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Characterization and control of oocyte large-scale chromatin configuration in different cattle breeds

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ABSTRACT

Differences in reproductive physiology between cattle breeds may help to explain distinct responses to assisted reproductive techniques and to define breed-specific protocols with improved efficiency. Germinal vesicle (GV) stage oocytes are characterized by increasing levels of chromatin compaction enclosed within the nucleus (graded from GV0 to GV3), associated with different developmental competence. The first objective of this study was to characterize chromatin configuration of GV stage oocytes recovered by OPU at random days of the estrous cycle from Nelore (*Bos indicus*) and Holstein (*Bos taurus*) cows. In Nelore 90% of the oocytes presented advanced stages of chromatin compaction associated with higher developmental competence (GV2 and GV3), while in Holstein, only 65% of the oocytes were at these stages. Then, aiming to obtain a more homogeneous population of oocytes in Holstein, we tested two synchronization protocols combining aspiration of all visible follicles at a random day (day 0), two IM injections of FSH 12 h apart on day 2, and OPU on day 4 (OPU/D4) or 5 (OPU/D5). The protocol OPU/D4 provided around 45% of the oocytes with low chromatin compaction (GV1), while the protocol OPU/D5 provided 70% of the oocytes at GV2 and 20% at GV3. Finally, we assessed the effects of a culture system known to prevent meiotic resumption on chromatin configuration of the GV2 enriched oocyte population obtained with the protocol OPU/D5. After 9 h of culture most oocytes transited from GV2 to GV3, with 90% of the oocytes at GV3 stage. This study demonstrates differences between Nelore and Holstein cows regarding patterns of chromatin configuration that may account for their different performance in IVM/IVF. In addition, it provides novel references for the design of protocols aiming to regulate oocyte quality before IVM for the optimization of IVF outcomes.

1. Introduction

Nelore (*Bos indicus*) and Holstein (*Bos taurus*) are major beef and dairy breeds that have been intensively subjected to assisted reproductive technologies (ART) in order to enhance genetic improvement and herd productivity. Among these strategies, embryo *in vitro* production (IVP), including oocyte *in vitro* maturation (IVM) and fertilization (IVF), has received particular interest. Physiological differences between these breeds, especially those concerning ovarian follicle population and dynamics, must be considered and protocols adapted for the optimization of ART outcomes. Nelore cows present larger numbers of antral follicles between 2 and 5 mm, smaller follicle diameters at deviation and ovulation and faster follicle turnover in relation to Holstein [1,2]. As a consequence of lower antral follicle numbers, ovum pick up (OPU) for IVP yields less oocytes in Holstein cows compared to Nelore. However, as indicated by the overall limited efficiency of standard IVP protocols, not only oocyte yield but also oocyte quality seems to be lower in Holstein compared to Nelore [3,4]. The causes determining lower oocyte developmental competence in Holstein are not fully understood, although they are thought to include metabolic challenges imputed by high milk yield and environmental and management induced stress [1].

In addition to breed differences, the efficiency of IVP is also troubled by the intrinsic heterogeneity of the oocyte population that resides in antral follicles. Oocyte developmental competence largely varies during nuclear differentiation, which is reflected by different

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degrees of chromatin compaction [5]. During the germinal vesicle (GV) phase, oocyte chromatin gradually condenses proceeding through stages classified as GV0, GV1, GV2 and GV3, while oocyte transcriptional activity decreases [6,7]. GV0 oocytes are still growing, present uncondensed chromatin and are unable to complete meiosis II, whereas GV1-3 oocytes are fully grown, present increasing degrees of chromatin compaction and are able to be fertilized. In addition, GV2 and GV3 oocytes present a higher developmental competence in relation to GV1 oocytes, although some GV3 cumulus-oocyte complexes show early signs of atresia [8,9]. Therefore, GV2 oocytes with an intermediate level of chromatin compaction and "fresh" GV3 oocytes with compacted chromatin but still no signs of atresia would represent the "gold standard" for IVP [9,10].

No study so far has characterized in any cattle breed the oocyte population obtained by OPU at a random day of the estrous cycle with regard to chromatin configuration. Nevertheless, data from abattoir ovaries suggest that this population is heterogeneous and contains oocytes from GV0 to GV3 [11]. Therefore, it is fair to speculate that breeds with different follicular patterns may also differ in terms of the distribution of GV stages (GV0-3) in the oocyte population. Most current *in vitro* maturation (IVM) protocols subject this likely heterogeneous population of oocytes to a culture system with supraphysiological gonadotrophic stimulus that interrupts cumulus-oocyte communication and precipitates meiosis completion [6], potentially causing asynchrony between oocyte nuclear, cytoplasmic and molecular maturation. The importance of controlling oocyte population *in vivo* before OPU to optimize IVM/IVF outcomes is well illustrated by the successful utilization of the "coasting protocol", which synchronizes follicle growth with dominant follicle aspiration and FSH treatment for three days, followed by a waiting interval of around 48 h before OPU [12].

In the present study, we first aimed to assess chromatin configuration patterns of oocytes recovered by OPU at a random day of the estrous cycle from Holstein (*Bos Taurus*) and Nelore (*Bos indicus*) cows. We hypothesized that overall heterogenous oocyte populations would be found and that different distribution patterns of GV stages would be observed in these two breeds. Then, we tested *in vivo* and *in vitro* protocols aiming to regulate oocyte chromatin configuration. First, we assessed the efficacy of two synchronization protocols combining follicle aspiration and FSH treatment to homogenize the oocyte population with regard to GV stage, and subsequently we evaluated the effects of a culture system known to prevent germinal vesicle breakdown on chromatin configuration of a GV2 enriched oocyte population.

2. Materials and methods

Nelore (*Bos indicus*) and Holstein (*Bos taurus*) cows were obtained from the experimental farms of the Sao Paulo State University (UNESP) campus located in Botucatu. Three to seven years old animals, kept on grass and with access to mineral salt and water *ad libitum* were used. Lactating Holstein cows were supplemented with corn silage and concentrate. Only cycling cows were included in the study, which was confirmed by the presence of a corpus luteum at an ultrasound examination.

All procedures were approved by the Institute of Biosciences - UNESP ethics committee (protocol 906). Chemicals and reagents were purchased from Sigma-Aldrich Brazil unless otherwise indicated; porcine pituitary-derived follicle stimulating hormone (Folltropin®-V) was obtained from Vetoquinol Brazil.

2.1. Experiment 1 - characterization of GV stage between breeds

To investigate the distribution pattern of GV classes in the different breeds, in the first experiment, a group of fifteen Holstein and eighteen Nelore cycling cows had all follicles at diameters equal or larger than 2 mm aspirated at a random day of the estrous cycle. The oocytes were mechanically separated from cumulus cells by repeated pipetting in Dulbecco's phosphate-buffered saline (DPBS), following fixation and staining to assess GV chromatin status as described below. All COCs regardless of their morphological classification were included in the study.

2.2. Experiment 2 - synchronization protocols in Holstein

In a second experiment, in order to obtain a homogenous population of recovered oocytes, two synchronization protocols were tested. In the first one, denominated OPU/D4, ten Holstein cows had all follicles at diameters equal or larger than 2 mm aspirated on a random day of the estrous cycle (day 0), then on day 2 they received two IM injections of FSH (Folltropin; 56 mg each dose) with a 12 h interval and were submitted to OPU on day 4 for recovery of the COCs (Fig. 1a). The second protocol, denominated OPU/D5, included six Holstein cows that had all follicles at diameters equal or larger than 2 mm aspirated on a random day of the estrous cycle (day 0). Subsequently, they received two IM injections of FSH (Folltropin; 56 mg each dose) with a 12 h interval on day 2, and were submitted to OPU on day 5 for recovery of the COCs (Fig. 1b). At the end of each synchronization protocol, oocytes were recovered to assess GV status; all recovered COCs were analyzed regardless of their morphological classification.

2.3. Experiment 3 - synchronization protocol associated with a pre maturation culture in Holstein

To assess the effect of a pre-maturation culture associated with a synchronization protocol, in a third experiment eleven Holstein cows were submitted to the synchronization protocol OPU/D5. The COCs were recovered by OPU, selected under a stereomicroscope and washed twice in TCM199 with Earle's salts and 25 mM HEPES, supplemented with 75 μg/mL amikacin and 4 mg/mL bovine serum albumin (BSA). All recovered COCs were transferred to tubes containing a defined pre-maturation (pre-IVM) medium described below, gas mixture (5% carbon dioxide, 5% oxygen and 90% nitrogen gas), and were transported to the laboratory.

In the laboratory, the COCs were cultured in four-well dishes at 38.5 °C and 5% CO_2 in humidified air for 9 h in the defined pre-IVM medium also named "follicular system" [13]. Briefly, medium was composed of TCM 199 containing Earle's salts, L-glutamine and $NaHCO_3$ supplemented with 0.4% fatty acid-free BSA, 22 mg/mL sodium pyruvate, 75 μg/mL amikacin, 100 nM natriuretic peptide type C (NPPC), 500 ng/mL 17β-oestradiol, 50 ng/mL progesterone, 50 ng/mL testosterone and 10^{-4} IU/mL human recombinant FSH. At the end of culture, COCs were processed for chromatin configuration assessment.

2.4. Follicle aspiration and cumulus-oocyte complex collection

In all experiments, COCs were recovered by ultrasound-guided transvaginal OPU from follicles at diameters equal or larger than 2 mm, after epidural anesthesia (5–7 mL lidocaine; Bravet, Brazil). The recovered fluid was deposited in a conical tube with DPBS and heparin, washed and filtered for further recovery and evaluation of COCs in a Petri dish (90 mm) under a stereomicroscope.

In experiments 1 and 2, COCs were mechanically separated from cumulus cells by repeated pipetting in DPBS, and oocytes were fixed and stained to assess GV chromatin status as described below. In experiment 3, COCs were transferred to tubes containing a defined pre-IVM medium and gas mixture and transported to the laboratory.

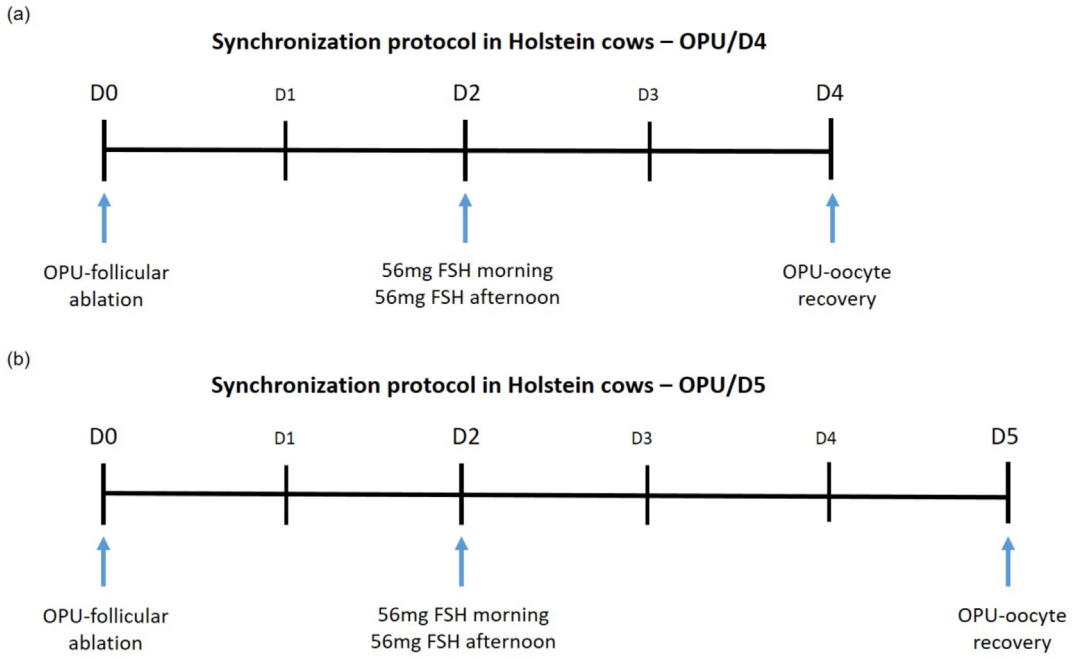

Fig. 1. Schematic representation of the synchronization protocols OPU/D4 (**a**) and OPU/D5 (**b**). Protocols consists of aspiration of all follicles at diameters equal or larger than 2 mm on a random day of the estrous cycle considered as day 0 (D0), two injections of FSH (56 mg each) with a 12 h interval on day 2 (D2), and OPU to recover the COCs on day 4 (OPU/D4) or on day 5 (OPU/D5).

2.5. Assessment of GV chromatin status

At the end of all experiments, GV chromatin was examined by fluorescence microscopy after mechanical denudation of oocytes in 500 mL DPBS and fixation with 60% methanol in DPBS for 30 min at 4 °C, followed by staining with 1 μg/mL Hoechst 33342. COCs were classified according to the integrity of the GV and degree of chromatin compaction according to Lodde et al., 2007 [7]: GV0 stage presenting a diffuse filamentous pattern of chromatin in the whole nuclear area, GV1 with few chromatin foci of condensation detected in the nucleus, GV2 showing a condensed chromatin into distinct clumps or strands, and GV3 with the chromatin condensed into a single clump within the nuclear envelope. Those oocytes with an irregular, partly degrading or absent GV were classified as GVBD and those which could not be identified at any of the stages above were classified as degenerated (DEG).

2.6. Statistical analysis

In all experiments, each cow represents one replicate. Data in the form of percentages were arcsine transformed. Shapiro-Wilk's test was used to verify normality. The analyzed data were nonparametric and were compared using the Kruskal-Wallis test. These analyses were performed using the JMP software (SAS Institute, Cary, NC, USA) and differences were considered significant at two-sided $P < 0.05$.

3. Results

In Holstein cows, OPU at a random day of the estrous cycle yielded a larger proportion of oocytes at the GV2 stage of chromatin compaction (47.44 ± 7.4%) in comparison with the GV1 (19.9 ± 4.7%) and GV3 (17.44 ± 3.3%) stages, which in turn were more frequent than GV0 (5.78 ± 4.5%), GVBD (2.31 ± 1.2%) and degenerated (DEG; 7.12 ± 3.5%) oocytes (Fig. 2a). On the other hand, in Nelore cows, the percentage of GV2 (50.2 ± 1.8%) and GV3 (39.66 ± 1.2%) oocytes

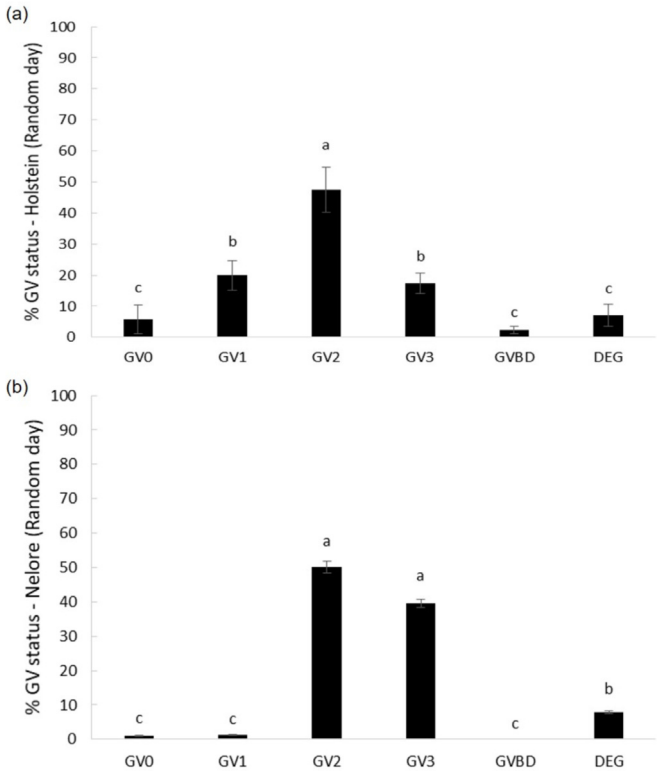

Fig. 2. Distribution patterns of germinal vesicle statuses according to chromatin configuration on a random day of the estrous cycle in (**a**) Holstein (n = 15 cows/140 COCs) and (**b**) Nelore cows (n = 18 cows/241 COCs). GV0 to 3 represent stages with increasing chromatin compaction; GVBD = germinal vesicle breakdown; DEG = degenerated oocytes. Different letters indicate statistical differences ($P < 0.05$).

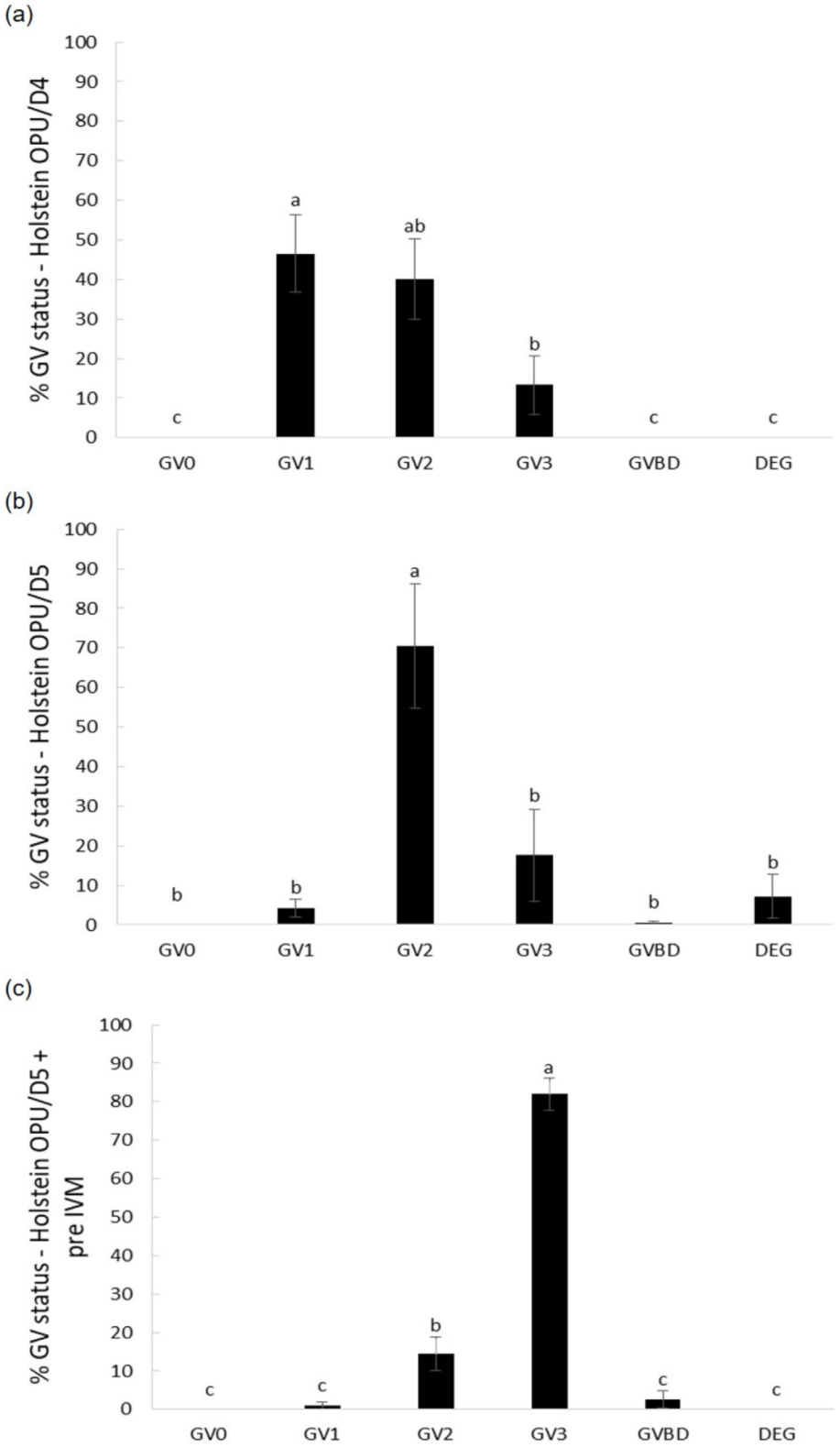

Fig. 3. Effects of synchronization protocols combining aspiration of all follicles at diameters equal or larger than 2 mm on a random day (day 0) and FSH treatment on day 2 on patterns of chromatin configuration of oocytes recovered by OPU from Holstein cows on day 4 (**a**; protocol OPU/D4; n = 10 cows/69 COCs) or on day 5 (**b**; protocol OPU/D5; n = 6 cows/63 COCs). (**c**) Effects of the combination of the synchronization protocol OPU/D5 with a culture period utilizing NPPC and steroids to control meiosis progression on chromatin configuration of oocytes recovered by OPU from Holstein cows (n = 11 cows/126 COCs). Different letters indicate statistical differences ($P < 0.05$).

recovered by OPU at a random day of the estrous cycle did not differ statistically, whereas markedly lower percentages of GV0 (1.04 ± 0.1%), GV1 (1.3 ± 0.2%) and DEG (7.8 ± 0.4%) oocytes were observed (Fig. 2b). GVBD oocytes were not recovered from Nelore cows. Oocyte yield per cow was on average 9.33 (n = 140 oocytes/15 cows) and 13.38 (n = 241 oocytes/18 cows) for Holstein and Nelore, respectively.

Since around 90% of the oocytes recovered from Nelore cows were at GV2 or GV3, stages previously suggested to hold higher developmental competence [17], protocols aiming at homogenizing oocyte population while increasing the proportion of GV stages with higher developmental competence were only tested in Holstein cows. The OPU/D4 protocol yielded 69 oocytes (6.9 oocytes/cow), most of them at GV1 (46.55 ± 9.9%) and GV2 (40.09 ± 10.1%) stages, and fewer oocytes at the GV3 stage (13.36 ± 7.33%). In contrast, the OPU/D5 protocol yielded 63 oocytes (10.5 oocytes/cow), 70.5% of which at the GV2 stage, whereas markedly lower percentages were observed at GV3 (17.59 ± 11.69%), GV1 (4.21 ± 2.29%) and GVBD (0.46 ± 0.46%). GV0 oocytes were not recovered from Holstein cows subjected to either of the synchronization protocols (Fig. 3).

Treatment of the GV2 enriched population of oocytes provided by the OPU/D5 protocol with a culture system previously developed in our laboratory and known to prevent meiotic resumption (n = 126 oocytes; 11 cows) [18] promoted the transition from GV2 to GV3; 81.94% (±4.4%) of the oocytes were at GV3 after 9 h of culture (Fig. 3).

4. Discussion

Differences in reproductive physiology between cattle breeds impact on fertility and require adaptations in ART protocols for optimal outcomes (reviewed by Refs. [1,9]). Nelore cows provide comparatively larger numbers of oocytes with apparently higher developmental competence, which has been recognized as the major cause of their successful performance in IVP [3,14,15]. Although previous data clearly show that chromatin status largely impacts on oocyte developmental competence [16,17], chromatin patterns of oocyte populations obtained by OPU for IVM/IVF have not been assessed yet. In the present study, we provide evidence of different distribution patterns of oocyte chromatin configuration between Nelore and Holstein that may help to explain their different performance in IVP. In parallel, we present new insights for *in vivo* and *in vitro* strategies aiming to control chromatin configuration to optimize IVP outcomes in cattle.

As chromatin compaction proceeds, transcriptional activity diminishes and oocyte meiotic and developmental competence are gradually acquired. In fact, oocytes at the GV0 stage hold very limited ability to resume meiosis, and only a small percentage of GV1 oocytes reach the blastocyst stage after IVM/IVF. In contrast, GV2 and GV3 oocytes present greater developmental competence, which is accompanied by specific changes of transcriptomic profiles [6–8,16]. We have recently reported that oocytes from Holstein cows appear to be more susceptible to meiotic resumption and GVBD upon COC removal from the follicle in relation to Nelore [13]. This suggests differences in the mechanisms that regulate oocyte nuclear maturation during follicular development. Chromatin remodeling is associated with morphological and functional changes in the oocyte [8]. In the present study, Nelore cows provided an oocyte population predominantly consisted of GV2 and GV3 oocytes at random days of the cycle, which is in agreement with their superior performance in IVP [7]. Since the same was not observed in Holstein cows, we tested two synchronization protocols aiming at controlling chromatin configuration patterns of oocytes

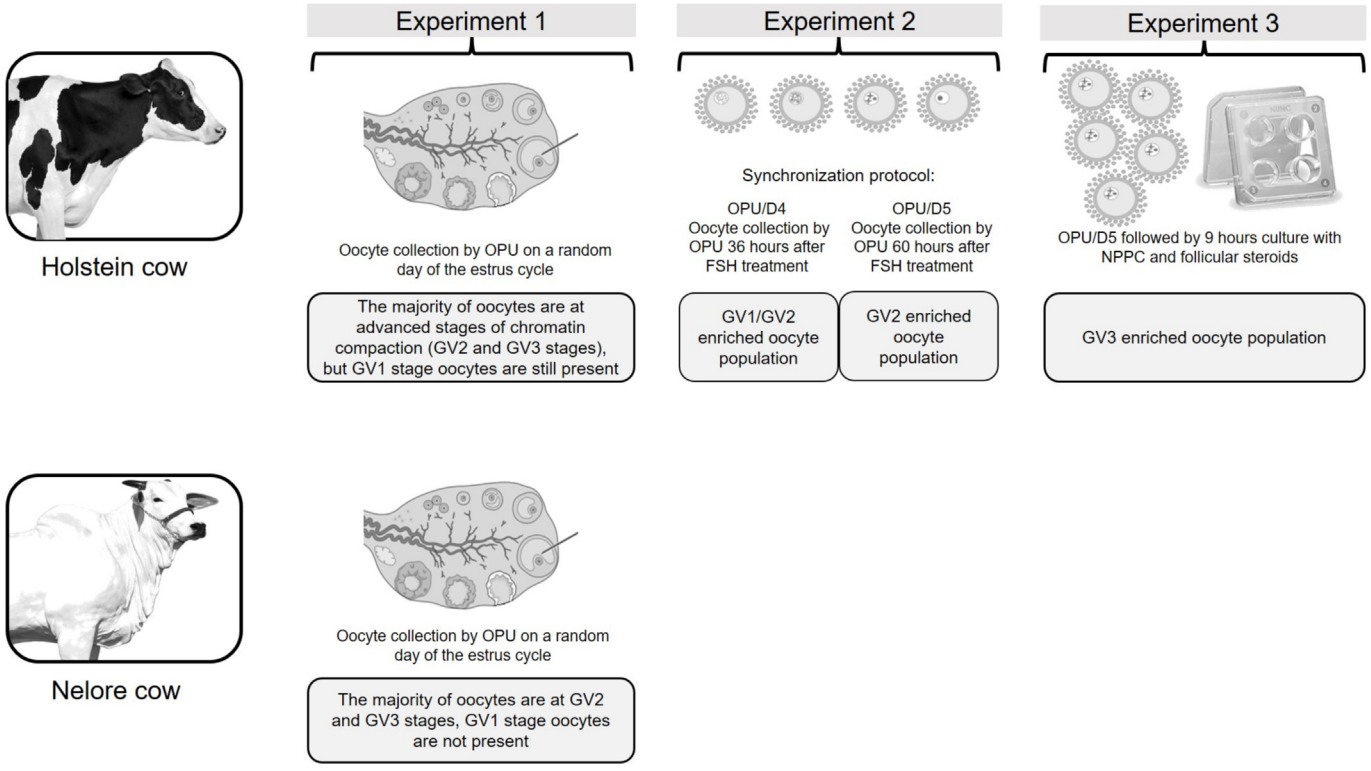

Fig. 4. Graphical abstract presenting the main findings. Chromatin configuration patterns on a random day of the estrous cycle differ between Nelore and Holstein cows (Experiment 1). Protocols combining follicle aspiration and FSH treatment can be utilized to control oocyte quality with regard to chromatin configuration in Holstein cows (Experiment 2). A culture strategy combining NPPC and steroids promotes the transition from GV2 to GV3 oocytes (Experiment 3).

obtained by OPU in this breed. These protocols combined follicle aspiration to induce a new follicular wave emergence, and FSH treatment, at lower and fewer doses (2 doses of 56 mg FSH) than those routinely utilized in superovulation protocols (200–400 mg), in order to enhance follicular recruitment [12,17–20]. The only difference between these two protocols was the interval between the second/last injection of FSH and OPU, which lasted 36 and 60 h for OPU/D4 and OPU/D5 protocols, respectively. While the OPU/D4 protocol yielded nearly 50% of the oocytes still at GV1, the OPU/D5 protocol led to the recovery of 70% of the oocytes at GV2, a stage previously suggested to hold higher developmental competence [9]. Previous studies have proposed a "coasting" period from 44 to 72 h between FSH treatment for 3 days (6 doses of 40 mg) and OPU, with optimal blastocyst rates obtained with intervals around 54 h [12,20–22]. In the present study, an interval between FSH treatment and OPU very similar to that recognized by the coasting protocol provided a GV2 enriched population of oocytes (around 70%; Fig. 4), despite the use of a milder/shorter FSH stimulation treatment. Further studies are required to assess whether this GV2 enriched population of oocytes does hold higher developmental competence and whether longer FSH treatment would alter chromatin patterns and developmental competence.

Culture systems aiming to delay meiotic resumption and prolong oocyte-cumulus communication have been proposed to better coordinate oocyte nuclear and cytoplasmic maturation, thus improving IVM/IVF outcomes [23]. We have recently developed a culture system containing natriuretic peptide C (NPPC) and intrafollicular concentrations of FSH and steroids, which is able to prevent GVBD for 9 h [13]. In this study, aiming to get insight on ways to control chromatin differentiation, we assessed the effects of our previously developed culture system on chromatin dynamics of the enriched GV2 oocyte population provided by the OPU/D5 protocol. Interestingly, the vast majority of the oocytes transited from GV2 to GV3 during culture, while GVBD was strongly suppressed, in agreement with our previous results. Previous studies have suggested that oocytes from large follicles with initial signs of atresia could present higher developmental competence for having been exposed to the follicular microenvironment for a longer time [5,24]. These oocytes would be expected to be at the GV3 stage of chromatin compaction [9]. Further studies are required to assess whether oocyte developmental competence is improved by *in vitro* transiton from the GV2 to the GV3 stage, before the induction of COC maturation.

In conclusion, our results indicate the occurrence of different patterns of chromatin configuration in the oocyte population recovered at a random day of the estrous cycle from Nelore and Holstein cows. These different patterns may help to explain, at least in part, the superior performance of Nelore donors in IVP schemes. Moreover, we report synchronization and culture protocols capable to regulate oocyte chromatin status before IVM (Fig. 4). Therefore, the present data contribute to elucidate differences in reproductive physiology and performance between cattle breeds and are potentially valuable for the design of new and more efficient IVM/IVF strategies in cattle.

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