis and 1:100 for E_2 analysis to avoid exceeding assay detection limits. The intra-coefficient of variation was <10% for all analyses. A human immunoassay (Quantikine ELISA, R&D Systems, Minneapolis, MN) was used for the analysis of FF IGF-1 and the procedure was performed according to the manufacturer's instructions. The sequence of human IGF-1 is identical to that of cattle (Honegger and Humbel, 1986). The detection limit of the assay was 0.026 µg/L, and the intra-assay coefficient of variation was 6.5%.

2.5. Ovarian follicular counts via histology

Following COCs collection, a 1 cm^2 of each ovary's cortex was cut with a scalpel blade and put in a tube containing 4% paraformaldehyde before being processed for histopathological examinations (hematoxylin and eosin-stained). Samples were examined by a single observer using a conventional light microscope (Kyowa Optical, Tokyo, Japan). The number of pre-antral follicle counts (primordial, primary, and secondary) was assessed in 3 random high power fields (magnification x 400) and divided into high or low FC (above or below the median count per ovary, respectively).

2.6. Cumulus oocyte complex expansion

The CE was measured from individually-matured oocytes as previously described (Azari-Dolatabad *et al.*, 2021). A digital microscope camera (ToupCam®, Hamamatsu, Japan) connected to a stereomicroscope, always under the same magnification (56 X) and with the lens focused on the zona pellucida, was used to take images of COCs before and after 22 h of maturation (Figure 1). For each COC picture, the distance between the zona pellucida and the external border of cumulus cells was measured in three places: the shortest, medium, and longest distances (Figure 1). Measurements before and after maturation were compared, and the difference between them was calculated based on the mean of three distances. (in μm) using the ImageJ software (Abràmoff *et al.*, 2004) (version 1.49 q; National Institutes of Health).

2.7. Embryo quality assessment by differential staining

As described by Wydooghe *et al.* (2011) differential staining was performed to evaluate the blastocyst quality. Briefly, blastocysts on day 8 were fixed in 2% paraformaldehyde for 20 min at room temperature and then stored in phosphate-buffered saline containing 0.5% BSA at 4 °C. In the first step, immunofluorescent staining with CDX2, a transcription factor exclusively expressed in trophectoderm (TE) cells, was performed (Figure 1). This was followed by active caspase-3 staining (for apoptosis) and further combined with 0.1% Hoechst 33342 staining (Figure 1). We assessed the number of TE cells, inner cell mass cells (ICM), the

total number of cells (TCN; TE + ICM), the ratio of ICM/TCN, and the total number of apoptotic cells (AC) and their ratio (ACR; AC/TCN). These assessments were done by fluorescence microscopy (Leica DM 5500 B) using a triple bandpass filter by a single observer.

2.8.Statistical analyses

Statistical analyses were performed in R-core (version 3.6.1; R Core Team, Vienna, Austria). Generalized mixed-effects models were used to fit the effects of the presence of a corpus luteum (yes vs no), corpus luteum stage (early vs late), the dominant follicle (yes vs no), and FC (low vs high) on developmental parameters (cleavage, day 7 blastocyst, day 8 blastocyst, and hatching rates). First, univariable models were fitted and variables associated with embryo development with $P < 0.2$ were identified. Then, multivariable models were built by manual stepwise backward elimination. Only variables and first-order interactions with $P < 0.05$ were retained in the final model. Similarly, mixed linear regression models were used to test the effects of each of the aforementioned fixed effects on CE and differential staining parameters (TCN, TE, ICM, ICM/TCN, AC, and AC/TCN). For all the models, the replicate number nested with the uteri ID was set as a random effect. Residuals for each model were analyzed by Shapiro-Wilk's test. If the distribution of model residuals was not normal, log10, ln, square root, square, or cubic transformations of the raw data were performed until visual inspection of the histogram and quantile plot of the residuals indicated the most-nearly normal distribution. The differences between treatment groups were assessed using Tukey's post hoc test. Results are expressed as least squares means and standard errors. The significance level was set at $P < 0.05$.

3. Results

3.1. *The effect of individual and group culture on embryo development and quality*

A total of 1,722 COCs over 12 replicates were individually cultured for the present paper (n = 193 COCs were individual control and n = 1,549 COCs were from the individual experimental group). The group control consisted of the culture of 605 COCs. The cleavage, day 7, and day 8 blastocyst rates were higher ($P < 0.01$) for group control (82.2 ± 2.2, 25.9 ± 2.1, and 37.5 ± 2.1%, respectively) compared to individual control (71.1 ± 3.9, 16.4 ± 2.7, and 24.8 ± 3.1%, respectively) or the individual experimental group (57.5 ± 2.8, 13.9 ± 1.1, and 17.2 ± 1.1%, respectively). The cleavage and day 8 blastocyst rates were higher in individual control than the in the individual experimental group ($P < 0.02$), but the day 7 blastocyst rate was similar ($P = 0.61$). Interestingly, the hatching rate was lower ($P < 0.03$) in the group control (23.6 ± 2.8%) compared to the individual control or experimental groups (41.7 ± 7.1 and 34.6 ± 2.8%, respectively). No differences in the hatching rate were found between individual control and the individual experimental group ($P = 0. 62$).

3.2. *Ovarian factors associated with embryo developmental parameters*

Results of univariable models of ovarian factors associated with embryo development are summarized in Table 1. Cumulus-oocyte-complexes collected from ovaries bearing a corpus luteum or dominant follicle >8 mm presented lower development (cleavage, day 7, and day 8 blastocysts) than COCs collected from ovaries without a corpus luteum or dominant follicle >8 mm ($P < 0.04$). Cumulus oocyte complexes collected from ovaries with high FC gave rise to embryos with higher cleavage rates ($P < 0.01$) than from ovaries with low FC, but the day 8 blastocyst rate was similar between groups ($P = 0.62$). Remarkably, COCs collected from ovaries without a corpus luteum or dominant follicle >8 mm presented the highest developmental parameter rates compared to all the other groups, as depicted in Table 2. No differences ($P < 0.35$) in developmental parameters were found for corpus luteum in early luteal or late luteal phase (52.0 ± 4.5 and 55.4 ± 3.5% for cleavage, 9.0 ± 2.0 and 12.6 ± 1.6% for day 7 blastocyst, and 9.9 ± 2.0 and 12.9 ± 1.4% for day 8 blastocyst, respectively). None of the

evaluated parameters affected the hatching rate ($P < 0.12$), which ranged from 28 to 38%. Follicles with >8 mm were the only dominant follicle category with effects on embryo developmental parameters, thus for further evaluations, only COCs collected from ovaries with dominant follicles> 8 mm were considered for further statistical analyses. We found a low

number of ovaries bearing dominant follicles (> 12 mm or > 15 mm) in the present experiment (Table 1).

Table 1. Results of univariable models for factors associated with *in vitro* embryo development in an individual culture system. Ovarian origin and their structures (corpus luteum (CL), dominant follicle (DF), and follicular count (FC)) from which oocytes were collected were tracked, and oocytes/zygotes were individually cultured. Results are expressed as least square means ± standard errors.

Variable		No. COCs	Cleavage	*P*-value	Day 7 blastocyst	*P*-value	Day 8 blastocyst	*P*-value
CL	Yes	851	54.4 ± 3.3	0.007	11.6 ± 1.4	0.006	12.1 ± 1.1	<0.0001
	No	728	61.3 ± 3.3		15.4 ± 1.8		23.2 ± 1.8	
DF (>8 mm)	Yes	561	54.0 ± 3.7	0.04	10.9 ± 1.6	0.01	14.0 ± 1.6	0.01
	No	1,018	59.7 ± 3.3		15.4 ± 1.7		19.0 ± 1.5	
DF (>12 mm)	Yes	239	53.9 ± 4.4	0.23	14.5 ± 2.5	0.74	16.1 ± 2.4	0.61
	No	1,340	58.2 ± 3.1		13.7 ± 1.3		17.4 ± 1.1	
DF (>15 mm)	Yes	64	55.9 ± 7.3	0.79	14.6 ± 4.6	0.86	15.2 ± 4.5	0.67
	No	1,515	57.7 ± 3.1		13.8 ± 1.3		17.3 ± 1.1	
FC	High	782	60.8 ± 3.2	0.01	15.0 ± 1.6	0.08	17.7 ± 1.4	0.62
	Low	797	54.0 ± 3.4		11.6 ± 1.3		16.7 ± 1.4	

Table 2. Results of multivariable models for factors associated with *in vitro* embryo development in an individual culture system. Ovarian origin and their structures from which oocytes were collected were tracked, and oocytes/zygotes were individually cultured. Dominant follicle is >8 mm in diameter. Results are expressed as least square means ± standard errors.

Variable	Corpus luteum YES		Corpus luteum NO	
	Dominant follicle YES	Dominant follicle NO	Dominant follicle YES	Dominant follicle NO
Cleavage	51.5 ± 4.0	56.7 ± 3.7	59.1 ± 4.8	62.1 ± 3.5
Day 7 blastocyst	11.4 ± 1.9[a]	11.8 ± 1.8[a]	10.1 ± 2.3[a]	18.6 ± 2.2[b]
Day 8 blastocyst	13.5 ± 1.9[a]	10.8 ± 1.5[a]	14.4 ± 2.7[a]	26.5 ± 2.3[b]

Within a row, different superscripts [a,b] indicate significant differences ($P < 0.05$).

3.3. *Ovarian factors associated with blastocyst quality parameters*

Ovarian factors associated with blastocyst quality parameters are summarized in Table 3. Neither corpus luteum nor FC affected differential nor apoptotic staining parameters ($P < 0.25$). Interestingly, blastocysts derived from COCs from ovaries with dominant follicles > 8 mm had lesser TCN and a higher number of apoptotic cells (greater AC and AC/TCN) than those blastocysts derived from ovaries with dominant follicles ≤ 8 mm ($P < 0.01$). No differences ($P < 0.11$) in blastocyst quality parameters were found for corpus luteum in early or late luteal phase.

Table 3. Results of univariable models for total cell number (TCN), trophectoderm cells (TE), inner cell mass (ICM), and apoptotic cells (AC) of day 8 differentially stained blastocysts. Ovarian origin and their structures (corpus luteum (CL), dominant follicle (DF), and follicular count (FC)) from which oocytes were collected were tracked, and oocytes/zygotes were individually cultured. Results are expressed as least square means ± standard errors.

Variable		No. of blastocysts	Cell numbers			Ratios (%)		
			TCN	ICM	TE	AC	ICM/TCN	AC/TCN
CL	Yes	134	97.0 ± 0.7	36.1 ± 0.7	60.9 ± 0.5	2.8 ± 0.07	37.0 ± 0.5	3.0 ± 0.08
	No	136	98.2 ± 0.7	36.6 ± 0.7	61.5 ± 0.5	2.8 ± 0.07	37.1 ± 0.5	2.9 ± 0.08
DF (> 8mm)	Yes	93	95.8 ± 9.0[a]	35.1 ± 0.8	60.7 ± 0.6	3.1 ± 0. 08[a]	36.5 ± 0.6	3.3 ± 0.09[a]
	No	177	98.5 ± 0.6[b]	37.0 ± 0.6	61.5 ± 0.4	2.7 ± 0.06[b]	37.3 ± 0.4	2.7 ± 0.06[b]
FC	High	129	98.7 ± 0.7	36.9 ± 0.7	61.8 ± 0.5	2.7 ± 0.07	37.2 ± 0.5	2.8 ± 0.08
	Low	141	96.6 ± 0.7	35.8 ± 0.6	60.8 ± 0.5	2.9 ± 0.07	36.9 ± 0.5	3.0 ± 0.08

Within a variable column, different superscripts [a,b] indicate significant differences ($P < 0.05$).

3.4. *Ovarian factors associated with cumulus expansion and follicular fluid hormonal concentrations*

Ovarian factors associated with CE are shown in Figure 2. Cumulus oocyte complexes collected from ovaries without a corpus luteum presented a greater ($P < 0.008$) CE (232 ± 4.4 µm) than COCs derived from ovaries bearing corpus luteum (216 ± 4.2 µm). Dominant follicle > 8mm or FC did not have an effect on CE ($P < 0.31$).

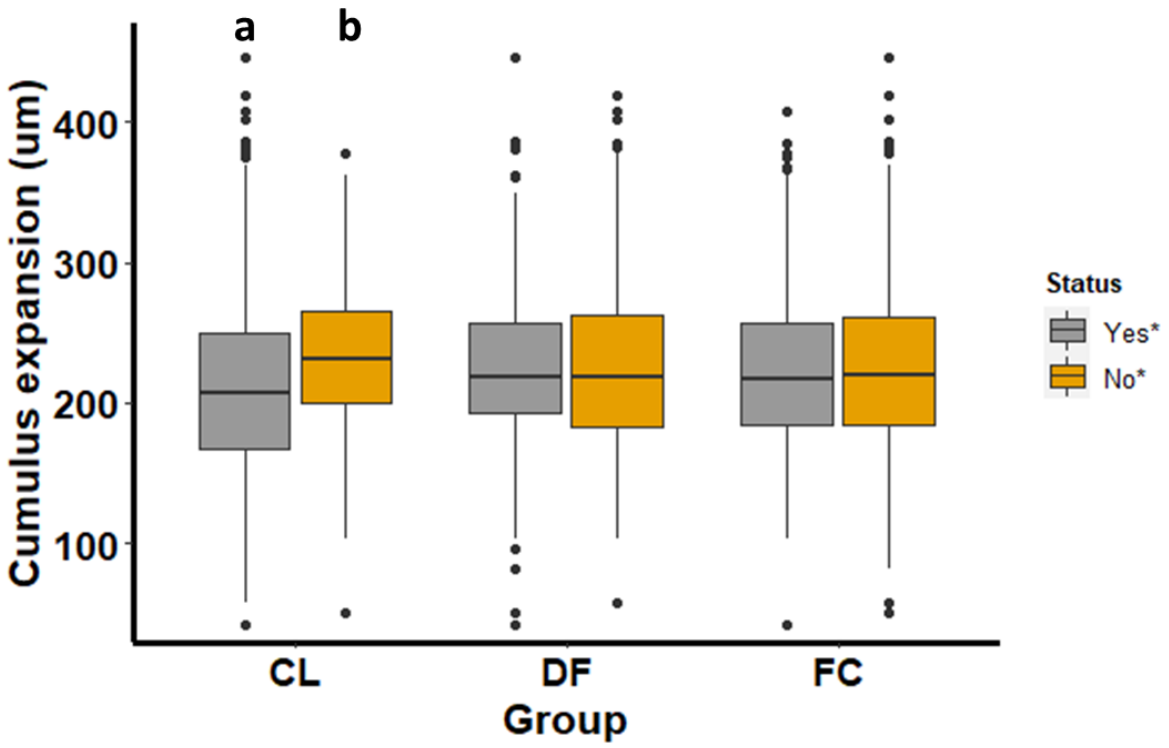

Figure 2. Boxplots showing cumulus expansions of oocytes after 22 h maturation. The cumulus expansion consisted of the difference between the distance between the zona pellucida and the extreme of the cumulus cells before and after maturation. Ovarian origin and their structures (corpus luteum (CL), dominant follicle (DF > 8 mm), and follicular count (FC)) from which oocytes were collected were tracked, and oocytes were individually matured. Different superscripts ($^{a, b}$) represent statistical differences ($P < 0.05$). *Yes and no represent high and low for FC, respectively.

To further explore potential factors associated with embryo development and blastocyst quality, we evaluated the hormonal profile of the FF from the pool of follicles (4 to 8 mm in diameter) of individual ovaries from which COCs were collected. Table 4 summarizes the E_2, P_4, and IGF-1 profiles of pooled FF from individual ovaries with or without a corpus luteum, the dominant follicle (> 8 mm), and high or low FC. Follicular fluid E_2 concentrations were not affected by ovarian structures ($P > 0.18$). However, ovaries bearing a corpus luteum had greater P_4 and IGF-1 FF concentrations ($P > 0.04$) in neighbouring follicles (4 to 8 mm in diameter) than ovaries without a corpus luteum. Remarkably, the P_4 concentration was the highest when a dominant follicle (> 8 mm) accompanied the corpus luteum in the same ovary (Table 5). Conversely, the IGF-1 concentration was the highest when no dominant follicle (> 8 mm) accompanied the corpus luteum in the same ovary (Table 5).

Table 4. Results of univariable models for factors associated with hormone concentrations of pooled fluid from follicles (4 to 8 mm in diameter) of individual ovaries from which oocytes were collected and individually cultured. Results are expressed as least square means ± standard errors.

Hormone	Corpus luteum			Dominant follicle (> 8 mm)			Follicular count		
	Yes	No	*P*-value	Yes	No	*P*-value	High	Low	*P*-value
Estrogen (ng/mL)	2.0 ± 7.9	3.5 ± 7.9	0.18	2.7 ± 1.0	2.7 ± 0.6	0.96	2.4 ± 0.9	2.9 ± 0.7	0.18
Progesterone (ng/mL)	102 ± 10.2	58.9 ± 10.2	0.005	104 ± 14.4	70.1 ± 9.1	0.05	64.7 ± 13.1	89.6 ± 9.9	0.14
IGF-1 (µg/L)	0.4 ± 0.05	0.2 ± 0.05	0.04	0.2 ± 0.07	0.3 ± 0.04	0.11	0.3 ± 0.07	0.3 ± 0.05	0.90

Table 5. Results of multivariable models for factors associated with hormone concentrations of pooled fluid from follicles (4 to 8 mm in diameter) of individual ovaries from which oocytes were collected and individually cultured. Dominant follicle is >8 mm in diameter. Results are expressed as least square means ± standard errors.

Hormone	Corpus luteum YES		Corpus luteum NO	
	Dominant follicle YES	Dominant follicle NO	Dominant follicle YES	Dominant follicle NO
Progesterone (ng/mL)	134.1 ± 16.4[a]	86.0 ± 11.6.8[b]	68.2 ± 18.4[b]	55.6 ± 11.1[b]
IGF-1 (µg/L)	0.21 ± 1.08[a]	0.50 ± 0.06[b]	0.23.8 ± 0.09[a]	0.24 ± 0.06[a]

Within a row, different superscripts [a,b] indicate significant differences ($P < 0.05$).

4. Discussion

We showed that the presence of a corpus luteum or dominant follicle (> 8 mm) within the ovary used for oocyte collection has adverse effects on subsequent CE and on resulting embryo development, and blastocyst quality. Hence, when performing *in vitro* embryo production in an individual culture system, ovarian intrinsic factors should be considered when the aim is to obtain the greater possible developmental rate with high blastocyst quality.

Concerning the effects (negative, positive, or no effect) of the presence of the corpus luteum on the developmental competence of oocytes matured *in vitro*, many inconsistencies have been reported in the literature (Boediono *et al.*, 1995; Sugulle *et al.*, 2008; Hajarian *et al.*, 2016), but all those studies were performed using group culture. We found that ovaries bearing a corpus luteum have lower COC developmental capacity and higher P_4 concentrations within follicles (4 to 8 mm in diameter) than ovaries without a corpus luteum. Other authors similarly found that oocytes collected from ovaries with a lower concentration of P_4 in FF resulted in higher blastocyst and hatching rates compared to ovaries with a higher concentration of P_4 in FF (Urrego *et al.*, 2015). Furthermore, Ben-Rafael *et al.* (1987) reported that oocytes collected from follicles with a high level of P_4 in FF resulted in abnormally fertilized oocytes and finally multipronuclear embryos in women. During the final stage of maturation, an ideal balance of FF hormone levels appears to be critical for the developmental competency of the oocyte (Aardema *et al.*, 2013). However, high P_4 concentrations in antral follicles at an initial stage of development seem to have carryover effects on further embryo development. Growing follicles

are sensitive to steroid hormonal concentration as they may mature or become atretic during the follicular wave (Ginther *et al.*, 1996, 2001). In that regard, the E_2/P_4 ratio may indicate the degree of atresia of a given follicle (Price *et al.*, 1995; De los Reyes *et al.*, 2006), and it also has been reported that the imbalance between these two hormones is associated with the apoptosis of granulosa cells (Yu *et al.*, 2004). In the present study, the ratio of E_2/P_4 of the FF in ovaries without a corpus luteum was 3-fold higher compared to ovaries bearing a corpus luteum. Thus, ovaries bearing an active corpus luteum may have more atretic follicles, containing COCs with lower developmental competence than ovaries without a corpus luteum. However, in contrast to our findings, some studies reported a positive effect of corpus luteum on the developmental competence of oocytes (Boediono *et al.*, 1995; Pirestani *et al.*, 2011; Argudo *et al.*, 2020). For example, Boediono *et al.* (1995) found beneficial effects of a corpus luteum when culturing immature oocytes in TCM-199 supplemented with FSH and superovulated cow serum. Likewise, Argudo *et al.* (2020) showed that the corpus luteum positively stimulated oocyte development in an IVM medium supplemented with fetal bovine serum, sodium bicarbonate, glutamine, rh-FSH, cysteamine, estradiol, and gentamicin. Discrepancies among results may be due to different culture systems (individual culture system in our study and group culture system in the other studies) or serum supplementation in the maturation medium. Furthermore, maturing oocytes in groups involve the secretion of cytokines and growth factors (Paria and Dey, 1990; Pavani *et al.*, 2022), restoring the adverse effects that high P_4 may have in individually cultured oocytes. Importantly, the fact that abattoir-harvested ovaries were used for assessments and in these types of studies, the developmental status of the follicles (growing or regressing) is not clear, in spite of the fact that follicles with comparable diameters were selected.

The presence of a dominant follicle >8 mm within the ovary from which oocytes were collected negatively affected the blastocyst rate. Hagemann *et al.* (1999) dissected follicles (≥

3 mm in diameter) from ovaries of synchronized dairy cows at different days of the estrous cycle (2, 7, 10, and 15 d post-ovulation). Oocytes from each follicle were matured, fertilized, and cultured individually to day 8 and the developmental competence of the oocytes was assessed. The proportion of oocytes that developed into a blastocyst was higher in the absence (days 2 and 10) than in the presence of a dominant follicle (days 7 and 15) (Hagemann *et al.*, 1999). It has been assumed that the inhibitory effect of the dominant follicle was related to the size of subordinate follicles and increased atresia of small follicles during the dominant phase (Guilbault *et al.*, 1992). Thus, increased amounts of E_2 and inhibin, which are mostly secreted by the dominant follicle, resulted in turning subordinates into atretic, regressing follicles (Ko *et al.*, 1991; Adams *et al.*, 1993a, b). It is crucial to mention that the action of E_2 and inhibin is mediated through suppression of FSH secretion by the pituitary gland. However, it cannot be discarded potential paracrine factors secreted by dominant follicles influencing the growth of adjacent follicles. For instance, in sheep, it has been demonstrated that a local inhibitory effect of the dominant follicle on small follicles in the ipsilateral ovary (Gonzalez-Bulnes *et al.*, 2004). Also, it was reviewed by Armstrong and Webb (1997) that the dominant follicle produces follicle growth inhibitory factors locally that affects the development of subordinate follicles. The fate of follicles adjacent to the dominant follicle is determined by direct communication between these follicles which causes functional alterations that enhance the endocrine dominance effects (Gonzalez-Bulnes *et al.*, 2004). Contrary to the above observations, other authors described that there was no negative effect of the presence of a dominant follicle on the developmental competence of oocytes from small antral follicles (Smith *et al.* 1996). Differences found among studies, owes to variations in the number of cows, breed, seasonal effects, culture method, and culture media (Varisanga *et al.*, 1998), as well as by the fact that most of the above-cited studies (and the present study) used abattoir-harvested ovaries (growing or regressing status of follicles is not clear) for their experiment. Unexpectedly, we did not see

any difference regarding the effect of the presence of follicles between 12-15 mm or larger than 15 mm on the developmental competence of the oocytes collected from neighbouring follicles. This lack of effect may be explained by the relatively low number of ovaries bearing dominant follicles (> 12 mm) found in the present experiment (Table 1).

We found the lowest developmental rate in oocytes collected from ovaries simultaneously bearing a corpus luteum and dominant follicle (> 8mm). Interestingly, the FF concentration of P_4 in adjacent follicles (4 to 8 mm) was the highest when these two structures were together within the same ovary, but the E_2 concentration was not higher in adjacent follicles of ovaries bearing a dominant follicle. Smith *et al.*, (1996) found that a dominant follicle does not affect the steroid concentrations of adjacent small follicles. However, their study may be biased since they aspirated FF from small follicles in each pair of ovaries (containing a young corpus luteum and with or without a large estrogen-active follicle) and pooled them to determine the concentration of steroid hormones. In our study, the lowest developmental rate in those ovaries simultaneously bearing a corpus luteum and dominant follicle may be because the ovaries bearing these two structures receive more blood flow, and subsequently, even higher P_4 diffusion to adjacent follicles within the same ovary, likely influencing the developmental capacity of COCs (as we previously discussed).

Cumulus expansion or mucification occurs before ovulation when the oocyte progress to metaphase II (Salustri *et al.*, 1989; Nuttinck *et al.*, 2008). Cumulus expansion is an important index for oocyte maturation, as well as for the acquisition of subsequent developmental ability (Dekel *et al.*, 1979; Eppig, 1979; Furnus *et al.*, 1998). The presence of growth factors in the FF like IGF-1 has been demonstrated and it has positive effects on CE and improves the maturation of bovine oocytes (Eden *et al.*, 1990; Lorenzo *et al.*, 1994; Nagyová *et al.*, 1999). However, in the present study, the CE was lower in COCs collected from ovaries with a corpus luteum even though their FF IGF-1 concentration was higher than in ovaries without a corpus luteum. *In*

vitro studies have revealed the secretion of IGF-1 by granulosa cells exhibits autocrine and paracrine effects on mitosis and steroidogenesis of granulosa cells (Adashi *et al.*, 1985; Hammond *et al.*, 1988; Schams *et al.*, 1988; Spicer *et al.*, 1988). Therefore, IGF-1 may only be considered an indirect activator, acting synergistically with other hormones to stimulate follicle growth and oocyte maturation (Lorenzo *et al.*, 1994). In this regard, co-supplementation of EGF and IGF-1 resulted in higher CE than their sole supplementation, suggesting that the effect of the two growth factors is additive (Lorenzo *et al.*, 1994; Rieger *et al.*, 1998). Furthermore, it is crucial to mention that when a positive effect of IGF-1 on oocyte maturation was found within follicles its concentration ranged from 23 to 168 ng/mL (Spicer *et al.*, 1988; Simpson *et al.*, 1994). The high concentration of IGF-1 is for preovulatory follicles, and it needs to be mentioned that any premature mucification would be unwanted and unnecessary in small follicles. Our data show that the concentration of IGF-1 ranged from 0.2 to 0.4 ng/mL. The IGF-1 FF concentration is low in follicles between 4 to 8 mm, and this concentration does not have carryover effects on further *in vitro* CE or embryo development.

To produce a live offspring, not only is important to increase the success rate of *in vitro* embryo production but also essential the produced embryos have the highest quality possible to ensure optimal pregnancy rates following transfer (Lonergan *et al.*, 2003). In this regard, embryos with high TCN, high ICM/TCN ratio, and low AC/TCN are considered to be of high quality and likely to be pregnant after embryo transfer (Knijn *et al.*, 2003). In the present study, although we did not see any effect of corpus luteum and FC on differential-apoptotic staining parameters, the presence of a dominant follicle (> 8 mm) had a negative effect on embryo quality in terms of lower TCN and higher AC and AC/TCN. The low quality of embryos in the dominant follicle group might be related to the length of dominance. Prolonging the period of follicular dominance has been reported to affect oocytes and reduce subsequent embryo quality

(Cerri *et al.*, 2009; Wiltbank *et al.*, 2011). In fact, embryo quality was inversely proportional to the length of follicular dominance (Santos *et al.*, 2010).

In *Bos taurus* cattle, the FC has been linked to female reproductive performance (Mossa *et al.*, 2012; Ribeiro *et al.*, 2014; Martinez *et al.*, 2016; Morotti *et al.*, 2017; Gobikrushanth *et al.*, 2019; Akbarinejad *et al.*, 2020). It has been reported that those animals with low antral FC have smaller ovaries compared with age-matched cows with a higher antral FC (Ireland *et al.*, 2008). Besides, compared with age-matched cattle with a higher antral FC, cattle with a low antral FC have a reduced response to superovulation (Singh *et al.*, 2004; Ireland *et al.*, 2007), lower circulating concentrations of progesterone, and reduced endometrial thickness from d 0 to 6 of the estrous cycle (Jimenez-Krassel *et al.*, 2009). Also, it has been shown that there is an association between low circulating progesterone concentrations and high rates of embryo mortality in cattle (Diskin and Morris, 2008). This might explain the lower fertility in cattle with a low antral FC, at least in some studies (Mossa *et al.*, 2012). Several reports suggest that high FC is associated with the total number of morphologically healthy follicles (Ireland *et al.*, 2008, 2009, 2011; Tessaro *et al.*, 2011; Sakaguchi *et al.*, 2018). Heifers with high FC had fewer healthy follicles than heifers with low FC (Ireland *et al.*, 2008). Nevertheless, no difference was observed in oocyte development competence *in vitro* between heifers with low and high FC (Ireland *et al.*, 2007; Tessaro *et al.*, 2011; Sakaguchi *et al.*, 2018). Other studies showed better pregnancy rates in cows with low FC (Santos *et al.*, 2013). For the present study, although the cleavage rate was higher in ovaries with high FC, this effect was not translated to higher blastocyst production. The impact and the mechanistic interplay between FC and *in vitro* embryo production remains to be elucidated.

5. Conclusion

The present study shows that P_4 produced by the corpus luteum might diffuse to adjacent follicles with possible carryover effects on *in vitro* CE, embryo development, and quality.

However, these results are not conclusive since the corpus luteum also produces a plethora of factors other than P_4. Furthermore, and although more subtle, the presence of a dominant follicle ≥ 8 mm has negative effects on embryo development and the quality of COCs collected from follicles (4 to 8 mm in diameter) within the same ovary. Consequently, structural classification of the ovaries before follicle aspiration should be considered as an indicator of the developmental competence of collected oocytes.

Declaration of interest

The authors have no conflicts of interest.

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CRediT authorship contribution statement

Nima Azari-Dolatabad: Conceptualization, Methodology, Data curation, Writing- Original draft preparation. **Camilla Benedetti, Daniel Angel-Velez, Andrea Fernandez Montoro, Hafez Sadeghi, and Gretania Residiwati:** Methodology. **Jo L.M.R. Leroy:** Conceptualization, Writing - review & editing. **Ann Van Soom:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing - review & editing. **Osvaldo Bogado Pascottini:** Conceptualization, Methodology, Data curation, Project administration, Supervision, Validation, Writing - review & editing. All authors read the final draft and agreed to its submission for publication.

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