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Serum progesterone profiles during the estrous cycle of dairy and beef cows determined using immunochemiluminescence (CLIA) and validated using enzyme-linked immunosorbent assay (ELISA)

Perfis da progesterona sérica durante o ciclo estral de vacas de leite e de corte determinados pela Imunoquimioluminescencia (CLIA) validados pela Imunoabsorção Enzimática (ELISA)

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Abstract: This study aimed to validate the methodology of immunochemiluminescence (CLIA) using an enzyme-linked immunosorbent assay (ELISA) to determine serum progesterone (P4) concentrations in non-pregnant multiparous dairy (12 Holstein cows) and beef (18 Nellore cows) cows during the estrous cycle. Cows with corpus luteum were chosen for estrus and ovulation synchronization by administering 500 mcg of cloprostenol. After luteolytic application, the animals were subjected to ovarian ultrasonography (US) daily to verify ovulation (day zero of the cycle) and two blood samples were collected for P4 determination using CLIA and ELISA. Samples were centrifuged to obtain serum and frozen at -20 °C for later measurement. ELISA and CLIA values were compared using the paired t-test, regression, analysis of variance, and coefficient of determination (R^2) to verify sensitivity and linear correspondence. The P4 concentration determined by both methodologies showed a similar profile; the P4 profiles were higher in beef cattle than in dairy cattle. The correspondence between the methodologies resulted in a high quotient for R^2 the P4 profiles. It was concluded that CLIA can be used for hormonal determinations of bovine serum P4. CLIA showed a high linear correspondence with ELISA values and can thus aid reproductive biotechnologies for hormone determination.

Keywords: Hormone dosage; Bovine Reproduction; Reproductive hormone; Nellore cow; Holstein cow.

Resumo: O objetivo do estudo foi validar a metodologia da imunoquimioluminescência (CLIA) através da imunoabsorção enzimática (ELISA), determinando a concentração sérica de progesterona em vacas de leite e de corte, durante o ciclo estral. Foram empregadas 30 vacas multíparas não-prenhes (12 da raça Holandesa Preta e Branca e 18 Nelore). Vacas com corpo luteo foram escolhidas com vistas

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à sincronização do estro e da ovulação, mediante aplicação de 500 mcg de cloprostenol (im). Após a aplicação do luteolitico, os animais foram diariamente submetidos a exames de ultrasssonagrafia (US) ovariana para verificação da ovulação (=dia 01 do ciclo), bem como eram colhidas 02 amostras de sangue para a determinação da P4 pela CLIA e ELISA. As amostras eram centrifugadas para a obtenção do soro, e congeladas a -20 graus para posterior dosagem. Os valores de ELISA e CLIA foram comparados entre si mediante teste t pareado, regressão, ANOVA e coeficiente de determinação (R2), visando a verificação da sensibilidade e correspondência linear. A concentração de P4 originou perfis similares entre as duas metodologias; os perfis de P4 foram mais elevados em bovinos de corte, que nos de leite. A correspondência entre as metodologias resultou em elevado quociente de R2 nos perfis de P4. Concluiu-se que a CLIA pode ser empregada nas determinações hormonais da P4 serica bovina; a CLIA mostrou elevada correspondência linear com os valores da ELISA; a CLIA pode auxiliar as biotecnicas da reprodução nas determinações hormonais.

Palavras-chaves: Dosagem hormonal; Reprodução Bovina; Hormonio reprodutivo; Vaca Nelore; Vaca Holandesa;

1. Introduction

Growing challenges related to animal production and reproduction are factors limiting the expansion of reproductive biotechnologies. Studies on improving reproductive efficiency have faced increasing research demands, including fixed-time artificial insemination (FTAI), fixed-time embryo transfer (FTET), and in vitro fertilization (IVF). To better understand the challenges of unsatisfactory reproductive efficiency in FTAI, it is necessary to know the progesterone (P4) hormonal profiles of animals subjected to the protocols[1]. Techniques for hormone determination using radioimmunoassays (RIA) have been accepted. However, the radioactive waste (radioactive iodine) originating from this technique dissuades adoption. To minimize this limitation, other methodologies have been developed, including enzyme immunosorbent assays (ELISA)[2] and enzyme immunoassays (EIA)[3], which employ enzymatic assays for hormone determination. ELISA is a promising methodology for determining several hormones and is frequently used for determining serum P4 in bovines [1].

Another method for hormone determination, that has been used as an alternative to RIA and ELISA due to its greater sensitivity, shorter execution time, and lower cost, is immunochemiluminescence assay (chemiluminescence assay; CLIA)[4,5,6,7]. In human medicine, CLIA is widely used to measure blood profiles of several hormones, including P4. CLIA can perform thousands of determinations per day owing to the advanced automated system flow, making the analysis process less costly and free from human interference (e.g., automatic pipetting of reagents, homogenization of reagents, and reactions). CLIA is based on the principle of light emission, which causes a chemical reaction[8] and generates light energy. It has high sensitivity, short analysis time, and reduced cost, without toxic effects[4,5]. In addition, hormone determination using CLIA does not require duplicate samples or the use of radioactive markers[6,7,9].

P4 regulates the function of the female reproductive system in the estrous cycle in animals. Ovarian follicular growth and metabolism and the succession of developmental

waves (follicular dynamics) are greatly influenced by circulating P4 concentrations[10]. The P4 concentration has been used to monitor the functional capacity of the corpus luteum[11,12], for the early diagnosis of pregnancy[11,13], and for the analysis of endocrine disorders in bovine females[14]. Thus, the measurement of P4 in the serum or plasma of cows is important in the research and clinical observations of reproductive aspects.

Cardoso[15] used CLIA as an analytical method to assess serum P4 concentrations in cyclic dairy heifers, established links with reproductive functions, and reported a positive correlation between corpus luteum (CL) diameter and P4 concentration (R^2=0.22, p <0.0001). The results obtained using CLIA were consistent with those obtained using RIA and ELISA and were able to determine the occurrence of estrous cycles and assess ovarian functionality.

The present study aimed to validate CLIA as an alternative methodology to RIA and ELISA as an auxiliary method for determining the parameters related to P4 for animal reproduction biotechniques. In this context, the present investigated the efficiency of CLIA in obtaining serum P4 concentrations in dairy and beef cows during the physiological estrous cycle and validated the methodology using ELISA.

2. Material and methods

This study was approved by the Committee on Ethics in the Use of Animals, Declaration of CEUA (PUCPR), no. 01686/2019.

The study was carried out on non-lactating and non-pregnant multiparous cows from two farms located at coordinates Latitude: 25º39'27"S; Longitude: 49º18'29"W; n=12), and Latitude: 26º10'13"S; Longitude: 53º21'41"W. Dairy cows (Holstein; n=12) were submitted to the free-stall management system, and were fed Leitemax 18 AE (Agraria Nutrição, Guarapuava, PR, Brazil), corn silage (*Zea mays*), hay (*Avena sativa*), and mineral salt (BellNutri 90, Bellman, São Paulo, Brazil), with water available ad libitum. Beef cows (Nellore; n=18) were maintained in pastures rotated with forage (*Brachiaria decumbens*) and mineral salt (Maxicorte 17, Agraria Nutrição, Guarapuava, PR. Brazil) and water *ad libitum*. All animals were vaccinated against the main diseases affecting them. At the gynecological examination prior to the start of the study (transrectal palpation and ultrasound examinations), all animals were confirmed free of reproductive disorders such as ovarian cysts (follicular or luteal), ovarian adhesions, endometritis of any degree, and other disorders.

Animal selection criteria

Cycling animals (presence of CL, confirmed by ultrasonography) out of the puerperium period were selected [16]. The dominant follicles (DF) measurements were performed by measuring the longitudinal diameter + the diagonal diameter and dividing by 2[17].

Ovulation was defined as the absence of a follicle measured on the previous day associated with medium echogenicity in the ovulation fossa. CL was considered when the gray tone content was verified in place of the former DF, which had an anechoic appearance.

Atresia of the DF was considered in the 1st. follicular wave when the follicle on the previous day experienced a reduction in dimensions (mm).

Estrus induction, ultrasonography, and data acquisition

For estrus induction, the animals were submitted to the application of 500 mcg of D-cloprostenol (intramuscularly) (Ciosin, Zoetis, São Paulo). Twenty-four hours after luteolytic application, ultrasound examinations were performed to monitor the DF. Day 0 of the estrous cycle was considered to be when ovulation of the DF was detected, and a complete estrous cycle was considered when the animals returned to the new manifestation of visible estrus (visible detection of signs of estrus during the day) with the presence of a preovulatory follicle. After verifying ovulation, the ovaries were scanned daily (afternoon period) using an ultrasound device (Mindway, Transducer 5.0, China) and the ovarian findings were recorded on specific sheets, followed by changes in the gonads over the interval between the two ovulations.

Blood collections and determinations of P4 profiles

Blood samples were collected daily on the day of ovulation (day 0). Samples were collected in Vacutainer tubes by puncturing the *Vena caudalis* and were stored in a refrigerator. At the end of the day's fieldwork, the blood was centrifuged and the serum was stored in Eppendorf tubes, identified using the animal number and day of sample collection, and stored in a freezer (-20 ºC). P4 was determined using ELISA and CLIA on the same day after the end of fieldwork.

Hormonal Determinations

Serum P4 levels were determined using ELISA (Kit; 96-well plates; Bio Medix diagnostica, São Paulo, Brazil) and a CLIA kit (Access P4 kit; Beckman Coulter, USA). The sensitivity of the CLIA kit for detecting P4 ranged from 0.10 to 40.0 ng/mL. Serum samples containing up to 5 mg/dL of bilirubin, 500 mg/dL of hemoglobin, and 450 mg/dL of triglycerides did interfere with the test (manufacturer's information) and the intra-assay variation was less than 10.0%, accepting the absolute values, based on routine laboratory measurement procedures for determining P4 (Curitiba, Paraná, Brazil).

For P4 measurements using CLIA, the samples were placed in a machine with automatic processing. The P4 measurements were performed using a Beckman Unicel DxI 800 instrument. The Access P4 Test is a competitive paramagnetic chemiluminescent immunoassay for the quantitative detection of serum P4 levels using Access Immunoassay Systems, No. Cat 33550, for 100 measurements (Beckman Coulter, INC, CA, USA).

Criteria for determining the follicular phase and luteal phase of the estrous cycle in cows

The follicular phase consists of proestrus (dominant follicle formation = > preovulatory) + external signs of estrus (sexual behavior and receptivity, vulva edema, etc.) and the luteal phase consists of the formation and presence of CL + luteolysis[18]. The proestrus and estrus

phases were marked by a low concentration of circulating P4, while the luteal, metestrus, and diestrus phases were marked by a gradual increase and high concentrations of P4 in the serum profile of the animals.

Statistical analysis

The ovarian findings were calculated using means and standard deviations. Serum P4 concentrations were determined using ELISA (ng/mL) and CLIA (ng/mL) methodologies in dairy and beef cows and are presented in the form of graphs according to the P4 concentration on the day of the estrous cycle. Serum P4 concentrations in each sample unit (cow) determined using ELISA and CLIA were compared using a paired t-test. The relationship between serum P4 concentrations measured using CLIA in dairy and beef cows was analyzed using linear regression (linear model) using the least squares method. The adherence to the original data was tested using analysis of variance (ANOVA) and the R^2 value, to estimate the actual serum concentration (obtained using CLIA) through ELISA. A significance of $p<0.05$ was considered significant.

3. Results

Table1 shows data on ovarian follicular dynamics by ultrasonography after administration of PGF2 alpha for estrus induction.

Table 1. Dimensions of the dominant follicle (DF), DF atresia (1st follicular wave), presence of the corpus luteum (CL) after ovulation, and CL size and preovulatory follicle size (2nd follicular wave) in Holstein and Nellore cows, monitored using ultrasound examinations.

Group	**Dimension of the DF 1st. follicular** wave (mm) (x ± s)	Atresia of the DF of the 1st. follicular wave (cycle day) (x ± s)	**Presence of CL after** of PGF2α (cycle day) (x ± s)	**Dimension of the POF of the 2nd.** follicular wave (mm) (x ± s)
Nellore	8.9 ±1.0	15.0±1.7	4.0 ± 0.5	11.5 ± 1.4
Holstein	11.41 ± 2.2	14.6±1.1	3.0 ±0.5	15.0 ± 1.5

The P4 profiles of Bos taurus and Bos indicus cows during the estrous cycle are shown in Figures 1, 3, and 5. The P4 values corresponded to each day of the monitored estrous cycle as determined using ELISA and CLIA. There was harmony in the P4 curves between the two methodologies, except that in the ELISA methodology, the values were higher than those in CLIA. This can be seen in figures 1, 3 and 5. The figures allowed the perception that there is a linear correspondence between the values obtained in dairy and beef cows, as verified by the high value of R^2, which ranged from 0.9473 to 0.9864 and was highly significant ($p < 0.00001$) (Table 2). For the same samples, CLIA showed lower concentrations of P4 than ELISA.

Table 2 Relationship between CLIA and ELISA in Bos taurus and Bos indicus cows, after linear regression tests and Least squares method.

Equation value related to CLIA and ELISA	Holstein	Nellore	Holstein and Nellore
a (angular coeficient)	0.6393	0.787	0.577
R^2	0.9864	0.9473	0.9643

*a: ELISA model coefficient= a * CLIA, where ELISA and CLIA are the sérum concentrations of P4; R^2: coefficient of multiple determination; P: p-value of ANOVA.*

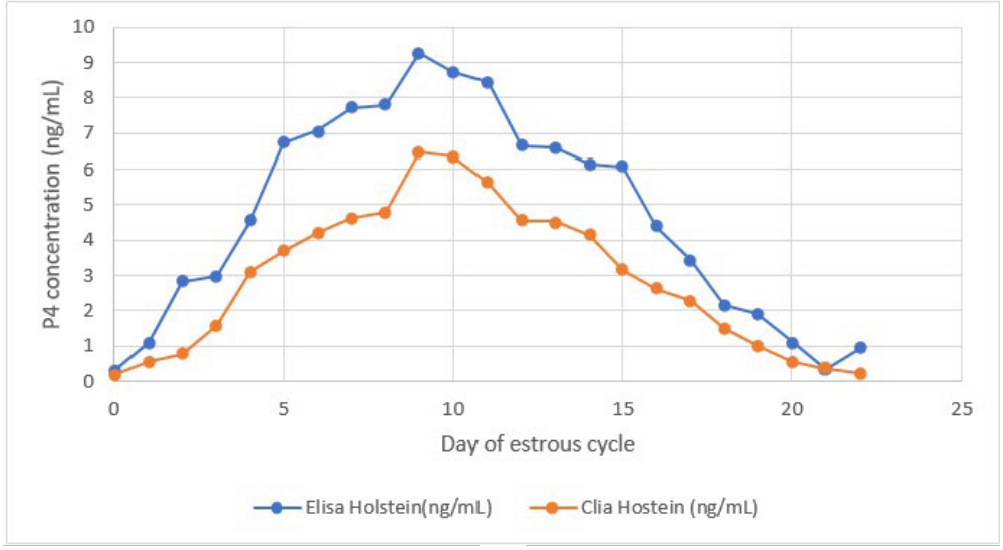

Figure 1 Profiles of serum P4 (ng/mL) in Holstein cow determined using CLIA and ELISA during the estrous cycle.

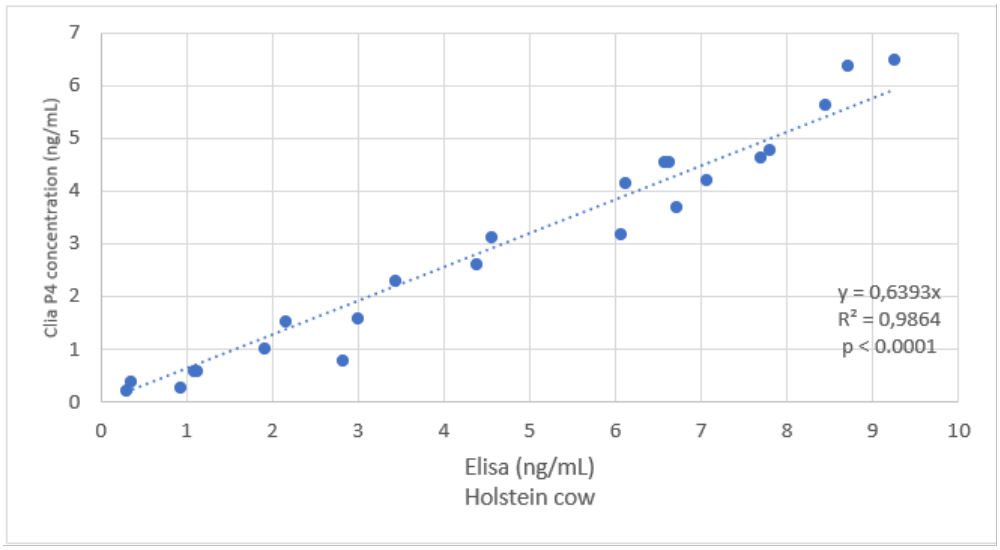

Figure 2 Determination of serum P4 using CLIA and ELISA during the estrous cycle in Holstein cows

Figures 2, 4, and 6 show a linear correspondence between the values obtained using the two methods, showing a high R^2 value. The figures show that ELISA overestimated the concentration of P4. The actual value (as obtained using CLIA) can be obtained by applying the equation (Clia = 0.6393 × ELISA). The adjustment was significant, and the coefficient contributed significantly to the equation.

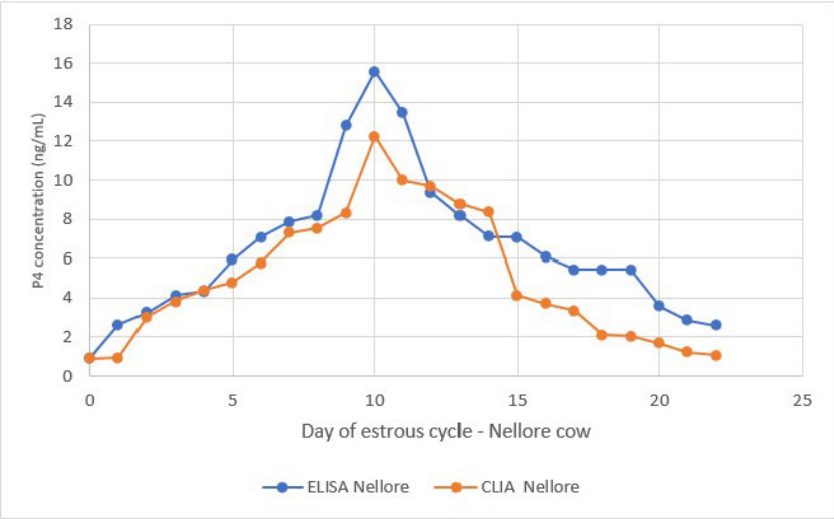

Figure 3 Profiles of P4 (ng/mL) in beef cows (Nellore) using CLIA and ELISA, during an estrous cycle.

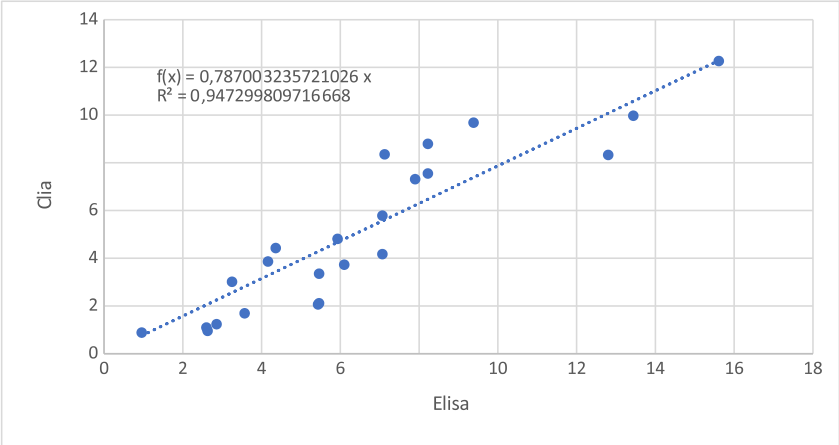

Figure 4 Determination of serum P4 using CLIA and ELISA during the estrous cycle in Nellore cows.

The actual value (as obtained using CLIA) was obtained by applying the equation (CLIA = 0.787 × ELISA).

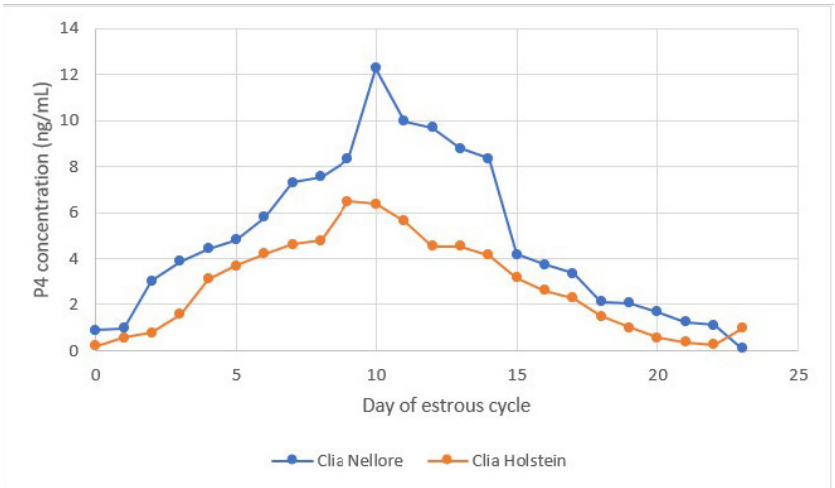

Figure 5 Profiles of P4 (ng/mL) using CLIA during the estrous cycle in Holstein and Nellore cows.

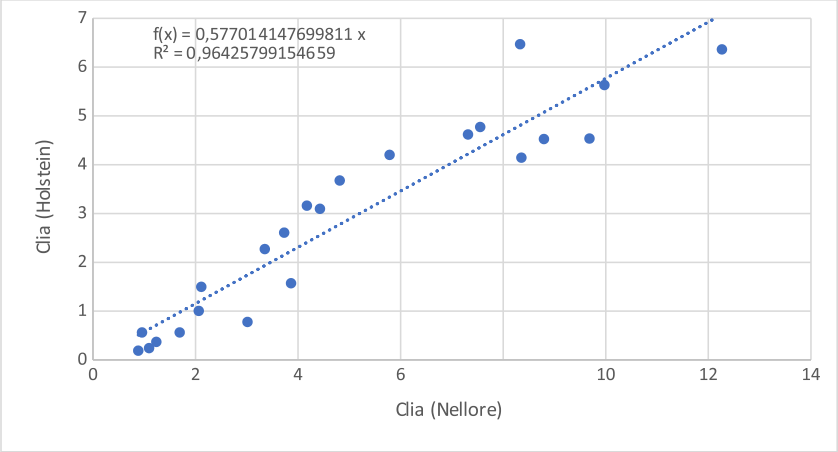

Figure 6 Determination of serum P4 using CLIA during the estrous cycle in Holstein and Nellore cows.

The relationship between the CLIA in dairy and beef cows is CLIA = 0.577 × ELISA.

4. Discussion

In the present study, actions were developed to monitor ovarian follicular dynamics through ultrasound examination after administration of PGF2α for estrus induction. CL was observed during days 3 and 4 days after ovulation in Holstein and Nellore cows, respectively (Table 1), corroborating the findings of Borges et al.[19] After ovulation and according to the evolution of the materialization of the hemorrhagic body, the gradual formation of the CL (early, medium, and late methaestrus phase) occurred, with full formation requiring 3–4 days, compatible with the verified levels of serum P4. The concentration of P4 immediately after ovulation (day 0) remained low and gradually increased as tissue reorganization occurred in the ovulation crater, progressing to complete formation. At the beginning of diestrus, the concentration of P4 gradually increased and remained high for 11–14 days in both methodologies.

We validated the CLIA methodology using ELISA. There was a linear correspondence between the measured values verified by the high R^2 (Table 2, Figures 2, 4 and 6), as demonstrated by Prus et al.[7], who studied crossbred mares during the breeding season. The blood profiles of P4 in the cows agreed with the findings obtained the ultrasonographic examinations, as also verified by Kozicki et al.[6] from their study on Jersey cows. The P4 concentration values from CLIA were lower than those from ELISA on the same day (Figure 1, 3, 5). The linear correspondence between the values was tested using ANOVA and R^2 and harmony was observed in the profile curves of the cows (Holstein and Nellore) formed using both methodologies, corroborating the reports of Prus et al.[7]. The CLIA profiles of dairy cows showed lower amounts of P4 during the estrous cycle than those of beef cows. The relationship between the CLIA in dairy and beef cattle yielded a value of 0.577 x ELISA. This could be explained by dairy cows having a more intense body metabolism than beef cows[20]. The relationship between CLIA and ELISA was significant, making it possible to estimate with high credibility that the concentration of P4 obtained using CLIA was similar to that obtained

using ELISA. The 02 values of p<0.0001, referring to ANOVA (Table 2) and linear regression, indicated that this coefficient could explain the CLIA variation from the ELISA values.

The metestrus phase (increase in P4 on days 1, 2, 3, and 4 post-ovulation) analyzed by both methodologies was similar. Both methodologies also similarly analyzed the diestrus phase, showing an elevation of the P4 profiles for values > 6 (ELISA) and >3 (CLIA) on day 5 of the estrous cycle, consistent with reports by Kozicki et al.[6].

Borges et al.[19] evaluated the follicular dynamics, luteal regression, and P4 concentrations in Gir and Nellore cows. The diameter of the ovulatory follicle observed was 11.0 ± 0.9 mm and the CL was detected for the first time around day 2.6 ± 0.7 after ovulation, results consistent with what was verified in the present study. At the end of the estrous cycle, the reduction in P4 concentration is not followed by a proportional decrease in the area of the CL[21], demonstrating that functional regression (reduction of serum P4) precedes the morphological regression of the CL[22]. P4 concentrations have been observed to peak following stabilization of the area and volume of the CL (day 9)[19], which was verified in our study, as the maximum values of P4 were observed between days 9 and 16 of the estrous cycle.

In cattle, between days 15 and 17 of the cycle, which is the onset of luteolysis, in the absence of a viable embryo, the endometrial secretion of PGF2α significantly increases[23], which explains the low levels of P4 (basal circulating levels (< 1.0 ng/mL) recorded at the end of the estrous cycle in this study. These findings were evidenced on days 20, 21, and 22, days before the next ovulation, corroborating the results of Duchens et al.[24] and Sunderland et al.[25]. The rapid alterations observed in the P4 secretion pattern during luteolysis were not accompanied by significant changes in the CL, while its volume was reduced by half after only 12h[26].

The data verified in the present study confirm the hypothesis that CLIA can be used to determine the concentration of P4 in the blood serum of bovines. It can indicate the presence or absence of CL, constituting an alternative method for P4 determination.

5. Conclusion

It was concluded that CLIA showed a high linear correspondence with the ELISA values, validating the methodology. CLIA can be used as a valuable method for the hormonal determination of bovine serum P4 and can thus aid reproductive biotechnologies (FTAI, ET, FTET, Superovulation, IVF) by determining the serum P4 concentration in the follicular and luteal phases of the estrous cycle in cattle.

Declaration of conflict of interest

The authors declare that there are no conflicts of interest.

Author contributions

Isabela da Silveira Padilha: Data curation - data management and execution activities.

Saulo Henrique Weber e Marcio Saporski Segui: Formal analysis - Application of statistical, mathematical, computational or other formal techniques to analyze or synthesize research data.

Marina de Pauli Thomaz: literature survey activities and updates.

Tacia Gomes Bergstein-Galan: Project management - Responsibility for managing and coordinating the planning and execution of the research activity.

Carlos Alberto Mayora Aita: Resources - Provision of study materials, reagents, materials, laboratory samples.

Luiz Ernandes Kozicki e Fernando Andrade Souza: Visualization - Preparation, creation and/or presentation of published work, specifically data visualization/presentation.

Jose Carlos dos Santos Breda: Preparation, creation and/or presentation of published work by those in the original research group, specifically critical review, commentary or revision - including pre- or post-publication stages.

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