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L.U. Gimenes, M.L. Ferraz, P. Fantinato-Neto, M.R. Chiaratti, L.G. Mesquita, M.F. Sá Filho, F.V. Meirelles, L.A. Trinca, F.P. Rennó, Y.F. Watanabe, P.S. Baruselli

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The interval between the emergence of pharmacologically synchronized ovarian follicular waves and ovum pick-up does not significantly affect *in vitro* embryo production in *Bos indicus, Bos taurus,* and *Bubalus bubalis*

L U Gimenes[a,*], M L Ferraz[b], P Fantinato-Neto[c], M R Chiaratti[d], L G Mesquita[e], M F Sá Filho[f], F V Meirelles[g], L A Trinca[h], F P Rennó[e], Y F Watanabe[i], P S Baruselli[f]

[a]Department of Preventive Veterinary Medicine and Animal Reproduction, FCAV – UNESP, Jaboticabal - Brazil

[b]Vida Reprodutiva Consultoria, Cravinhos - Brazil

[c]Department of Surgery, FMVZ - USP, Pirassununga - Brazil

[d]Department of Genetics and Evolution, CCBS – UFSCar, São Carlos - Brazil

[e]Department of Animal Nutrition and Production, FMVZ – USP, Pirassununga - Brazil

[f]Department of Animal Reproduction, FMVZ – USP, São Paulo - Brazil

[g]Department of Veterinary Medicine, FZEA - USP, Pirassununga - Brazil

[h]Department of Biostatistics, IB – UNESP, Botucatu - Brazil

[i]Vitrogen, Cravinhos - Brazil

*Corresponding author. Email: gimeneslu@fcav.unesp.br. Phone number: +55 16 3209-7320

Abstract

The aim of the present study was to determine the optimal phase of the follicular wave to perform ovum pickup (OPU) for *in vitro* embryo production (IVEP) in various genetic groups. For this purpose, 27 heifers - nine *Bos taurus* (Holstein), nine *Bos indicus* (Nelore) and nine *Bubalus bubalis* (Mediterranean) - were maintained under the same nutritional, management and environmental conditions. Heifers within each genetic group were submitted to six consecutive OPU trials with 14-day intersession intervals, at three different phases of the pharmacologically synchronized follicular wave (Day 1, Day 3 or Day 5 after follicular wave emergence), in a 3 x 3 crossover design., when OPU was performed at different phases of the pharmacologically synchronized follicular wave (Day 1, Day 3 or Day 5), no differences were found in the percent of oocytes recovered (70.5% ± 3.1, 75.0% ± 3.1, 76.0% ± 3.2, respectively; P = 0.41) or blastocyst production rates (19.4% ± 2.9, 16.6% ± 2.9, 15.9% ± 2.6, respectively; P = 0.36). Comparing genetic groups, *Bos indicus* showed a higher blastocyst rate (28.3%^{a} ± 2.8; P < 0.01) than *Bos taurus* and *Bubalus bubalis* (14.1%^{b} ± 2.9 and 10.2%^{b} ± 2.0, respectively). However, only *Bos indicus* heifers showed a variation in the number of visualized follicles and the total and viable oocytes along consecutive OPU sessions. In conclusion, different phases of the pharmacologically synchronized ovarian follicular wave did not affect OPU-IVEP in *Bos indicus*, *Bos taurus*, and *Bubalus bubalis* heifers. Additionally, *Bos indicus* heifers showed greater OPU-IVEP efficiency than did the other genetic groups, under the same management conditions.

Additional keywords: Nelore, Holstein, buffalo, oocyte, embryo

1. Introduction

Over the past 20 years, a sharp increase of bovine *in vitro* embryo production (IVEP) from oocytes retrieved by ovum pick-up (OPU) has been reported [1]. *In vitro* embryo production along with other important advancements (i.e., animal genetic improvement) has enabled a significant enhancement of both dairy and beef livestock during this period. In buffaloes, OPU associated with IVEP is particularly important as an embryo production technologies [2-5], because multiple ovulation and embryo transfer programs show very low efficiency and few commercial applications [6-8].

Many studies have focused on improving the efficiency of OPU-IVEP programs, enabling an even broader use of these reproductive techniques. Many factors are known to influence OPU-IVEP outcomes, including follicular size [9-11], oocyte diameter [11, 12], the phase of the follicular wave [13-20], the genetic group [4, 21-23], and the animal category [24].

Previous studies have shown that the acquisition of developmental competence by oocytes (e.g., the ability of the oocyte to reach the blastocyst stage) is associated with follicular growth. Developmental competence continues to be enhanced as follicular diameter increases and approaches the LH surge [9,25], which is strongly linked to oocyte growth and mRNA and proteins being stored in the oocyte [26]. This buildup of RNA is essential to sustain the first few cell cycles of early embryo development [27]. However, oocytes obtained from follicles of the same diameter can differ in blastocyst yield. This can be partially attributed to the influence of the phase of the follicular wave when the oocytes are recovered. During the bovine [28] or buffalo [29] estrous cycle, 2 or 3 follicular waves can be identified. During each follicular wave, dominant and subordinate follicles can be in a growth, dominance, static or regressing phase [28, 30]. Several studies have demonstrated that oocyte quality, recovery, cleavage and blastocyst rate can be

77 influenced by these phases, as described below . In the regression phase, a severe level
78 of atresia can be observed in follicles, reducing oocytes developmental competence [18,
79 31]. However, an improvement of the blastocyst rate was demonstrated when oocytes
80 were collected from follicles with a mild level of atresia [19, 31, 32]. Conversely, other
81 authors have shown that including a growth phase [21] before follicle selection (i.e., follicle
82 deviation) improves the results of OPU-IVEP in cattle. Therefore, there is no consensus
83 about the most appropriate phase of the follicular wave to harvest oocytes to increase
84 oocyte recovery and quality for blastocyst production.

85 *Bos indicus* and *Bubalus Bubalis* females show some differences in their
86 reproductive behaviors and responses compared to *Bos taurus* [4, 23, 33, 37]. For this
87 reason, one cannot assume that outcomes obtained in *Bos taurus* would be the same in
88 *Bos indicus* or in *Bubalus bubalis*. *Bos indicus* cattle have a greater number of follicles
89 recruited per follicular wave and a lower diameter of the dominant follicle at deviation and
90 at ovulation compared to *Bos taurus* [35-39]. Additionally, buffaloes have an intermediate
91 follicle size at deviation and ovulation [8, 37,40]. These details could impact OPU-IVEP
92 outcomes because a greater number of recovered oocytes and a greater number of
93 blastocysts produced per OPU session were reported in *Bos indicus* compared to *Bos*
94 *taurus* cattle [41, 42]. Furthermore, poorer blastocyst rates (19.9% vs. 29.7%) following
95 IVEP programs have been reported in *Bubalus bubalis* compared to *Bos taurus* cattle [4].
96 In addition, as nutritional and environmental conditions can impact reproductive
97 parameters, it is important to analyze the effects of genetic background in animals
98 maintained under similar conditions.

99 In response to the lack of information on the efficiency of OPU-IVEP programs in
100 *Bos indicus*, *Bos taurus*, and *Bubalus bubalis* raised under similar conditions, we designed
101 the present study to evaluate: 1) the best time to perform OPU (Day 1, Day 3 or Day 5

after synchronized follicular wave emergence) for optimal IVEP in each genetic group, and 2) differences in OPU-IVEP outcomes between genetic groups.

2. Material and Methods

2.1. Animals

The present study was approved by the Animal Experimentation Ethics Committee of the University of Sao Paulo (protocol number 1070/2007).

A total of 27 cycling heifers - 9 *Bos taurus* (Holstein), 9 *Bos indicus* (Nelore), and 9 *Bubalus bubalis* (Mediterranean) - weighting a mean of 409.5 , 467.7 and 579.0 kg, respectively, and aging between 22 and 29 months - were maintained at FMVZ – USP (Pirassununga, São Paulo, Brazil). Each genetic group was maintained in pens of 0.3 hectares, with the same environmental and shading conditions. The study was conducted from July to October 2008, with an adaptation period in April 2008. Heifers had free access to water and were fed twice daily with corn silage plus corn and soy concentrate (as described by Gandra e*t al.*[43]) at 2% of the body weight, which was adjusted based on the previous day. During the adaptation and experimental periods, management, environmental conditions and nutrition were controlled to offer the same conditions to all heifers.

2.2. Experimental design

This study employed a 3 x 3 crossover design (3 genetic groups x 3 phases of pharmacological synchronized ovarian follicular wave), performed with a 14-day interval

between OPU for 6 consecutive sessions(i.e., replicates). Number of replicates assured our study being conducted before summer to avoid compromise ourresults.

2.3. Pharmacological synchronization of follicular wave emergence

All heifers were submitted to the same standard synchronization protocol for the pharmacological induction of a new follicular wave before each OPU according to a previously published method [44]. Briefly, the induction of a new follicular wave was done by the injection of 50 mg of progesterone (P4; Progestar®, Syntex, Buenos Aires, Argentina) and 2 mg of estradiol benzoate (EB; RIC-BE®, Agener Uniao Quimica, Sao Paulo, SP, Brazil) administered during insertion of a norgestomet ear implant (containing 5 mg of Norgestomet; Crestar®, MSD Animal Health, Sao Paulo, Brazil). The emergence of the new follicular wave occurred 4.4 ± 0.2 days (mean ± S.E.M.) after the beginning of the treatment, regardless of the genetic group [44]. To avoid the presence of a corpus luteum (CL) at the OPU procedure, all heifers received an additional treatment of 150 µg of d-cloprostenol (Preloban®, MSD Animal Health) at insertion of the ear implant. Immediately before each OPU procedure, the ear implant was removed. Therefore, because the new follicular wave was expected to emerge 4 days after the onset of the synchronization protocol as described also by other authors [34] and to perform all OPU-IVEP on the same day, the animals in each genetic group were subjected to the OPU procedure 5, 7 or 9 days after hormone treatment for groups Day 1, Day 3 or Day 5, respectively. In each replicate, the onset of the synchronization protocol was normalized within each genetic group, aiming to submit all animals to OPU in the same day . Resynchronization of follicular wave emergence for the next OPU procedure was initiated 9, 7 or 5 days after OPU for groups Day 1, Day 3 or Day 5, respectively.

152

153 *2.4. OPU procedure*

154

155 Animals were submitted to 6 consecutives OPU procedures with 14-day
156 intersession intervals, performed by the same operator. For oocyte collection, heifers were
157 contained in a chute and an epidural anesthesia was administered (4 mL of lidocaine
158 hydrochloride 2%, Lidovet®, Bravet, Brazil) to facilitate the handling of the ovaries through
159 the rectum. The perineal area was cleaned using water and alcohol prior to each OPU
160 session.

161 All visible follicles were counted and aspirated by transrectal ultrasonography using
162 a portable scanner (Aloka SSDV 500; Aloka, Tokyo, Japan) with a 5 MHz convex array
163 transducer housed in a plastic vaginal probe with a stainless steel needle guide connected
164 to aspiration equipment and a vacuum system. Follicular aspirates were recovered via a
165 1.1-mm i.d. by 120-cm length circuit (Watanabe Tecnologia Aplicada, Cravinhos, SP,
166 Brazil), directly connected to a disposable 1.7 mm x 48 mm 16-gauge needle (BD
167 Angiocath, São Paulo, SP, Brazil) and a 50-mL conical tube containing 15 mL of 0.9%
168 NaCl supplemented with 1% fetal calf serum (FCS; Gibco Life Technologies, Grand Island,
169 NY, USA), plus 50 ng mL^{-1} penicillin, 50 ng mL^{-1} streptomycin, 100 ng mL^{-1} neomycin and
170 5,000 IU mL^{-1} sodium heparin (Parinex, Hipolabor, Belo Horizonte, MG, Brazil) at 35 to
171 37℃. The vacuum connected to the needle was set at a 12-15 mL $minute^{-1}$ water flow
172 rate. The conical tube containing the follicular aspirate was transported to a field
173 laboratory and cumulus-oocyte complexes (COCs) were washed using a 75-µm filter
174 (Watanabe Tecnologia Aplicada) and the same warmed solution used during OPU. COCs
175 were morphologically evaluated under a stereomicroscope and classified as viable or
176 unviable based on oocyte cytoplasm characteristics and the number of cumulus cell layers
177 (adapted from Leibfried and First 45]). The same criteria were used to evaluate bovine and

buffalo oocytes. Compact COCs with more than 3 layers of cumulus cells and oocytes with homogeneous cytoplasm, compact COCs with at least one layer of cumulus cells and oocytes with slightly heterogeneous cytoplasm, and partially denuded COCs and oocytes with heterogeneous cytoplasm were considered viable for IVEP and were used in the study. Denuded or degenerated oocytes and COCs with expanded cumulus cells were considered unviable for IVEP and excluded from the study. Viable COCs were transported to the IVEP laboratory within 2 h after OPU in 1.2-mL cryotubes containing HEPES-buffered tissue culture medium 199 (TCM-199; Gibco, USA) with 10% FCS (Gibco), sodium pyruvate (22 μg mL^{-1}) and amikacin (83.4 μg $μl^{-1}$; Neo Química, Brazil) at 35ºC.

2.5. In vitro embryo production

All chemicals and reagents used were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise stated. *In vitro* experimental procedures were performed in humidified incubators maintained at 38.5°C in air with 5% CO_2, always by the same operator.

Prior to *in vitro* maturation (IVM), oocytes were washed once in HEPES-buffered TCM-199 with 10% FCS, sodium pyruvate (22 μg mL^{-1}) and amikacin (83.4 μg/μl) and three times in IVM medium, composed of bicarbonate-buffered TCM-199 (Gibco, USA) supplemented with 10% FCS, sodium pyruvate (22 μg mL^{-1}), amikacin (83.4 mg/μl), human chorionic gonadotropin (hCG; 50 μg mL^{-1}; Chorulon®, MSD Animal Health, Brazil), follicle-stimulating hormone (FSH; 0.5 μg mL^{-1}; Folltropin®, Bioniche, Canada), estradiol (Estradiol 17β; 1 μg mL^{-1}), cysteamin (50 μM) and cystine (0.3 mM). COCs from each heifer at each phase of the follicular wave (Day 1, Day 3 and Day 5) were separately cultured for 24 h in drops of IVM medium under mineral oil. The ratio of oocyte: medium was maintained at 1:3-5 μl in all steps of IVEP.

After IVM, COCs were washed three times in IVF medium and submitted to IVF in drops of IVF medium under mineral oil. The IVF medium was Tyrode albumin lactate pyruvate (TALP) [46] supplemented with heparin (10 µg mL^{-1}), sodium pyruvate (22 µg mL^{-1}), amikacin (83.4 µg mL^{-1}), fatty acid-free BSA (6 mg mL^{-1}) PHE solution (2 µM penicillamine, 1 µM hypotaurine and 0.25 µM epinephrine). A single batch of frozen-thawed semen from a *Bos indicus* (Nelore) and a *Bubalus bubalis* (Murrah) bull of known fertility in previous IVEP programs was used for bovine and buffalo oocytes, respectively. For IVF, straws were thawed for 30 s in a 37℃ wate r bath. Semen was deposited on a 90% to 45% Percoll gradient prepared with sperm wash medium (modified Tyrode medium) and centrifuged at 320 x g for 30 min to separate the morphologically normal spermatozoa and to remove the diluents and the seminal plasma. Afterwards, the sperm pellet was evaluated for motility and concentration. Each fertilization drop received 10 µL of sperm, to achieve a final concentration of 2 x 10^6 live sperm mL^{-1} and was incubated for 20 h.

After IVF, presumptive zygotes were denuded of cumulus cells by gentle pipetting in 2% hyaluronidase, followed by washing in HEPES-buffered TCM199 and *in vitro* culture (IVC) medium. Groups of presumptive zygotes were co-cultured on a monolayer of cumulus cells that had attached to the plate surface during IVM. The IVM medium was changed to SOF [47] medium supplemented with 2% FCS, BSA (6 mg mL^{-1}), sodium pyruvate (22 µg mL^{-1}), amikacin (83.4 µg mL^{-1}), and essential and non-essential amino acids to be used for IVC drops.

On the third day of IVC, one third of the IVC medium was replaced with fresh IVC medium. At this time, the proportion of cleaved oocytes (the number of embryos with two or more cells divided by the total number of structures in the culture) was also recorded. The blastocyst rate (the total number of blastocysts divided by the total number of embryos in the culture) was recorded on the seventh day and the hatching rate (number of hatched

blastocysts divided by the number of blastocysts) was recorded on the ninth day of IVC. Embryos were classified according to IETS criteria [48]. Hatched blastocysts were fixed in 2% paraformaldehyde plus 0.1% polyvinyl-pyrrolidone (PVP) for 1 h and maintained in PBS with 0.1% PVP at 4ºC to be used for cell number determination. Embryos were stained in PBS with 10 µg mL^{-1} Hoechst 33342 for 5 min. The blastocysts were mounted on a glass slide and nuclei were counted using a fluorescence microscope (Axioplan, Carl Zeiss, Zeppelinstrasse, Germany) and AxioVs40 software (V4.6.1.0; Carl Zeiss). Oocyte competence was assessed by the ability of the female gamete to produce a viable blastocyst. Blastocyst viability was assessed by embryo morphology (i.e., clear blastocoel and a well-defined inner cell mass), blastocyst hatching rate and blastocyst cell number.

2.6. Statistical analyses

The experimental unit was each oocyte within each genetic group. All variables (number of visualized follicles, number of recovered oocytes, oocyte recovery rate, number of viable oocytes, percentage of viable oocytes, number of cleaved structures, cleavage rate, number of blastocysts, blastocyst rate, number of hatched blastocysts and hatching rate) were analyzed by ANOVA using a MIXED procedure in SAS version 9.2 (SAS/STAT, SAS Institute Inc., Cary, NC). Genetic group (*Bos taurus*, *Bos indicus* and *Bubalus bubalis*), phase of follicular wave (Day 1, Day 3 and Day 5), replicate, and interactions among the previous variables were considered as fixed effects in the statistical model. The variably 'animal' within each genetic group was included as a random effect. The variable 'number of nuclei in hatched blastocysts' was analyzed by ANOVA using the GLM procedure in SAS. Tests for normality of residuals and homogeneity of variances were conducted for each variable. Data, which did not fulfill the assumptions for ANOVA, were transformed using square root or square root arcsine. Differences between experimental

groups were tested by Tukey tests at a 5% significance level. Data are presented as the means ± S.E.M.

3. Results

The phase of the follicular wave did not affect any of the variables evaluated in the present study when pharmacological synchronization of follicular wave emergence was done (Tables 1 and 2). There was no interaction between genetic group, phase of follicular wave and replicate, genetic group and phase of follicular wave or phase of follicular wave and replicate for any of the variables analyzed (Tables 1 and 2). However, an interaction between genetic group and replicate was found for the number of visualized follicles ($P < 0.01$), the number of total oocytes recovered ($P = 0.03$), the number of viable oocytes ($P < 0.01$), and the number of cleaved embryos ($P < 0.01$; Table 2 and Fig. 1). Overall, *Bos indicus* (Nelore) heifers showed a greater number of visualized follicles and a greater number of total oocytes per OPU session than *Bos taurus* (Holstein) and *Bubalus bubalis* (Mediterranean) heifers over all replicates. However, *Bos indicus* heifers showed a drop in these same variables in the last replicate (Fig. 1). This effect of the cumulative OPU procedures on the number of visualized follicles and the total oocytes was not observed in *Bos taurus* and *Bubalus bubalis* heifers, whose values remained constant through the replicates (Fig. 1).

No effect of phase of follicular wave was found for: Recovery rate (D1: 70.5% ± 3.1, D3: 75.0% ± 3.1, D5: 76.0% ± 3.2; $P = 0.41$), Percentage of viable oocytes (D1: 60.6% ± 2.6, D3: 64.3% ± 2.0, D5: 62.4% ± 2.2; $P = 0.48$), Number of blastocysts (D1: 2.9 ± 0.7; D3: 3.0 ± 0.7; D5: 2.8 ± 0.6; $P = 0.76$, Blastocyst rate (D1: 19.4% ± 2.9, D3: 16.6% ± 2.9, D5: 15.9% ± 2.6; $P = 0.36$, Number of hatched blastocysts (D1: 1.4 ± 0.4, D3: 1.6 ± 0.4,

D5: 1.3 ± 0.3; P = 0.56, Hatching rate (D1: 36.8% ± 6.9, D3: 38.9% ± 6.6, D5: 41.2% ± 7.0; P = 0.68), Nuclei of hatched embryos (D1: 176.4 ± 10.0, D3: 187.6 ± 8.2, D5: 170.6 ± 8.3; P = 0.35).

Although the phase of the follicular wave did not affect the remaining variables tested, a significant effect of genetic group was observed for most of these variables, as follows. Recovery rate was not affected by genetic group (Bos indicus: 82.3 ± 2.5, Bos taurus: 66.8 ± 2.8, Bubalus bubalis: 72.5 ± 3.6; P = 0.07). Compared to *Bos taurus* and *Bubalus bubalis*, *Bos indicus* heifers had a greater percentage of viable oocytes (57.7^{b} ± 2.1, 60.9^{ab} ± 2.7, 68.8^{a} ± 1.8, respectively; P = 0.01), and these developed with better number of blastocysts (1.1^{b} ± 0.2, 0.7^{b} ± 0.1, 7.3^{a} ± 0.9, respectively; P < 0.01), blastocyst rates (14.1^{b} ± 2.9, 10.2^{b} ± 2.0, 28.3^{a} ± 2.8, respectively P < 0.01), and number of hatched blastocysts (0.4^{b} ± 0.1, 0.3^{b} ± 0.1, 3.7^{a} ± 0.5, respectively; P < 0.01). Nonetheless, this did not result in an effect on the hatching rate (24.9 ± 7.1, 34.7 ± 9.2, 49.0 ± 4.9, respectively ; P = 0.13) or the number of nuclei in hatched blastocysts (168.9 ± 13.7, 206.1 ± 23.1, 176.6 ± 5.3, respectively; P = 0.35).

4. Discussion

Here, we provide evidence that when OPU is performed up to the fifth day after pharmacologically synchronized follicular wave emergence, there is no effect on follicle number, oocyte recovery, oocyte morphology, cleavage rate, blastocyst rate and number of cells of the hatched blastocysts. However, despite the lack of effects of the phase of the follicular wave on OPU-IVEP, *Bos indicus* (Nelore) heifers yielded more oocytes and produced more blastocysts per OPU procedure than *Bos taurus* (Holstein) and *Bubalus bubalis* (Mediterranean) heifers. Nonetheless, a decrease in these same variables was

306 noted over the consecutive OPU sessions in *Bos indicus* but not in *Bos taurus* or *Bubalus*
307 *bubalis* heifers.

308 According to previous reports, we expected the phase of the follicular wave to
309 affect OPU-IVEP yields, as the phase of the follicular wave has been shown to affect
310 oocyte recovery rates [17] and competence to develop *in vitro* [16]. When OPU is
311 performed soon after the emergence of a follicular wave, better recovery and blastocyst
312 rates are obtained compared to when OPU is performed in later stages. Furthermore,
313 some studies have reported higher developmental rates *in vitro* when oocytes were
314 retrieved during the dominance phase [19,48]. Based on these reports, we designed the
315 present experiment to determine the optimal day of the pharmacologically synchronized
316 follicular wave to perform OPU to achieve better yields of OPU-IVEP in different genetic
317 groups. However, unexpectedly, the present study is in disagreement with the above
318 findings, and suggests that OPU can be carried out at any day between the first and the
319 fifth day after a pharmacologically synchronized follicular wave emergence with no
320 negative impact on the OPU-IVEP efficiency in *Bos indicus*, *Bos taurus* and *Bubalus*
321 *bubalis* heifers.

322 An important aspect which could contribute to this divergence is different
323 experimental conditions done in those studies. In almost all of them [15,16,19], the source
324 of oocytes was from animals slaughtered (*post mortem*), and animals were synchronized
325 with prostaglandin and/ or had follicles ablated to initiate the experiment. In the other two
326 works [14,17], oocytes were obtained *in vivo*, however with differences in the method of
327 synchronization of follicle wave emergence (prostaglandin and norgestomet plus estradiol
328 valerate, respectively) and also in the day 0 (estrus or emergence, respectively). Perhaps
329 the unexpected results regarding a day effect on OPU-IVEP variables could be attributed
330 to the method of synchronization of follicular wave emergence (pharmacological
331 synchronization) in comparison to follicular ablation. Although our purpose was to provide

a more practical method of synchronization of follicular wave, the pharmacological induction of follicular wave emergence could result in a cumulative follicle population containing follicles under regression together with the new follicle cohort. More studies must be conducted to clarify this matter.

Nevertheless, collectively, these works are not conclusive about a better day or a better follicular wave stage to perform the OPU, nor about which variables (follicles, oocytes, embryos produced) are constantly affected by collection day. Although our results are in discordance with the previously mentioned findings, there are other studies corroborating similar data to ours. For instance, no effect of day was reported by other authors regarding the number of visualized follicles, total oocytes and recovery rate [24], oocyte quality [17,19,20], cleavage [20] and blastocyst yield/ rate [32]. Despite this, there is a consensus concerning the later phases of the follicular wave (from 7 days of follicular wave emergence), which negatively affect oocyte competence, possibly due to advanced atresia [18, 31]. However, this long interval from the follicular wave emergence and the OPU procedure was not evaluated in the present trial. Therefore, we propose that oocyte recovery be performed one day after pharmacologically synchronized follicular wave emergence, which could shorten the interval between OPU sessions.

Overall, our results support the greater response of *Bos indicus* heifers when subjected to OPU-IVEP programs than *Bos taurus* and *Bubalus bubalis* heifers. Pontes et al [41] also reported similar data in *Bos indicus* (Gir), *Bos taurus (*Holstein) and *Bos taurus* x *Bos indicus* (cross-bred) cows. These better outcomes can be explained by genetic adaptability to tropical regions (i.e., thermotolerance) [21,49,50] and by the size of the antral follicle population [35,37,38 in *Bos indicus* compared to *Bos taurus*. Several reports also support a role for the IGF system in these physiological differences between *Bos indicus* and *Bos taurus* [34,51,52]. The higher number of follicles present in *Bos indicus*

cattle is associated with increased blood levels of IGF-I compared to *Bos taurus*. Some genes of the IGF system are expressed in oocytes [53] and can accelerate nuclear maturation *in vitro* [54]. The proteins encoded by these genes can also affect oocyte developmental competence *in vivo* [55]. Therefore, higher IGF-I concentrations might also be associated with better oocyte quality in *Bos indicus* cows compared to *Bos taurus* cows.

Concerning the data on buffaloes, the poorer performance in OPU-IVEP compared to *Bos indicus* might be explained by the smaller follicular pool found in buffalos than in cattle [37,56,57]. Fragile zona pelucida [58,59] and junctions between granulosa cells and the oocyte [2] are additional factors that could correlate with the poorer performance of buffalo heifers in OPU-IVEP. In our study, the number of follicles, oocytes and cleaved embryos did not differ between Hosltein and buffaloes. In work performed by Neglia et al. [4], the breed of bovine used was not mentioned, but probably was beef *Bos taurus*, and maybe the different performances reported can be attributed to this factor. In a recent study carried out in Brasil [37], the number of follicles among buffaloes and Holstein heifers did not differ, similar to our work. However, information about number of oocytes or cleaved embryos among these genetic groups under the same management was not found in the current literature. In this point of view, our trial is the first to report differences in OPU-IVEP between two breeds of bovine and buffaloes in contemporary conditions.

In our study, we tried to provide all conditions to avoid a distress in the animals. We choose as experimental model only heifers, because lactation and different milk production levels could differentially impact the results. Also this category has a better adaptation to climates changes than cows [24]. Additionally, in the present study we tried to minimize the environmental stressors, performing the OPU only in the morning, providing shadowing during management and in installations. Also, during all trial there was a control of the dry matter intake with total mixed ratio feeding management. It is important to reinforce that all

383 animals were maintained in the same conditions, and that these conditions were carefully
384 checked before and during all experimental period, in order to minimize bias which could
385 affect our results.

386 Regarding the decrease in the number of follicles visualized and in the total
387 oocytes that we observed in *Bos indicus* heifers after several OPU sessions, this finding
388 disagrees with some studies in which these variables remained constant in *Bos taurus*
389 [60,61] or in *Bubalus Bubalis* [62]. Possibly, OPU promotes ovarian lesions when
390 performed continually and with a short interval between sessions. This effect seems to be
391 well correlated with the number of follicles punctured in each OPU session, which may
392 explain the difference between genetic groups, as both *Bos taurus* and *Bubalus bubalis*
393 heifers performed poorly in OPU-IVEP compared to *Bos indicus* heifers. Another possibility
394 is the diameter of the needle used in our study (16 G), which could have favored more
395 lesions due the higher number of punctures performed in this genetic group.
396 In the present experiment, the OPU procedures were carried out 14 days apart; perhaps a
397 longer interval between OPU sessions should be studied, at least in *Bos indicus* heifers, to
398 avoid decreasing OPU-IVEP yields.
399

400 **5. Conclusions**

401

402 OPU performed at different phases of the pharmacologically synchronized follicular
403 wave did not alter oocyte yields or *in vitro* embryo production. Additionally, *Bos indicus*
404 (Nelore) heifers showed a greater efficiency in OPU-IVEP programs than *Bos taurus*
405 (Holstein) and *Bubalus bubalis* (Mediterranean) heifers. Furthermore, in *Bos indicus*
406 (Nelore) heifers, more than five consecutive OPU procedures with 14 day intersession
407 intervals negatively affected the number of follicles suitable to puncture and the embryo
408 yield per OPU procedure.

409

410 **Declaration of interest**

411 The authors declare that there is no conflict of interest that could affect the impartiality of
412 this scientific work.

413

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428

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616 **Table 1.** P Values of main effects (genetic group – GEN; time of OPU relative to follicular wave emergence – DAY; and replicate – REP) and
617 their interactions.

	P Value						
	GEN	**DAY**	**REP**	**GEN*DAY**	**GEN*REP**	**DAY*REP**	**GEN*DAY*REP**
Number of visualized follicles	<0.01	0.82	0.08	0.88	<0.01	0.93	0.99
Number of total oocytes	<0.01	0.56	0.05	0.90	0.03	0.37	0.39
Recovery rate (%)	0.07	0.41	0.52	0.30	0.64	0.49	0.23
Number of viable oocytes	<0.01	0.06	0.01	0.46	<0.01	0.16	0.44
Percentage of viable oocytes (%)	0.01	0.48	0.01	0.38	0.05	0.28	0.45
Number of cleaved embryos	<0.01	0.15	0.02	0.68	<0.01	0.53	0.45
Cleavage rate (%)	0.02	0.62	<0.01	0.65	0.01	0.62	0.06
Number of blastocysts	<0.01	0.76	<0.01	0.62	0.28	0.37	0.48
Blastocyst rate (%)	<0.01	0.36	<0.01	0.56	0.36	0.32	0.68
Number of hatched blastocysts	<0.01	0.56	0.01	0.62	0.20	0.69	0.85
Hatching rate (%)	0.13	0.68	0.02	0.66	0.15	0.76	0.94
Nuclei of hatched embryos	0.35	0.35	-	0.11	-	-	-

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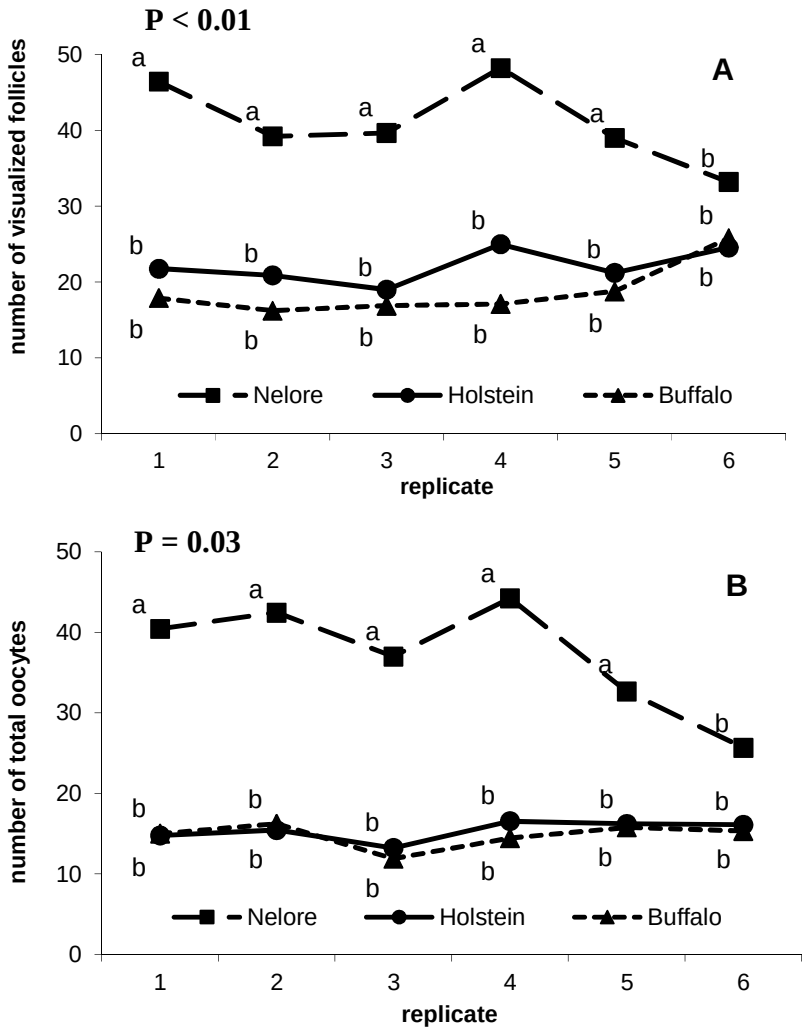
Table 2. Effect of OPU performed at different times after follicular wave emergence (D1, D3 or D5) in three different genetic groups (Nelore – NEL, Holstein – HOL, and buffalo – BUF) on oocyte recovery, quality, and developmental competence.

	D1			D3			D5			P VALUE			
	NEL (n=3)	HOL (n=3)	BUF (n=3)	NEL (n=3)	HOL (n=3)	BUF (n=3)	NEL (n=3)	HOL (n=3)	BUF (n=3)	GEN	DAY	GEN* DAY	GEN* REP
Number of replicates		6			6			6					
Number of visualized follicles	38.8 ± 3.0	23.1 ± 2.6	18.1 ± 1.4	42.2 ± 4.4	21.7 ± 2.3	19.4 ± 1.5	42.0 ± 3.6	21.5 ± 2.2	18.8 ± 1.6	<0.01	0.82	0.88	<0.01
Number of total oocytes	35.2 ± 4.8	13.8 ± 1.8	14.7 ±1.9	37.5 ± 4.4	16.2 ± 2.2	13.9 ± 1.6	38.5 ± 4.3	16.2 ± 2.3	15.7 ± 2.0	<0.01	0.56	0.90	0.03
Recovery rate (%)	79.5 ± 4.7	57.3 ± 4.5	74.8 ± 5.4	83.4 ± 3.8	73.4 ± 4.5	68.4 ± 6.9	83.9 ± 4.4	69.6 ± 4.7	74.4 ± 6.7	0.07	0.41	0.30	0.64
Number of viable oocytes	24.7 ± 3.5	7.1 ± 1.1	7.9 ± 0.9	25.9 ± 3.2	9.5 ± 1.5	9.6 ± 1.4	26.2 ± 2.7	10.6 ± 1.8	8.7 ± 1.3	<0.01	0.06	0.46	<0.01
Percentage of viable oocytes	68.6 ± 4.1	53.8 ± 4.0	59.5 ± 5.1	68.6 ± 2.7	58.0 ± 3.6	66.3 ± 3.8	69.1 ± 2.6	61.2 ± 3.3	56.8 ± 4.8	0.01	0.48	0.38	0.05
Number of cleaved embryos	20.6 ± 3.2	4.1 ± 0.6	4.5 ± 0.5	21.6 ± 2.7	5.5 ± 1.1	5.9 ± 0.8	21.1 ± 2.3	5.9 ± 1.0	5.1 ± 0.9	<0.01	0.15	0.68	<0.01
Cleavage rate (%)	82.4 ± 3.6	62.5 ± 5.8	61.4 ± 5.4	85.1 ± 2.2	57.4 ± 7.0	68.1 ± 5.0	80.3 ± 2.5	59.7 ± 5.9	61.1 ± 5.0	0.02	0.62	0.65	0.01
Number of blastocysts on D7	7.2 ± 1.7	1.1 ± 0.4	1.1 ± 0.3	7.7 ± 1.5	1.2 ± 0.3	0.4 ± 0.1	6.9 ± 1.5	1.1 ± 0.4	0.6 ± 0.2	<0.01	0.76	0.62	0.28
Blastocyst rate (%)	28.1 ± 5.6	16.6 ± 5.2	14.5 ± 4.0	28.4 ± 4.7	15.7 ± 5.9	6.3 ± 2.3	28.4 ± 4.8	10.0 ± 3.7	10.0 ± 3.6	<0.01	0.36	0.56	0.36
Number of hatched blastocysts	3.7 ± 1.1	0.4 ± 0.3	0.4 ± 0.2	4.1 ± 1.0	0.3 ± 0.2	0.4 ± 0.2	3.4 ± 0.7	0.3 ± 0.1	0.2 ± 0.1	<0.01	0.56	0.62	0.20
Hatching rate (%)	40.7 ± 9.1	28.9 ± 14.9	38.3 ± 14.5	52.9 ± 8.4	22.2 ± 8.8±	28.6 ± 18.4	52.5 ± 8.3	23.3 ± 13.7	35.7 ± 18.0	0.13	0.68	0.66	0.15
Nuclei of hatched embryos	173.0± 9.1	116.5± 23.0	225.1± 35.3	185.1± 9.0	199.5± 16.0	191.3± 38.6	168.9± 9.1	175.5± 17.2	183.5± 73.5	0.35	0.35	0.11	-

Data are shown as the means ± S.E.M. Percentages were calculated as the number of structures/ donor/ replicate, except for nuclei of hatched embryos



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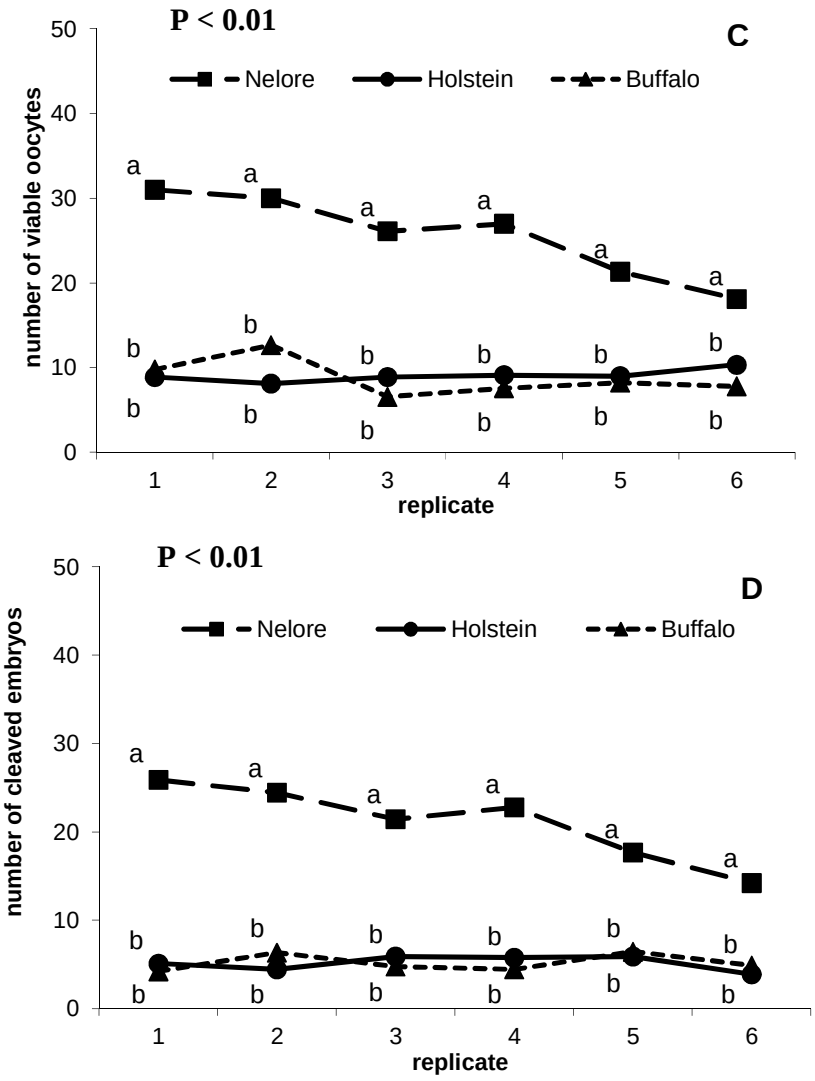

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628 **Fig. 1.** Interaction between genetic group and replicate for the number of visualized follicles (**A**), the
629 number of total oocytes (**B**), the number of viable oocytes (**C**), and the number of cleaved embryos
630 (**D**). Although *Bos indicus* (Nelore) had a greater number of visualized follicles, total oocytes, viable
631 oocytes, and cleaved embryos than *Bos taurus* (Holstein) and *Bubalus bubalis* (Mediterranean)
632 heifers, note the decrease in the number of visualized follicles and in the number of total oocytes in
633 the 6th replicate for *Bos indicus*. $a \neq b$: $P < 0.01$

634