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Effect of follicle ablation before ovum pick-up in Girolando cattle

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ABSTRACT

The present study characterized the effect of follicle ablation (FA) intervals on the follicular population and the quantity and quality of cumulus-oocyte complexes (COCs) from 1/2 and 1/4 Girolando donors (n=125 and n=110, respectively) at *Ovum* Pick-Up (OPU). The timing for that FA has not been definitively established. Our hypothesis was that FA in 1/2 and 1/4 blood Girolando donors performed 48 hours prior to OPU provided more and higher quality COCs than at 72 hours post-FA. To test this hypothesis, FA performed 48 h or 72 h before OPU, and the ovarian follicular population (grouped by size) and quality (grade I-IV) of COCs recovered from OPU were evaluated. The FA 48 h and 72 h groups had a greater estimated number of medium-sized follicles (more developed) compared with the control group without FA (18.39±1.42, 14.98±1.42, and 10.84±1.42 follicles, respectively, $P<0.01$) at OPU. Furthermore, there was a greater number of highest-quality COCs at FA 48 h than at FA 72 h and control (2.09±0.31, $P=0.004$, 1.11±0.31, and 0.92±0.31 COCs, respectively). In comparison, 1/2 Girolando had greater counts of medium (18.3±2.02, $P=0.05$) and total follicles (33.34±2.67, $P=0.02$) and grade IV COC (5.52±0.64, $P<0.01$) than 1/4 Girolando (11.35±2.02, 22.56±2.67, 0.77±0.64, respectively). The association of FA with OPU on 1/2 and 1/4 Girolando has advantageous effects, resulting in the aspiration of more developed follicles and better-quality oocytes. Besides that, 1/2 Girolando has a greater follicular count than 1/4 Girolando, although these crosses respond similarly to FA. Overall, FA improves OPU outcomes.

KEYWORDS: Antral follicle count, Follicular development, *Bos taurus*, *Bos indicus*, *Ovum* pick-up, Cumulus-oocyte complex grade

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INTRODUCTION

In most tropical areas of the planet, there is a shortage of animal-sourced food supply due to insufficient animal production, aggravated by global warming and climate change (Nardone *et al.*, 2010). A suggested strategy to approach this issue is to exploit *Bos indicus* endurance traits, expanding the knowledge of zebu and its crossbreed (e.g., *B. taurus* X *B. indicus*) characteristics for systems' sustainability and production increment (Cooke *et al.*, 2020). An example of a successful crossbreeding plan is the Girolando breed, a cross between Holstein and Gir breeds, which has gained great representativity in the dairy sector (Daltro *et al.*, 2020; Silva *et al.*, 2022).

Girolando is a well-established breed from Brazil that has demonstrated significant potential to supply the demands of the dairy industry, especially after years of research, genetic selection, and management efforts focused on the breed's particularities (Silva *et al.*, 2022). As a result, the country produces about 35 billion liters of milk annually (IBGE, 2021), 80% from Girolando cows (Silva *et al.*, 2022). In addition, differences are observed for reproductive traits (i.e., age at first calving and calving interval) according to the percentage of Holstein (H) or Gir (G) in the animal, which could relate to the heterosis effect resulting from the *Bos taurus* X *Bos indicus* cross (Vieira *et al.*, 2022).

A great tool to accelerate genetic gain and the dissemination of these superior genetics is *in vitro* embryo production (IVEP) (Viana *et al.*, 2012; Bó & Mapletoft, 2018). In addition, the quantity and quality of the oocytes recovered for IVEP directly affect its outcomes. By aspirating follicles during the growth phase, more viable oocytes are expected, significantly improving embryo production rates (Machatkova *et al.*, 2004). Furthermore, the use of follicle ablation (FA) synchronizes follicular growth by removing inhibiting factors (estradiol and inhibin) released by the dominant follicle over the subordinates (Bergfelt *et al.*, 1994).

For taurine animals, studies report comparable findings for the timing of follicle-stimulating hormone (FSH) peak and follicular emergence (considered when follicles are >4 mm) when follicle ablation is performed at either random (Bergfelt *et al.*, 1994) or controlled days (6 d post-ovulation) (Gomez-León *et al.*, 2019) of the estrus cycle. This timeline is related to the FSH peak that happened at about 1 d, and the follicular surge was 1.5-2 d post-ablation (Bergfelt *et al.*, 1994; Gomez-León *et al.*, 2019). Although little has been reported about the timing of these events on Girolando crosses, a new follicular wave on 5/8 Girolando (5/8 H: 3/8 G) is characterized by the presence of follicles of 3-5 mm diameter (Filho *et al.*, 2001), which will keep growing until about 3.5 d when dominance is established.

Controversial findings have been reported about the differences between Girolando crosses as oocyte donors, with either differences in total and viable oocytes recovered (Pontes *et al.*, 2010; Cardoso *et al.*, 2019; Adona *et al.*, 2020) or similarities in antral follicle count, oocyte grade, and viability (Monteiro *et al.*, 2018). Therefore, we hypothesize that introducing the FA technique before OPU within an appropriate time interval improves OPU yield in Girolando cattle and that the FA responses vary according to breed composition. The timing for that FA has not been definitively established in Girolando crosses. Our hypothesis was that FA in 1/2 and 1/4 blood Girolando donors performed 48 hours prior to OPU provided more and higher quality COCs than at 72 hours post-FA. To test that hypothesis, the present study was designed to test the effects of FA-OPU intervals (48 h and 72 h) on OPU yields, characterizing the follicular population (number of small, medium, large, and total structures) and the quantity and quality of cumulus-oocyte complexes (COCs) from 1/2 (1/2 H: 1/2 G) and 1/4 (1/4 H: 3/4 G) Girolando donors.

MATERIALS AND METHODS

The study was performed on a commercial farm in the Southeastern United States in October, November, and December 2020 and 2021. The studies were conducted under approved protocols by the University of Illinois Institutional Animal Care and Use Committee (IACUC Protocols # 19186 and 22147).

The heifers and cows studied were 1/2 Girolando (n=125, 36.09±7.79 months old) and 1/4 Girolando donors (n=110, 21.96±1.32 months old) with body condition scores ranging from 3.0 to 4.0 (on a 1.0 to 5.0 scale (Wildman *et al.*, 1982)). These cows and heifers were non-pregnant, presented regular estrous cycles, and had no reproductive abnormalities at the time of enrollment in the study. The animals were raised in a shared environment under similar management conditions, on a continuous grazing system (*Paspalum notatum*), supplemented with hay and grain, and water and mineral mixture *ad libitum*.

Experimental Design

The animals were randomly assigned to three groups on the first day of the experiment (Day 0): i) one control group (no FA), which was equally split for OPU on Day 2 and Day 3; ii) one FA 48h group (OPU on Day 2); and iii) one FA 72h (OPU on Day 3). On Day 0, all animals were evaluated by ultrasound scanning to determine each donor's ovarian follicular population, and the donors in the FA groups had any follicle with a diameter ≥5 mm aspirated. On Day 2, the corresponding control and the FA 48h groups underwent ovarian scanning, had their follicular population counted by size (small, medium, and large), and were checked for the presence of *corpus luteum* (CL), followed by OPU and subsequent grading of the COCs recovered. On Day 3, the other control and the FA 72h groups underwent the same steps described for Day 2.

Follicular Evaluation and Aspiration and Cumulus-Oocyte Complexes Grading

Ultrasound scanning was performed by the same experienced technician using a real-time B-mode machine (EXAPad, IMV Imaging, Minneapolis, MN) with a transvaginal convex transducer (6 MHz) adapted for OPU procedures. For follicular evaluation and count, follicles with a diameter estimated to be <3 mm were classified as small, 3-6 mm as medium, and >6 mm as large. For FA and OPU, the ultrasound scanner was combined with a follicular aspiration system, which consisted of a vacuum line connected to a vacuum pump (Watanabe Applied Technology, São Paulo, Brazil). A 1 1/2" 18 G hypodermic needle is placed in the needle guide and attached to flexible tubing on the transducer end. The flexible tubing passed through an aluminum stopper into a 50 mL conical tube, with a second connection to the vacuum pump set for 90 mmHg. Right before and after both ovaries were aspirated, the system was rinsed with flush solution (ABT Complete Flush Solution, ABT 360, Pullman, WA) with 0.1% sodium heparin (10,000 UI/mL, SAGENT, Schaumburg, IL).

The oocytes collected in the 50 mL conical tube were counted and evaluated under a stereomicroscope at 60X. The oocytes were graded from I (highest quality) to IV (lowest quality) according to morphological characteristics. Briefly, grade I oocytes had homogenous dark ooplasm and dense (>4 layers) cumulus; grade II oocytes had fewer layers but still compact cumulus, with possibly a few uneven spots on the ooplasm; grade III oocytes had reduced cumulus cover, with a slight level of expansion and some rough spots on the ooplasm; and finally, grade IV oocytes were partially or entirely denuded, with or without expanded cumulus and coarse ooplasm.

Data handling and statistical analyses

Since no previous estrous synchronization was performed, and the females were at random days of the estrous cycle, the dataset was filtered so that only observations from donors presenting at least one larger follicle (≥5mm) at Day 0 were considered for the statistical analysis. The response variables included the number of follicles and the quality of the COC. The model included the fixed effects of treatment, breed composition, and the interaction of treatment and breed composition, and age (in months) was fitted as a covariate. Donor and donor interaction, period (October, November, and December), and year (2020 and 2021) were considered random effects.

Preliminary analyses indicated no significant effects of CL and interaction with treatment, and these effects were removed from the final model. The mixed effects model was analyzed using the MIXED procedure of SAS (Version 9.4). Results are reported as least squares mean (LSM) ± standard error of the means (SEM). Statistical significance was set at $P \leq 0.05$, and tendency was set at $0.05 > P \leq 0.10$.

RESULTS

In total, 235 OPUs were performed, and 212 observations were analyzed because the diameter of at least one follicle was ≥5 mm on Day 0. This finding implies that FA use is applicable to most animals considering the described farming system if randomly assigned. The comparison of the treatment LSM and characterization of the treatment effect is presented in Table 1. The introduction of FA before OPU resulted in a significantly lower count of small follicles with a 48h interval, compared with 72 h and control. However, both FA groups had higher counts of more developed (medium) follicles ($P<0.01$) than the control, with a tendency to be higher with a shorter interval (48 h vs. 72 h). Additionally, fewer large follicles were observed in FA 48 h (0.22 follicles +0.24, $P=0.002$) than in the control group (1.21 follicles +0.24). The number of large follicles in FA 72 h was similar to that of FA 48 h and the control group. Regarding COC quality and quantity, treatment affected the highest-quality COCs (grade I), with FA 48 h having a greater number of grade I COCs (2.09 follicles+0.31, $P=0.004$) than control (0.92 follicles+ 0.31) and FA 72 h (1.11 follicles+0.31).

Table 1: Least squares mean (LSM) and corresponding standard error of the mean (SEM) for follicle size (diameter) and cumulus-oocyte complex quality grade of Girolando donors with no (Control) previous follicle ablation (FA), or 48 h (FA 48 h) and 72 h (FA 72 h) after FA

Variable	n	Control	FA 48h	FA 72h	SEM	P-value
Follicle size (diameter)						
Small (<3 mm)	212	15.15[a]	8.97[b]	12.47[a]	1.19	0.0002
Medium (3-6 mm)	212	10.84[a]	18.39[b]	14.98[b]	1.42	<0.01
Large (>6 mm)	212	1.21[a]	0.22[b]	0.56[ab]	0.24	0.002
Total	212	27.67	27.69	28.57	1.63	0.73
Cumulus-oocyte complex (number)						
Grade I[1]	171	0.92[a]	2.09[b]	1.11[a]	0.31	0.004
Grade II	171	3.20	3.83	3.14	0.42	0.24
Grade III	171	8.89	10.09	8.57	0.98	0.30
Grade IV	171	3.90	2.46	3.08	0.52	0.15
Total	208	18.66	19.03	17.58	1.25	0.41

[ab]Values within rows with different superscripts differ ($P \leq 0.05$).
[1]Grades correspond to morphological cumulus-oocyte complex grading, grade I being the highest quality and grade IV the lowest quality

Table 2: Least squares mean (LSM) and corresponding standard error of the mean (SEM) for the ovarian response, OPU yield, follicle size, and cumulus-oocyte complex quality grade of 1/2 and 1/4 Girolando donors

Variable	n	1/2 Girolando	1/4 Girolando	SEM	P-value
Follicle size (diameter)					
Small (<3 mm)	212	14.44	9.96	1.65	0.12
Medium (3-6 mm)	212	18.13[a]	11.35[b]	2.02	0.05
Large (>6 mm)	212	1.08	0.25	0.27	0.08
Total	212	33.34[a]	22.56[b]	2.67	0.02
Cumulus-oocyte complex (number)					
Grade I[1]	171	1.35	1.39	0.45	0.96
Grade II	171	3.32	2.95	0.61	0.73
Grade III	171	7.43	10.93	1.53	0.19
Grade IV	171	5.52[a]	0.77[b]	0.64	<.0001
Total	208	20.61	16.23	1.99	0.20

[ab]Values within rows with different superscripts differ ($P \leq 0.05$).
[1]Grades correspond to morphological cumulus-oocyte complex grading, grade I being the highest quality and grade IV the lowest quality

Breed composition, presented in Table 2, significantly affected medium and total follicles and grade IV COC, with 1/2 Girolando presenting a greater number for the three variables. Grade I, II and III COC while numerically different were not statistically different.

DISCUSSION AND CONCLUSION

The present research focused on understanding the ovarian response to prior FA of 1/2 and 1/4 Girolando donors at OPU and identifying the best interval to its application according to the animal categories we were working with. The follicular growth happens in a wave-like pattern characterized by the surge of a uniform pool of 3-5 mm antral follicles induced by FSH (Ginther *et al.*, 2010). These structures continue to grow (growth phase) until the future dominant follicle deviates its growth pattern and induces the atresia (dominant phase) of the subordinates, about 2 d after the follicular surge (Ginther *et al.*, 1989; Gomez-León *et al.*, 2019). Furthermore, obtaining oocytes with greater competence is a primary goal in implementing assisted reproductive technologies (ARTs), and factors like follicle size and development status relate to COC competence (Sirard *et al.*, 1999; Blondin *et al.*, 2012).

Oocyte competence is the capacity of an oocyte to successfully mature, be fertilized, and become a blastocyst, resulting in a healthy pregnancy (Watson, 2007). Moreover, oocytes recovered from smaller follicles (diameter <3 mm) have reduced competence compared with larger (diameter ≥3 mm) ones (Lonergan *et al.*, 1994; Blondin & Sirard, 1995). In addition, follicular atresia is first observed on the granulosa cells and later on the cumulus cells and oocyte (Kruip & Dieleman, 1982). When COCs are recovered from larger follicles, with minor or no signs of atresia, these complexes have comparable higher competency despite the follicular phase (Vassena *et al.*, 2003; Machatkova *et al.*, 2004).

In the present study, the use of FA at both 48 h and 72 h intervals was responsible for shifting the follicular population profile at OPU. While the lowest number of small follicles is described with FA 48 h, the highest number of medium follicles are described for FA 48 h followed by FA 72 h. In addition, more large follicles are present in the FA 72 h and control groups than in the FA 48 h, although the total number of follicles is similar for all groups. This finding reveals that FA induced the development of small follicles into medium follicles and that the follicular population was likely starting to deviate from its growth pattern, with the regression of subordinate medium follicles after 72 h, since the number of small follicles increased from the 48 h to the 72 h intervals. This result may indicate the establishment of a dominant phase, corroborated by the slight increase in large follicle count at 72 h.

Corroborating these findings, Viana *et al.* (2010) performed OPU of all follicles >3 mm in diameter every 96 h on Gir (zebuine) cows and described a quick regeneration of the follicular population, with the follicular count at 48 h after OPU similar to the count at the following OPU, 48 h later. Additionally, Filho *et al.* (2001), working with 5/8 Girolando cows without estrous manipulation, described the establishment of follicular dominance about 84 h after the follicular wave emergence. This is an approximate time to that described in the present study, where follicle regression was detected within 72 h, considering that at FA (0 h), the follicular population was already established, and only larger (≥5 mm) follicles were ablated.

Further, on the understanding of a dominant phase establishment within 72 h post-FA, the greatest count of grade I (best quality) COC was higher on FA 48 h. It probably relates to the inhibiting effects of dominant follicles over the subordinate ones, resulting in lower quality COC on FA 72 h and control groups. According to de Wit *et al.* (2000) there is a strong correlation between follicle and COC quality, with most of the highest quality COC derived from follicles with no sign of atresia.

Besides the heterosis influence on the follicular population, it is valid to discuss how the levels of the founding breeds (Holstein and Gir) might affect these variables since specific distinctions of physiological conditions have been reported for taurine and zebuine animals (Alvarez *et al.*, 2000; Sartori *et al.*, 2010, 2016). For example, Alvarez *et al.* (2000) describe greater levels of FSH in taurine (0.62 ng/mL) than in zebuine (0.45 ng/mL) breeds. Since FSH is responsible for inducing follicular growth (Adams, 1999), an animal with more taurine influence (1/2 Girolando) might have greater levels of circulating FSH and a higher follicular growth rate than a more zebuine-like (1/4 Girolando), which could explain the presence of more medium and total follicles on 1/2 Girolando within a similar interval.

In the study performed by Pontes *et al.* (2010), a higher number of COC was also recovered from 1/2 (31.4±5.6) than 1/4 (20.4±5.8) Girolando cows, even though the follicular population was not evaluated. Besides that, like our study, a greater number of low-quality (grade IV) COC was recovered from 1/2 Girolando, where 82.33% of the total COC collected from 1/4 Girolando donors are viable (grades I-III), decreasing to 77.39% on 1/2 Girolando (Pontes *et al.*, 2010).

The method of COC aspiration, including factors like needle gauge, vacuum pressure, and follicle size, may affect OPU success (Wrenzycki, 2018). The previous studies hypothesize that a COC with a larger mass of cumulus cells is more susceptible to disruption when passing through the OPU tubing system. This impact could decrease the difference between a COC with an initially larger cell cover (initially greater quality) and a COC with a reduced cover (initially lower quality). Lastly, for a better estimation of the impact of FA on OPU-IVEP schemes, embryo production information (commonly cleavage and blastocyst rate) should be considered for a thorough system evaluation.

In the present study, a preliminary analysis, including the effect of CL and its interaction with FA, was considered in the model. Previous studies report variable results on the influence of CL on COC quality and embryo production, ranging from improvement, positive effect (Penitente-Filho *et al.*, 2015), or no effect (de Wit *et al.*, 2000). In the current conditions, the presence of a CL did not affect or interact with the donor's response to FA, reinforcing the indication for the introduction of FA before OPU with no previous synchronization or donor management required. In addition, the reported absence of significant interaction between treatment and breed composition indicates that the intervals tested are suitable for both genetic groups.

In conclusion, removing follicles with ≥5 mm in diameter promotes more developed follicles at OPU when performed at 48 h or 72 h in advance in 1/2 and 1/4 Girolando donors. Moreover, the 1/2 Girolando had the greatest follicular count. This result indicates a certain schedule flexibility if introduced on a commercial system. Although both intervals are appropriate for the animal groups evaluated, the 48 h interval had increased highest quality COC and tended to have more medium follicles.

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STATEMENT OF ANIMAL RIGHTS

The studies were conducted under approved protocols by the University of Illinois Institutional Animal Care and Use Committee (IACUC Protocols # 19186 and 22147).

AUTHOR STATEMENT

MBW, BL: Conceptualization, Methodology, Data Collection. PVM, SRZ: Data Collection, Data Curation, Data Analysis. PVM: Data Interpretation, Writing- Original Draft preparation. MBW: Supervision. MBW, PVM, BL, SRZ: Writing - Reviewing and Editing.

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