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Validation of an immunoassay system for progesterone dosage and physiological responses of postpartum cows treated with long-acting injectable progesterone

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Dissertation presented to obtain the degree of Master in Science. Area: Animal Science and Pastures

Piracicaba
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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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DEDICATION

To my biggest supporters: mom, dad, sis, and bro. Without you I would never have made it this far.

“*A man ceases to be a beginner in any given science and becomes a master in that science when he has learned that he is going to be a beginner all his life*.”
Robin G. Collingwood

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SUMMARY

RESUMO

Validação de um sistema de imunoensaio para dosagem de progesterona e respostas fisiológicas de vacas no pós-parto tratadas com progesterona injetável de longa ação

Estudos foram desenvolvidos com o objetivo de validar um kit para dosagem de P4 em bovinos e avaliar o perfil de progesterona (P4) circulante e dinâmica ovariana após tratamento com progesterona injetável de longa ação (P4i) em vacas pós-parto. No primeiro estudo, foram realizados testes para validação de um kit comercial baseado em tecnologia quimioluminescente, Immulite 1000 Progesterone (LKPW1), para dosagem de P4 em bovinos. Para tal, curvas padrão foram produzidas a partir de amostras de plasma de garrotes castrados enriquecidas com concentrações conhecidas de P4, e a precisão, exatidão, efeito de anticoagulante e efeito residual foram avaliados. O método se mostrou exato para amostras com concentração de P4 entre 0.3 e 10.0 ng/mL e preciso para amostras entre 0,5 e 20,0 ng/mL. Além disso, os resultados fornecidos pelo kit são fidedignos às concentrações nas amostras, sejam elas soro ou plasma, não sendo necessária a análise de curvas padrão a cada ensaio. No segundo estudo, primíparas Nelore (n = 28) em anestro pós-parto foram aleatoriamente designadas a receber 1 mL de veículo oleoso (Con; n = 14) ou 1 mL de veículo com 150 mg de P4i (P4i; n = 14) no D0. A partir de então, durante 21 d, foram realizadas colheitas de sangue e ultrassonografia ovariana em todos os animais, além de observação da expressão de cio entre D8 e D15. De modo geral, nenhuma vaca expressou cio, permanecendo anovulatórias até o D20. No D1, vacas do grupo P4i tiveram um pico de P4 circulante (3,0 ng/mL), permanecendo acima de 0,5 ng/mL por 7 d, enquanto no grupo Con a P4 circulante se manteve abaixo de 0,2 ng/mL durante todo o estudo. Vacas do grupo P4i apresentaram maior diâmetro do folículo dominante durante o estudo (12,8 ± 0,5 vs. 10,6 ± 0,6 mm), maior intervalo entre ondas (9,4 ± 1,0 vs. 6,5 ± 0,7 d) e mais dias com folículos com capacidade ovulatória nos ovários em comparação ao grupo Con (12,5 ± 0,5 vs. 8,3 ± 1,3 d). Sendo assim, apesar de ineficiente na indução de ciclicidade em vacas pós-parto, o tratamento com P4i promoveu alterações na dinâmica ovariana e perfil circulante de P4 que podem servir de base para novos estudos e estratégias de uso. Além disso, o kit testado apresentou alto potencial para uso na quantificação de P4 circulante em bovinos. Contudo, ainda são necessários outros testes e análises de maior número de amostras para confirmar sua robustez.

Palavras-chave: Progesterona, Anestro, *Bos indicus*, Quimioluminescência, Immulite

ABSTRACT

Validation of an immunoassay system for progesterone dosage and physiological responses of postpartum cows treated with long-acting injectable progesterone

Studies were conducted to validate a kit for measuring P4 in cattle and to evaluate the circulating progesterone (P4) profile and ovarian dynamics after treatment with long-acting injectable progesterone (P4i) in postpartum cows. In the first study, tests were conducted to validate a commercial kit based on chemiluminescent technology, Immulite 1000 Progesterone (LKPW1), to measure P4 in cattle. For this purpose, standard curves were generated from plasma samples of steers enriched with known concentrations of P4, and the precision, accuracy, anticoagulant effect, and residual effect were evaluated. The method proved to be accurate for samples with a P4 concentration between 0.3 and 10.0 ng/mL and precise for samples between 0.5 and 20.0 ng/mL. In addition, the results provided by the equipment are reliable to the concentrations in the samples, whether using serum or plasma, and it is not necessary to analyze standard curves for each assay. In the second study, Nelore primiparous (n = 28) in postpartum anestrus were randomly assigned to receive 1 mL of oily vehicle (Con; n = 14) or 1 mL of oily vehicle with 150 mg of P4i (P4i; n = 14) on D0 . Thereafter, for 21 d, blood samples and ovarian ultrasonography were performed in all animals, in addition to observation of expression of estrus between D8 and D15. In general, no cow expressed estrus, remaining anovulatory until D20. On D1, cows in the P4i group had a peak of circulating P4 (3.0 ng/mL), remaining above 0.5 ng/mL for 7 d, while in the Con group, circulating P4 remained below 0.2 ng/mL throughout the study. Cows in the P4i group had a larger diameter of the dominant follicle during the study (12.8 ± 0.5 vs. 10.6 ± 0.6 mm), a longer interval between waves (9.4 ± 1.0 vs. 6.5 ± 0.7 d) and more days with follicles with ovulatory capacity in the ovaries compared to the Con group (12.5 ± 0.5 vs. 8.3 ± 1.3 d). Thus, despite being inefficient in inducing cyclicity in postpartum cows, treatment with P4i promoted changes in ovarian dynamics and circulating profile of P4 that could serve as a basis for further studies and use strategies. Furthermore, the tested kit showed high potential for use in the quantification of circulating P4 in cattle. However, further tests and analyses of a larger number of samples are needed to confirm its robustness.

Keywords: Progesterone, Anestrus, *Bos indicus*, Chemiluminescence, Immulite

1. INTRODUCTION

The reproductive efficiency in beef and dairy cattle systems is strongly influenced by the service rate and conception rate of dams exposed to breeding. In this sense, one of the main factors that negatively impact reproductive efficiency is postpartum anestrus, a condition in which females remain for an extended period without ovulation [1,2]. This situation is even more harmful in systems that use only the natural mating, since cows in anestrus do not express estrus and, therefore, are not mated, resulting in delayed conception and increased calving interval [3–5].

In this sense, studies have been developed in search of strategies that anticipate the return to cyclicity in the postpartum period [6]. As reviewed by Yavas and Walton [7], one of these strategies is based on the use of intravaginal progesterone (P4) devices, resulting in ovulation and a reduction in the occurrence of short cycles. Those effects are probably related to a reduction in the negative feedback of estradiol (E2) on gonadotropin releasing hormone (GnRH) release, reflecting an increase in the frequency of luteinizing hormone (LH) pulses [8]. Similarly, administration of long-acting injectable progesterone (P4i) has been a strategy for inducing cyclicity in prepubertal beef heifers [9,10]. However, there are no studies evaluating this strategy in postpartum anestrus cows.

Progesterone is one of the main hormones involved in the regulation of the estrous cycle and maintenance of pregnancy in bovine females [11], in addition to being frequently used in association with other hormones or individually, aiming at synchronizing ovulation [12] and inducing cyclicity, as cited above. In this sense, analytical methods capable of quantifying this hormone in cattle with high reliability and low cost are extremely necessary. Currently, the most used method for this purpose is the radioimmunoassay, which is based on the use of radioisotopes in a competitive assay for the detection of the analyte [13]. Several methods have been developed and improved for measuring P4, including immunoassays based on chemiluminescence, with low cost and practicality, such as the Immulite 1000 Progesterone LKPW1 kit. However, there are no reports of tests proving the ability of this kit to adequately quantify for P4 in bovine matrix.

Thus, considering the effects of P4 in the regulation of the hypothalamic-pituitary-gonadal axis as well as the need for a test that is reliable for its quantification in studies related to the understanding of reproductive physiology and response to exogenous P4, this study includes two experiments that aim to validate the Immulite 1000 Progesterone

(LKPW1) kit for measuring P4 in bovine plasma and to evaluate physiological responses of cows in anestrus after treatment with P4i.

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2. VALIDATION OF A CHEMILUMINESCENT IMMUNOASSAY PROTOCOL FOR MEASURING CIRCULATING PROGESTERONE IN BOVINE

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Abstract

Progesterone (P4) is one of the main hormones involved in the regulation of the estrous cycle and maintenance of pregnancy in animals. Its quantification is of significant importance for the elucidation of physiological aspects as well as for the application of biotechnologies and is often performed by radioimmunoassay (RIA), especially in cattle. However, this analytical method has some disadvantages such as the requirement for adequate facilities, risk to the health of the handlers and short half-life of the radioactive isotopes. Therefore, the aim of this study was to evaluate the efficiency and reliability of a competitive immunoassay protocol with chemiluminescent technology (LKPW1), for P4 measurement in bovine plasma. For this purpose, standard curves were constructed, and the precision, accuracy, anticoagulant effect, and linearity of the kit were evaluated, using samples from steers added to known concentrations of P4. The protocol showed accuracy for samples with concentrations between 0.3 and 10.0 ng/mL and good precision (CV < 15%) for standards with 0.5, 1.0, 1.5, 5.0 and 20.0 ng/mL. Furthermore, it was found that the analyte can be quantified both in bovine plasma and serum, without compromising the results. In conclusion, this kit demonstrated a high potential for P4 quantification. However, analysis of a larger number of samples and comparison with other methods can be useful for establishing this immunoassay system as a routine method.

Keywords: Progesterone; Animal reproduction; Immunoassay; Hormone; Immulite.

2.1. Introduction

Progesterone (P4) is one of the main hormones involved in the regulation of the estrous cycle of female cattle. This steroid is mainly produced by the corpus luteum (CL), and it is essential for the establishment and maintenance of pregnancy [1].

Circulating P4 concentrations vary during the estrous cycle, increasing after ovulation due to CL formation and decreasing around the 18th day of the cycle due to luteolysis naturally induced by the release of prostaglandin F2 alpha [2]. In addition, concentrations can be altered by dietary manipulations and hormonal treatments, such as those

used in timed-artificial insemination (TAI) protocols and in vivo embryo production. Therefore, the quantification of circulating P4 can be useful to determine the stage of the estrous cycle, to identify alterations in hormone profile and to elucidate the effects associated with such alterations [3].

Currently, the radioimmunoassay [4,5] is the most reliable method used to measure P4 in bovine females [6–10] because it has high sensitivity, specificity and it is an open system that allows assays adjustments to minimize matrix interference. However, the RIA has some disadvantages, such as the requirement for a specific license and adequate facilities to conduct the tests due to the manipulation of radioactive isotopes. In addition, in Brazilian scenario, the lengthy period for importing kits reduces the time for their use, since the I^{125} has a half-life of just 60 days [11].

In this sense, some studies have reported the use of enzyme-linked immunosorbent assay [ELISA; 12,13], liquid chromatography coupled with tandem mass spectrometry [LC-MS/MS; 14], enzyme immunoassay [EIA; 15] and chemiluminescent immunoassays [CLIA; 16] for P4 detection in plasma or serum of bovine females. The CLIA are based on obtaining light energy through a chemical reaction, replacing the radioisotopes used in RIA by chemiluminescent markers [17]. Thus, one of the main advantages is not using radioactive materials, as well as greater practicality and agility in obtaining results. This method was developed in 1977 by the association of chemiluminescence (CL) to the immunoassay, showing high sensitivity in the detection of bacteria [18].

Currently, several CLIA systems are commercially available, including Immulite 1000®, an automated system used for dosing P4 with the LKPW1 kit, a competitive solid-phase immunoassay with enzyme-labeled chemiluminescent technology, in which antibody-impregnated plastic beads are used as the solid phase, alkaline phosphatase as the reagent and an adamantyl dioxetane phosphate as a luminogenic substrate [19,20]. This system is frequently used for hormonal dosage in humans and dogs [21–23]. However, although there are reports of its use for measuring P4 in cattle [16,24–26], there are still no studies that adequately demonstrate its precision, accuracy and sensitivity in bovine, parameters necessary for validation of an analytical method.

Thus, considering the low cost per sample and the practicality provided by this commercial kit (Immulite 1000 Progesterone, LKPW1), the aim of this study was to verify its effectiveness as an analytical method to quantify plasma P4 in cattle. In this sense, the proposed hypothesis was that this immunoassay protocol meets the requirements for quantifying plasma P4 in cattle, providing reliable results.

2.2. Material and Methods

The study was carried out between March and October 2021 in the Animal Reproduction Laboratory of the Department of Animal Science of the Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo, Piracicaba, SP, Brazil.

2.2.1. Pre-analytical step

Blood samples were collected from steers, by puncture of the jugular vein, into 9 mL evacuated tubes containing sodium heparin to obtain plasma and with coagulation activator to obtain serum. The tubes were then centrifuged at 1800 x *g* for 15 minutes at 4 °C and stored as pools of plasma and serum at -20°C in in conical bottom tubes (15 mL). The plasma from steers was used as a dilution matrix because, when subjected to chemiluminescence analysis, it presented concentrations below the detection limit of the equipment (< 0.2 ng/mL) not interfering with the results.

2.2.2. Chemiluminescence assay

Assays were performed using the Immulite 1000 Progesterone (LKPW1, Diagnostic Product Corporation, Caernarfon, UK), a competitive solid-phase immunoassay with enzyme-labeled chemiluminescent technology. The system automates nearly the entire assay process. However, before assay, the samples were thawed at room temperature, homogenized in a vortex, then 200 µL were pipetted into the sample cups, which in turn were added to the equipment loading platform, followed by a Test Unit. From then on, the entire process is automated, including sample and reagent pipetting in the test unit, incubation, washing the bead, adding the chemiluminescent substrate, reaction, and signal reading. Results were extracted as concentration in nanograms per mL (ng/mL) and CPS (count per second).

Before starting the tests and every 15 d thereafter, the equipment underwent calibrations according to the manufacturer's instructions, using the high and low adjusters included in the commercial kit, and low, medium, and high controls (0.566 - 1.38; 5.46 - 12.3; 14.1 - 32.3 ng/mL, respectively) acquired in parallel (Lyphochek® Immunoassay plus control, Bio-Rad Laboratories, Irvine, CA, USA). The adjusters are produced by the manufacturer from human serum enriched with P4 and are used to construct the standard curve of the system, using a four parameter logistic (4PL) equation to fit the curve. Instrument calibration

provides a working range of 0.2 to 40.0 ng/mL for P4 measurements. In addition, all routine maintenance procedures assigned by the operator's manual were performed, including water testing, probe cleaning, daily and monthly equipment cleaning [20].

2.2.3. Tests for method validation

The tests were performed in accordance with the standards for validation of bioanalytical methods established by resolution 899 of 2003 [27], resolution 27 of 2012 [28] and the guidelines on validation of analytical methods of document DOQCGCRE-008 of 2016 [29].

Standard curve - Considering that the standard curve of the Immulite 1000 system is built based on P4 standards in human serum and aiming to evaluate the protocol's ability to adequately determine P4 concentrations, standard curves were constructed and subjected to quantification. Initially, the dilution of pure lyophilized P4 (≥ 99%; P0130; Sigma-Aldrich, St. Louis, MO, USA) in methanol was performed, resulting in a solution at 10.000 ng/mL. Sixteen microliters of this solution were added to 4 mL of plasma from steers resulting in the first standard of the curve with 40.0 ng/mL, from which serial dilutions were made to obtain the other standards (20.0; 10.0; 5.0; 2.5; 1.25; 0.63; 0.31 and 0.15 ng/mL). Furthermore, samples without the addition of P4 were used as a blank sample, at a concentration of 0.0 ng/mL.

This concentration range (0.15 to 40.0 ng/mL) was chosen as it covers the physiological concentrations of P4 in the distinct phases of the estrous cycle in *Bos indicus* and *Bos taurus* females [30], as well as concentrations promoted by treatments with progestins [10] or formation of several CL after superovulation protocols.

The expected concentrations of the standards and the mean CPS were plotted on a graph and, considering that the behavior of the curve is non-linear and that the device itself uses 4PL regression to calculate the standard curve and then determine the concentration in ng/mL based on the CPS, the same method was used to calculate P4 concentrations based on the curve produced in bovine plasma. Calibration curve data were plotted on a graph (X axis = standard concentrations in Log10; Y axis = CPS) and 4PL was applied. The regression equation is:

$$Y = \frac{Bottom + (Top - Bottom)}{1 + 10((X\text{-}LogIC50)*Hill\ slope)}$$

Using the described curve, the concentrations of standards from a commercial RIA kit (ImmuChem Coated Tube P4125 RIA Kit, MP Biomedicals) with known P4 concentrations (0.15; 0.5; 1.0; 5.0; 20.0 and 80.0 ng/mL), were calculated. Then, expected concentrations (determined by the supplier), experimental concentrations (determined by Immulite), and calculated concentrations were compared.

Accuracy - As there is no availability of certified reference material (MCR) in the laboratory, the accuracy test of the method was performed by recovery tests with standards produced in the laboratory. Similar to the construction of the standard curve, P4 diluted in methanol was added to plasma from steers resulting in samples with 40.0; 20.0; 10.0; 5.0; 2.5; 1.25; 0.62 and 0.31 ng/mL. Such dilution was done twice independently, so that at each concentration level two samples were produced. Each of these samples was analyzed in duplicate in different assays.

In addition, the standards from the commercial RIA kit (ImmuChem Coated Tube P4 125 RIA Kit, MP Biomedicals, Costa Mesa, CA) were subjected in duplicate to P4 quantification by chemiluminescence. These standards are part of the RIA Kit and are based on human serum added with P4, in the following concentrations: 0.0; 0.15; 0.5; 1.0; 5.0; 20.0 and 80.0 ng/mL.

Precision - From the pool of plasma from steers and working solution of P4 in methanol, samples were produced with 5.0 and 20.0 ng/mL concentrations, characterizing the intermediate and high concentrations of the range of interest. These were submitted, in replicates (n = 10/sample), to three independent assays with an interval of 7 d, to evaluate the repeatability (intra assay) and intermediate precision (inter assay), being expressed by the coefficients of variation (CV). In the period between assays, samples were kept in -20ºC. Subsequently, three other samples were produced with the following concentrations: 0.5, 1.0, and 1.5 ng/mL and then were subjected in replicate (n = 10/sample) to one assay to obtain the intra-assay CV. Considering that Immulite is a closed system, and the same lot of the commercial kit was used, the variation between assays refers only to the time and different analyst responsible for the homogenization and pipetting of the samples.

Anticoagulant effect – To detect the possible effect of anticoagulant (heparin) on P4 dosage results, three independent dilutions of P4 were performed in plasma and serum from steers to reach the following concentration levels: low (0.6 ng/mL), intermediary (2.5 ng/mL), and high (20.0 ng/mL) according to the range of interest. These samples were analyzed in triplicate, totaling 54 sample units (three dilutions x two matrices x three levels x three replicates per sample).

Carryover - Considering that the residual effect corresponds to changes in the result of samples caused by contamination with a previous sample and that the equipment used in this study works as an automated carousel system, the evaluation of this parameter was carried out through the consecutive analysis of three blank samples (plasma without addition of P4), followed by three samples with P4 concentration at the superior limit of quantification (SLQ; 40 ng/mL) and finally, two other blank samples. The results were visually evaluated.

2.2.4. Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC) and GraphPad Prism (Version 9.2.0 for Windows, GraphPad Software, San Diego, California US).

To assess the accuracy of the analytical method, the relative standard error (RSE), recovery rate and average difference in ng/mL were calculated. Then Pearson correlation and linear regression were performed. Precision was determined by evaluating inter- and intra-assay CVs.

Continuous data (P4 concentrations and differences between expected and CLIA P4) were tested for normality of studentized residuals using the UNIVARIATE procedure of SAS according to the Shapiro-Wilk test. The homogeneity of variances was evaluated with the Levene test using the Hovtest and Welsh methods. Analysis of were performed using the GLIMMIX procedure fitting a Gaussian distribution. To evaluate the effect of anticoagulant, the model included P4 level, anticoagulant and their interaction as fixed effect, and replicate as random effect. Tukey honest significant difference post hoc test was performed to determine differences. Values are presented as means ± SEM. Significant differences were declared when $P < 0.05$.

Correlation coefficient between expected values of P4 and those obtained using CLIA, and serum and plasma P4, were calculated using the PROC CORR procedure of SAS, while the intercept and slope of the equations were obtained using the option solution in the GLIMMIX procedure.

2.3. Results and discussion

The CLIA kit used in this study is validated and widely used in the quantification of hormones in humans and, due to its practicality and low cost of analysis per sample, its use for hormone measurement in cattle has been widespread. However, for a method or protocol to be considered appropriate for the intended purpose and for the results to be reliable, it is necessary to evaluate it regarding several parameters [29].

Standard curve – As shown in Fig. 1A, the method presented the CPS variation of the standard curve with non-linear behavior and, therefore, 4PL regression was applied (Fig. 1B).

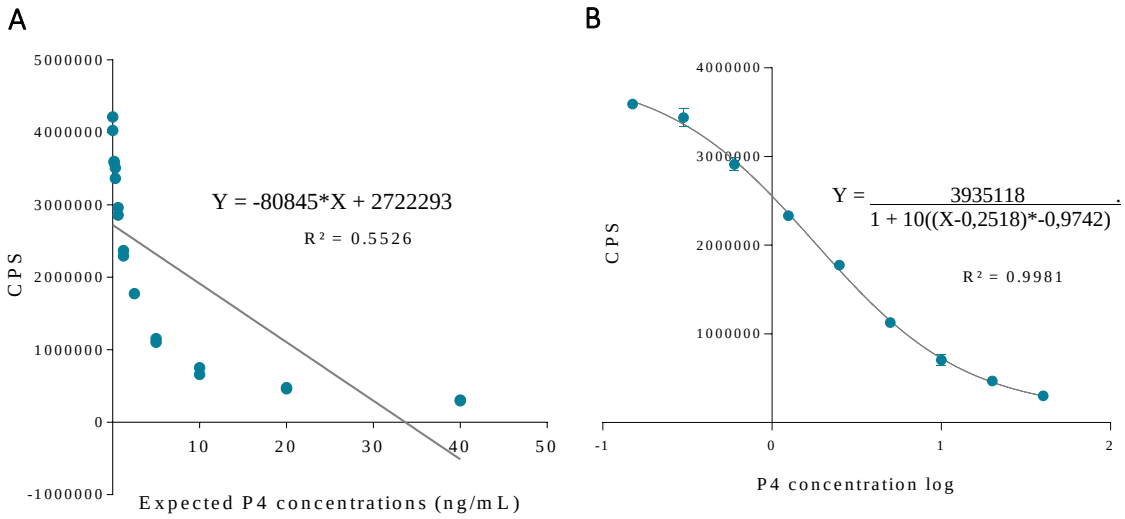

Figure 1. Relationship between P4 concentrations and the CPS response produced by each point on the standard curve after (A) linear regression; and (B) four-parameter logistic regression.

Using the presented curve, the concentrations of the standards from the RIA kit were calculated and compared to the concentrations determined by the CLIA. At the 0.15ng/mL concentration level, the RSE was high, as the CLIA kit was not able to quantify the analyte in the sample, possibly due to the loss of sensitivity. At concentrations 0.5; 1.0; 5.0 and 20.0 the RSE remained within the limits established as acceptable (± 15%), indicating slight variation between the calculated concentrations and those determined by the Immulite 1000 Progesterone (Table 1). As expected, the standard with 80 ng/mL of P4 could not be quantified by the kit and, when calculated based on the curve described above, it had a very high concentration. However, this value should not be considered, as it exceeds the limit determined by the supplier, requiring dilutions and application of a correction factor for an

adequate quantification. In addition, there was a high correlation between calculated and kit-determined concentrations (r = 0.99), as well as calculated and expected (determined by the supplier) concentrations (r = 0,99), reaffirming the kit's ability to adequately quantify analyte concentrations.

According to Hubert et al. [31], the linearity criterion must be applied to the results, evaluating the relationship between the experimental concentration and the expected concentration. In this case, the Immulite 1000 system provides a response in ng/mL that behaves linearly with respect to expected concentrations, as shown in Fig. 2.

Table 1. Relative standard error (RSE) for calculated (based on the standard curve) and experimental (determined by the kit) concentrations of P4 in RIA commercial kit standards.

Concentrations (ng/mL)			**Relative standard error (%)**
Expected	Experimental	Calculated	Calculated vs. Experimental
0.15 (n=2)	0.32 ± 0.07	0.18 ± 0.09	-46.11 ± 15.92
0.50 (n=2)	0.61 ± 0.00	0.56 ± 0.00	-8.68 ± 0.13
1.00 (n=2)	1.06 ± 0.01	1.10 ± 0.01	4.55 ± 0.35
5.00 (n=2)	5.40 ± 0.00	6.02 ± 0.00	11.40 ± 0.09
20.00 (n=2)	21.55 ± 1.45	24.66 ± 1.81	14.40 ± 0.72
80.00 (n=2)	> 40.0*	197.20 ± 4.73	.

*Above the kit's upper detection limit.

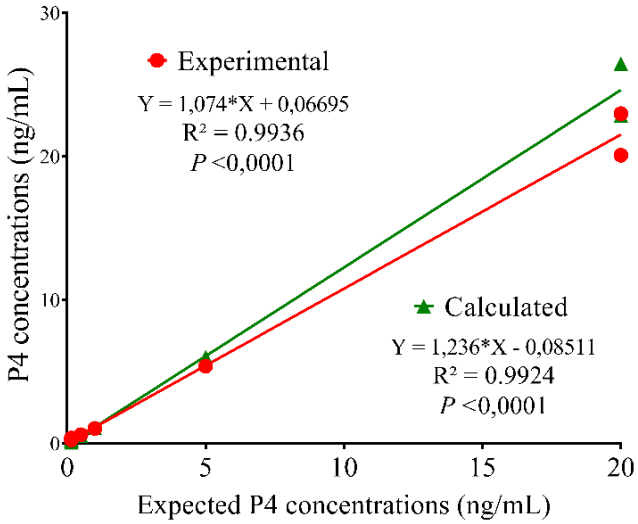

Figure 2. Relationship between calculated (based on the standard curve), experimental (determined by the CLIA kit) and expected (determined by suplier) concentrations of P4 in the standards of a commercial RIA kit (n = 10). Samples at 80.0 ng/mL level were not included in this analysis since they are above the upper limits of quantification of the CLIA kit.

Precision – Results are presented in Table 2. The inter- and intra-assay CV were less than 15% for the 5.0 and 20.0 ng/mL levels, indicating good reproducibility and repeatability [28,32]. Also, in the samples assayed just once, the intra-assay CV was lower than 15%, proving the precision of the method in the range of interest for physiology and biotechnology studies in bovine females.

Table 2. Inter- and intra-assay CV for plasma samples at three levels of P4 concentration submitted to three independent assays and intra-assay CV for three other samples submitted to only one assay.

	Mean ± SEM	**Inter-assay CV (%)**	**Intra-assay CV (%)**
0.5 (n=10)	0.61 ± 0.62	.	14.3
1.0 (n=10)	1.11 ± 0.01	.	5.0
1.5 (n=10)	1.78 ± 0.04	.	6.5
5.0 (n = 30)	4.53 ± 0.05	6.5	5.0 ± 0.9
20.0 (n = 30)	15.75 ± 0.32	11.2	6.6 ± 2.4

Accuracy – Data from standards with known P4 concentrations are presented in Table 3. The overall mean difference between expected and experimental P4 concentrations was high and negative (-2.09 ± 0.34 ng/mL), this indicates that CLIA kit is quantifying the analyte in the sample at concentrations below the actual value. However, when the average difference is observed in each of the concentration levels, it is clear that the higher concentration levels (20.0 and 40.0 ng/mL) are responsible for raising the average difference, since the difference in ng/mL was low and similar for the standards between levels of 0.3 and 10.0 ng/mL. Figure 3 shows that the higher the expected concentration, the greater the observed difference.

At the lowest concentration level (0.3 ng/mL), CLIA had higher than the expected results, represented by the recovery rate and RSE above the recommended levels. However, the recovery rate was not significantly different when compared with 0.6 and 1.25 levels of concentration, and the difference in ng/mL was similar to that obtained for samples between 0.6 and 10.0 ng/mL.

Table 3. Mean P4, relative standard error (RSE), recovery rate and difference between expected (determined by manufacturer) and experimental (determined by the CLIA kit) P4 concentrations.

Expected P4, ng/mL (n)	Experimental P4, ng/mL	Relative standard error (%)	Difference, ng/mL	Recovery rate, %
Overall 10.15 ± 1,29 (102)	8.06 ± 1.00	-10.07 ± 1.98	-2.09 ± 0.34	89.9 ± 2.0
0.30 (11)	0.35 ± 0.01	15.9 ± 4.61	0.05 ± 0.01[a]	115.9 ± 4.6[a]
0.60 (13)	0.61 ± 0.05	2.1 ± 7.80	0.01 ± 0.05[a]	102.1 ± 7.8[ab]
1.25 (13)	1.19 ± 0.07	-4.6 ± 5.61	-0.06 ± 0.07[a]	95.4 ± 5.6[abc]
2.50 (13)	2.14 ± 0.09	-14.3 ± 3.77	-0.36 ± 0.09[a]	85.7 ± 3.8[bc]
5.0 (13)	4.33 ± 0.16	-13.3 ± 3.28	-0.67 ± 0.16[a]	86.7 ± 3.3[bc]
10.0 (13)	8.26 ± 0.36	-17.4 ± 3.59	-1.74 ± 0.36[a]	82.6 ± 3.6[c]
20.0 (13)	15.60 ± 0.70	-22.0 ± 3.51	-4.40 ± 0.70[b]	78.0 ± 3.5[c]
40.0 (13)	30.78 ± 1.01	-23.0 ± 2.53	-9.2 ± 1.01[c]	77.0 ± 2.5[c]

[a, b, c] Values within the same column differ ($P < 0.05$).

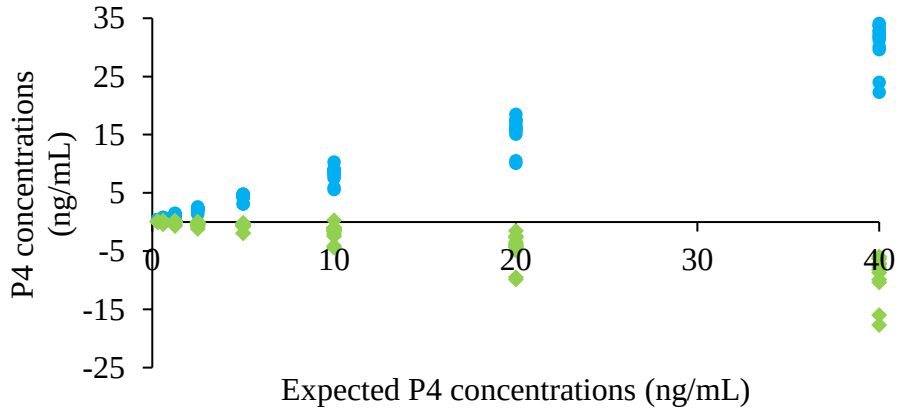

Figure 3. Mean experimental concentrations of P4 (●) and mean difference (♦) between expected and experimental concentrations for plasma samples with known concentrations of P4 subjected to CLIA measurement.

At higher levels (1.25 to 40.0 ng/mL), the RSE was negative, and the recovery rate was less than 100%, indicating that CLIA determined concentrations below expectations. However, the test behavior was similar to that expected and the correlation between concentrations was high (Fig. 4).

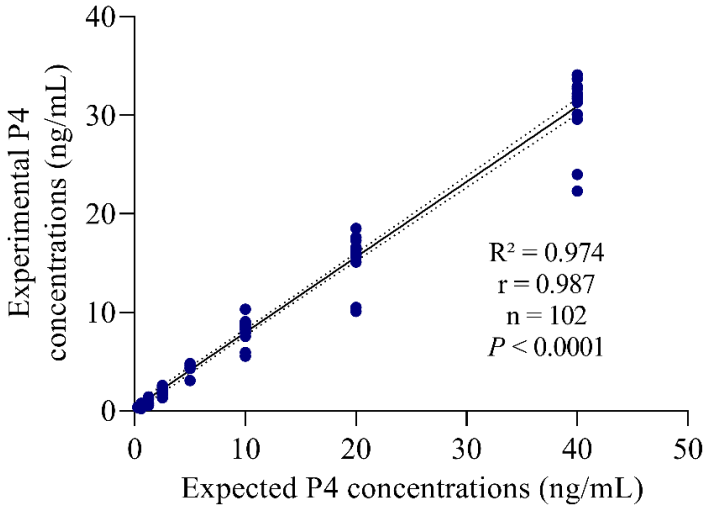

Figure 4. Relationship between concentrations determined by chemiluminescence and expected P4 concentration for plasma samples with known concentrations of P4.

According to ANVISA [28], RSE values between ± 15% of the nominal value are admitted, so CLIA seems to be an accurate method to measure samples with P4 concentration between 0.6 and 5.0 ng/mL. On the other hand, considering the analyte concentration in the sample according to the AOAC [32], an average recovery rate of 80 to 110% is acceptable, so that the method is considered accurate also for samples with 10 ng/mL of P4 concentration (82.6 ± 3.6% recovery), similar to the results obtained by Fernandes [14], who reported an accuracy of 90 to 103.2% for samples with concentrations between 0.25 and 8.0 ng/mL when using LC-MS/MS to quantify bovine P4.

Considering the working range is the interval between the lowest and the highest analyte concentration in which good precision and accuracy are observed, in this study the working range determined for the CLIA kit was 0.5 to 10 ng/mL. At the 20.0 ng/mL level, the protocol showed good precision (CV < 15%) but the accuracy was unsatisfactory. Samples at 0.3 ng/mL level had acceptable accuracy but the precision at this level was not tested. Within the established working range, the mean difference between expected and experimental concentration was -0.56 ± 0.11 ng/mL and, specifically for samples at the 0.3 level, the mean difference was only 0.05 ± 0.01 ng/ml. Such difference will probably not have clinical significance.

The P4 concentrations measured by CLIA, and the concentrations determined by the RIA KIT supplier had a high correlation (r = 0.99; Fig. 2). At the 0.15 and 0.5 ng/mL levels, the RSE and the recovery rate were quite high (Table 4), however, this is probably an effect of

the small number of replicates (n=2). In the range between 1.0 and 20.0 ng/mL CLIA presented acceptable RSE and recovery, confirming the accuracy of the method.

Table 4. Mean P4, relative standard error, and recovery for concentrations determined by chemiluminescence and expected P4 concentration in the standards of the standard curve from a commercial RIA kit.

Expected concentrations (ng/mL)	Experimental mean concentration (ng/mL)	Relative standard error (%)	Recovery rate (%)
0.15 (n = 2)	0.32 ± 0.07	116.0 ± 43.3	216.0 ± 43.3
0.50 (n = 2)	0.61 ± 0.00	22.9 ± 0.3	122.9 ± 0.3
1.00 (n = 2)	1.06 ± 0.01	5.5 ± 0.5	105.5 ± 0.5
5.00 (n = 2)	5.40 ± 0.00	8.0 ± 0.00	108.0 ± 0.0
20.00 (n = 2)	21.55 ± 1.45	7.75 ± 7.25	107.8 ± 7.3
80.00 (n = 2)*	> 40	.	.

* Concentration above kit quantification limit

Anticoagulant Effect – Progesterone concentrations did not differ between the two matrices (Table 5) and there was no interaction between P4 level and anticoagulant effect (*P* = 0.97). Furthermore, the difference in ng/mL (0.06 ± 0.39 ng/mL; *P* = 0.97) and the recovery rate was not significant (106.8 ± 4.42 %; *P* = 0.73). The RSE between P4 concentrations determined in serum and plasma are shown in Fig. 5A. The concentrations determined in plasma and serum had a high correlation (r = 0.96; *P* < 0.0001; Fig. 5B), however, when the relationship between plasma and serum P4 was assessed within each concentration level, the correlation between both was reduced (r = 0.10; 0.52 and 0.51, for low, intermediate, and high P4 levels, respectively).

Table 5. Mean concentration and P4 level difference between serum and plasma P4 quantified by chemiluminescence.

P4 level	Plasma (n=27)	Serum (n=27)	*P* value	Difference, ng/mL	Recovery rate, %
0.6 ng/mL	0.58±0.05	0.53±0.02	0.94	0.05±0.06	110.2±11.1
2.5 ng/mL	2.41±0.02	2.23±0.04	0.76	0.17±0.05	108.1±2.5
20.0 ng/mL	16.48±0.68	16.51±0.72	0.95	-0.03±1.22	102.0±7.5

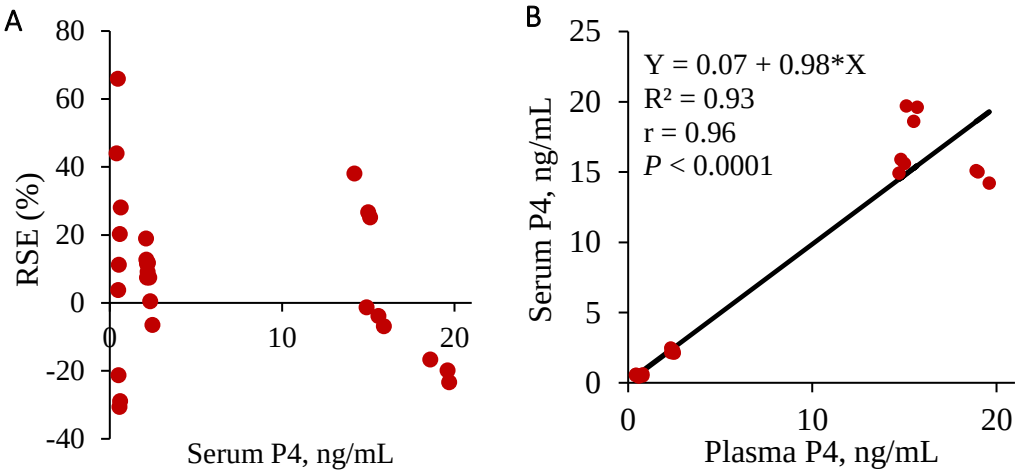

Figure 5. Analysis of anticoagulant effect in plasma and serum samples submitted to P4 quantification by chemiluminescence. A) Relative standard error between the concentrations determined in plasma and serum; and B) Relationship between P4 concentrations quantified in both matrices.

According to the information from the supplier, the use of EDTA as anticoagulant may interfere with the results [33] due to its ability to inhibit alkaline phosphatase (AP) activity through the chelation of zinc ions present in its chemical structure [34]. In this sense, the reduction in AP activity results in quantifications higher than the actual concentration of the hormone in the sample, as it is a competitive assay that promotes light emission in an amount inversely proportional to the concentration of the analyte. On the other hand, no effect of using sodium heparin as anticoagulant for P4 dosage in human samples using Immulite 1000 Progesterone was reported. Despite that, comparisons between plasma and serum samples were conducted, suggesting that, as in humans, the use of sodium heparin as an anticoagulant does not interfere in the quantification of P4 in cattle. However, it is important to emphasize that the samples used in this study were obtained from steers, which may have masked some possible effect of this anticoagulant on the concentration of P4, since the analyte was added after blood processing.

Carryover - There was no significant difference (P = 0.36) between the CPS of blank samples dosed before or after running assay with samples containing a concentration of 40 ng/mL. Also, the results provided were compared and there was no evidence of interference caused by previously dosed samples, since the kit did not detect the analyte in the blank samples (<0.2 ng/mL).

The Immulite 1000 Progesterone kit uses an enzyme-amplified reaction that allows the light produced in the chemiluminescent reaction to be prolonged and the amount of light detected is inversely proportional to the concentration of the analyte in the sample, as it is a

competitive assay with high sensitivity [20]. This CLIA kit was previously compared with other analytical methodologies for measurement of bovine P4, such as ELISA [3] and RIA [26], with a high correlation between CLIA and the aforementioned methods (r = 0.95 in both comparisons). However, correlation between results does not always indicate that the assay is reliable, and the results are accurate and/or precise. Therefore, further testing and detailed analysis of the data obtained is necessary.

During the estrous cycle of bovine females, the physiological concentrations of P4 vary and generally differ between *Bos indicus* and *Bos taurus* cattle [30] and can reach 6 to 10 ng/mL in dairy females [2]. Also, in this sense, administration of long-acting injectable P4 or insertion of intravaginal P4 devices are strategies used to increase circulating concentrations of P4 [10]. The commercial protocol presented in this study demonstrated good accuracy and precision at levels that include P4 concentrations in the situations mentioned above, proving useful for studies aimed to monitor the hormonal profile during the estrous cycle and/or after hormonal treatments.

In conclusion, the Immulite 1000 Progesterone (LKPW1) kit demonstrated high potential for quantifying P4 in samples between 0.5 and 10.0 ng/mL, coinciding with the range of interest. However, the analysis of a larger number of samples under different circumstances and the comparison of this CLIA protocol with RIA, especially in samples below 1 ng/mL of P4, will certainly be of immense value to establish it as a routine method, and this is a of the next steps to be taken.

Acknowledgments

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3. PROGESTERONE PROFILE AND FOLLICULAR DYNAMICS OF ACYCLIC PRIMIPAROUS NELORE COWS TREATED WITH LONG-ACTING INJECTABLE PROGESTERONE

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Abstract

The study aimed to evaluate the ovarian dynamics and circulating progesterone (P4) of postpartum anestrus beef cows after treatment with long-acting injectable progesterone (P4i) and to verify the efficacy of this treatment as a cyclicity inducing agent in this category. Postpartum primiparous Nelore cows (n = 28) were randomly assigned into one of two groups: Con = cows (n = 14) received 1 mL of oily vehicle im on D0, and P4i = cows (n = 14) received 1 mL of oily vehicle with 150 mg of P4i im on D0, at 36.0 ± 1.4 d postpartum. Daily, from D0 until D20 after treatment, all cows were submitted to blood collection and ovarian ultrasonography to evaluate circulating P4 profile and follicular dynamics, respectively. Also, cows received a tail-head adhesive patch as an estrus detection aid and were observed in the field twice a day for 1 hour between D8 and D15 to detect for standing estrus. Cows in both groups were not detected in estrus and remained anovulatory until D20. There was a peak of circulating P4 in cows treated with P4i at D1 (3.0 ± 0.5 ng/mL), remaining above 0.5 ng/mL for 7 d, while in the Con group circulating P4 remained below 0.2 ng/mL. The mean diameter of the dominant follicle (DF) during the study was greater for cows in the P4i group than in the Con group (12.8 ± 0.5 vs. 10.6 ± 0.6 mm), but no differences were observed in the follicular growth rate of the first and second wave. Cows in the P4i group had a longer inter-wave interval (9.4 ± 1.0 vs. 6.5 ± 0.7 d) and more days with follicles with ovulatory capacity in the ovaries in comparison with Con group (12.5 ± 0.5 vs. 8.3 ± 1.3 d). In conclusion, although 150 mg of P4i does not induce cyclicity in postpartum Nelore cows, this treatment promoted changes in daily plasma P4 profile and ovarian dynamics that open up possibilities for the strategic use of this pharmaceutical.

Keywords: *Bos indicus*; Anestrus; Follicle, Cattle.

3.1. Introduction

Postpartum anestrus is the period between calving and the first ovulation, characterized in beef cows by the presence of several waves of follicular growth without ovulation [1]. This pattern is due to the negative feedback of estradiol (E2) on the hypothalamus on the release of gonadotropin releasing hormone (GnRH) and, consequently, on luteinizing hormone (LH) pulse frequency [2]. The main factors linked to the long duration of postpartum anestrus are lactation/presence of the calf and undernutrition and this condition is even more striking in primiparous, as they are still under development [3–6].

When it comes to Nelore females raised on pasture, it is known that even with adequate body condition (BCS ≥ 3.0) the percentage of cyclicity in early postpartum is very low. Alves et al. [7] reported only 7.6% (43/565) of primiparous and 23.1% (311/1349) of multiparous cows with corpus luteum (CL) at 50 d postpartum. This study also indicates that part of the females that do not become pregnant return to anestrus, since at the time of pregnancy diagnosis 30 days after a timed artificial insemination (TAI) protocol the percentage of non-pregnant cows with CL was still low (23.6% [86/364] and 49.0 % [266/543] for primiparous and multiparous, respectively [7]. In this sense, *Bos indicus* cows without hormonal treatment and submitted to natural service have reduced reproductive efficiency, as most cows do not express estrus and have a longer interval between calving and conception [8,9], conceiving only at the end of the breeding season or not conceiving at all, mainly due to postpartum anestrus. In Brazil, this is one of the main factors involved in low reproductive efficiency, since about 80% of beef cattle are exclusively mated by natural breeding [10].

As previously reviewed [11,12], several strategies to induce cyclicity in anestrus beef and dairy cows have been studied, such as, short exposure to progesterone (P4) with or without another hormonal treatment. Intravaginal P4 devices are able to induce cyclicity and reduce the occurrence of short cycles in *Bos taurus* beef cows [13], and currently have been used as a method for induction of cyclicity in prepubertal heifers [14–16], resulting in a high percentage of females with CL 12 d after removal of an intravaginal P4 releasing device (IVD). Furthermore, a recent study [17] showed equivalent results using long-acting injectable P4 (P4i; 82.6% [228/276] vs. 80.8 % [227/281] of heifers with CL, after treatment with P4i or with IVD, respectively).

It is known that P4 is one of the main hormones responsible for regulating the estrous cycle, affecting LH pulse-frequency. When circulating P4 concentrations are high, LH pulse-

frequency is reduced due to the negative feedback in the hypothalamic GnRH release, whereas, when circulating P4 is low, there is an increase in LH pulse-frequency and increase in circulating E2 [18,19]. According to Caraty and Skinner [20], P4 is able to increase the responsiveness of the basal middle hypothalamus to E2, which is the main site of action for regulating the expression of estrus and LH release in sheep [21]. Therefore, prior exposure to P4 may anticipate the first postpartum ovulation, increasing LH pulsatility and circulating E2, which in turn, will induce the occurrence of a preovulatory GnRH-induced LH peak. Another effect of P4 is the reduction in the incidence of silent estrus and short cycles, often observed associated with the first postpartum ovulation [13,22].

The use of hormonal protocols prior to exposure to bulls allows for a reduction in the interval between calving and conception [8], with a greater number of pregnant cows at the beginning of the breeding season. In this sense, administration of P4i in Nelore cows in postpartum anestrus could be a strategy for inducing cyclicity in those animals. By administering 150 mg of P4i to Nelore cows 10 d before the onset of a TAI protocol, Simões et al. [23] obtained an increase on the diameter of the dominant follicle and on pregnancy per AI (P/AI), so that cows pretreated with P4i were 1.68 times more likely to become pregnant after TAI.

However, there are still no studies evaluating the efficacy of this treatment with P4i as inducer of cyclicity in Nelore cows in postpartum anestrus. Therefore, the objectives of this study were: 1) To evaluate ovarian dynamics, plasma P4 profile and estrus expression in acyclic Nelore (*Bos indicus*) primiparous cows treated or not with P4i; 2) To test the efficacy of P4i as a cyclicity inducing agent in lactating Nelore cows in early postpartum.

The two main hypotheses were that: 1) Nelore cows in postpartum anestrus treated with 150 mg of P4i would develop larger follicles and have longer inter-wave intervals compared with untreated cows, also, P4i cows would present estrus and ovulation within a 20-d period after treatment; 2) Treatment with P4i would promote an increase in plasma concentration of P4, remaining above 1 ng/mL for, at least, 10 d.

3.2. Material and Methods

3.2.1. Location

The Experiment was approved by the Animal Research Ethics Committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo (Protocol

6176030920) and was conducted at the Department of Animal Reproduction of the University of São Paulo, Pirassununga, SP, Brazil.

3.2.2. Cows and management

Twenty-nine primiparous Nelore (*Bos indicus*) cows, at 36.0 ± 1.4 d postpartum, weighing 416.9 ± 8.3 kg, and with body condition score of 2.8 ± 0.1 (BCS; on a scale of 1 to 5 [24]) were used. Cows were maintained on a *Brachiaria decumbens* pasture, supplemented with mineral salt, corn silage and a corn plus soybean-based concentrate. Cows had ad libitum access to water during the experimental period.

3.2.3. Experimental design

The cows were evaluated for CL presence at two moments prior to experiment initiation with a 7-d interval (D-7 and D0). Cows without CL were classified as anovulatory (28/29) and were randomly distributed into one of two experimental groups: Con = cows (n = 14) received 1 mL of oily vehicle im on D0, and P4i = cows (n = 14) received 1 mL of oily vehicle with 150 mg of long-acting P4i (Sincrogest injetável, Ourofino Saúde Animal, Cravinhos, SP, Brazil) im on D0 (Fig. 1).

On D0 cows in both groups received a tail-head adhesive patch as an estrus detection aid (BOViFLAG; Bovitime Animal Products LTD, Stellenbosch, South Africa). These patches were checked daily, and the cow was considered in estrus when the paint of the device had been removed. Additionally, between Days 8 and 15 after treatment, cows were observed in the field twice a day (7 AM to 8 AM and 5 PM to 6 PM) to check for standing estrus.

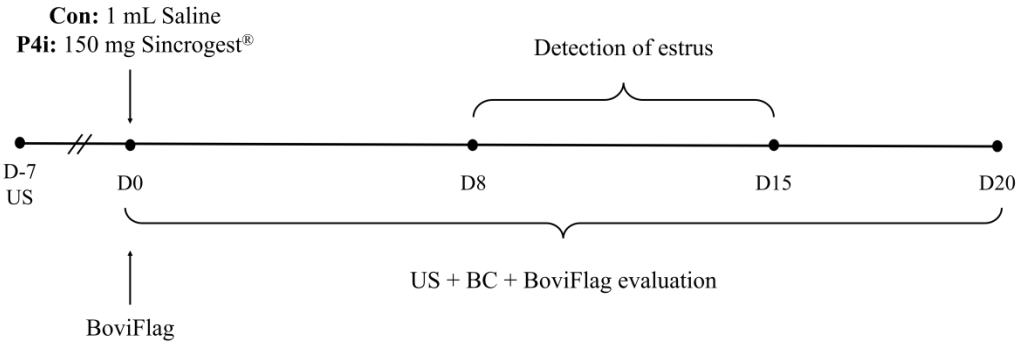

Figure 1. Experimental design. US = ovarian ultrasound evaluations; BC = blood collection; D-7 = US evaluation for CL presence; Day 0 = group distribution on a random day of the estrous cycle and administration of P4i (long-acting P4i [n = 14]) or saline (n = 14); Days 8 to 15 = field observations for signs of estrus.

3.2.4. Ultrasound evaluations

Transrectal ultrasound examinations of the ovaries in B-mode with a 5-10 MHz linear transducer (MyLab Delta, Esaote Healthcare, Genova, Italy) were performed by a single operator on D-7 and 0 in order to evaluate the presence or absence of CL and daily from D0 to D20 after treatment in order to monitor follicular and luteal dynamics. All follicles that had a diameter ≥ 5 mm were measured and recorded. Besides, videos were saved at each exam. The diameter of the follicles was calculated by the mean of the maximum length and width using the caliper function. Thirty-nine days (D39) after treatment cows were submitted to another US evaluation for CL presence.

Follicular wave emergence was defined retrospectively by an evaluation of the dominant follicle (DF) to the time when it was about 4 mm. Turn-over was considered when the DF stopped growing (i.e., reached a plateau) or decreased in size and a new follicular wave emerged. The follicular growth rate (GR) of the future dominant follicle was calculated as follows:

$$GR = \frac{\textit{final follicular diameter - initial follicular diameter}}{\textit{growth days}}$$

Retrospectively, the presence of follicles with ovulatory capacity was evaluated, considering only those larger than 8 mm [25] during their growth period until the emergence of a new wave. So, follicles that had already reached the plateau or were in atresia were not counted. Follicular growth patterns for all cows were evaluated individually in order to identify the most frequent pattern after each treatment.

3.2.5. Blood collection and hormonal assays

Blood samples were collected on D0 just before the treatments started and daily until D20 post treatment. Samples were collected by puncture of the jugular vein into 9 mL heparinized evacuated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and instantly placed on ice. Tubes were centrifuged at 1800 x *g* for 15 min at 4 °C and the plasma was stored at –20 °C for subsequent measurements.

Plasma P4 concentrations were determined using a competitive solid-phase immunoassay with enzyme-labeled chemiluminescent technology (Immulite 1000 Progesterone, LKPW1, Diagnostic Product Corporation, Caernarfon, UK) which has a sensitivity of 0.2 ng/mL. The intra assay coefficients of variation were 1.3% and 1.7% for the low and high adjusters, respectively.

3.2.6. Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC). Data were tested for normality of residuals using the UNIVARIATE procedure of SAS according to the Shapiro-Wilk test and the homogeneity of variances was evaluated by Levene's test using the Hovtest and Welsh methods.

Analysis of binomial (presence of DF on D7 and D10) and continuous variables (diameter of DF, DF growth rate, number of waves, days for first and second wave emergence, interval between emergence and plateau) were performed using the GLIMMIX procedure fitting a binomial distribution for binomial variables and a Gaussian distribution for continuous variables. Plasma P4 concentrations throughout time were analyzed using the MIXED procedure as repeated measures using day as the repeated statement. The model included effects of group, day, and group by day interaction. The covariance structure that provided the lowest value of corrected Akaike's information criterion was the Compound Symmetry (CS). The slice command was used to study the interaction between group and day.

Tukey honest significant difference post hoc test was performed to determine differences. Values are presented as means ± SEM for continuous variables and as percentage for binomial variables. Significant differences were declared when $P < 0.05$, whereas tendencies were considered when $0.10 \geq P \geq 0.05$.

3.3. Results

There were no differences ($P > 0.1$) between Con and P4i groups on the interval between calving and the beginning of the experiment (36.2 ± 1.9 vs. 35.8 ± 2.1 d), body weight (416.0 ± 9.6 vs. 417.8 ± 13.9 kg) and BCS (2.7 ± 0.1 vs. 2.8 ± 0.1) on D0. Additionally, in average, cows in both groups lost weight during the study (-16.9 ± 3.0 kg; $P = 0.4$).

Data from two cows, one from the P4i group and one from the Con group, were removed from the analysis of ovarian dynamics due to difficulties in identifying and tracking the dominant and subordinate follicles.

No signs of estrus were observed during the field observation period and the patches were not activated, indicating that no cow, either in the Con group or in the P4i group, expressed estrus. Furthermore, all cows remained anovulatory until D20 and CL presence at D39 after treatment did not differ ($P = 0.4$) between groups (21.4 [3/14] vs. 35.7% [5/14], for Con and P4i, respectively).

There were significant differences in plasma P4 concentrations between cows in the Con and P4i groups ($P < 0.0001$; group per day effect) from D1 to D14 (Fig. 2). Mean P4 concentrations remained basal (< 0.2 ng/mL) in the Con group cows, while in the P4i group there was a peak on Day 1 after treatment (3.0 ± 0.5 ng/mL, ranging from 1.2 to 6.4 ng/mL), with the average remaining above 0.5 ng/mL for at least 7 d. Although there was no difference between groups from D15 on, only on D18 after treatment all cows in the P4i group had plasma P4 concentration below 0.4 ng/mL.

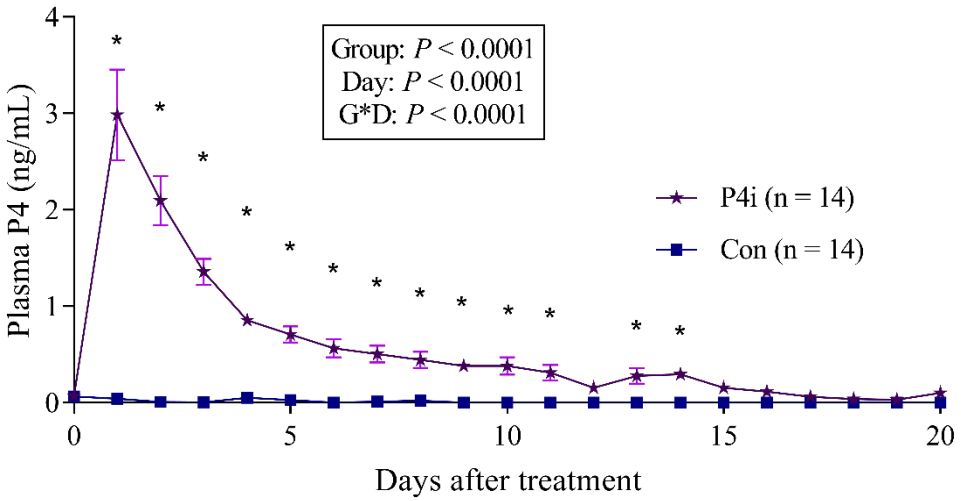

Figure 2. Circulating progesterone (P4) concentrations during 21 d in anestrus primiparous cows receiving saline (Con) or 150 mg of long-acting injectable P4 (P4i). * Indicate difference between Con and P4i within time.

Ovarian dynamics data are described in Table 1. Cows in the P4i group had a greater DF diameter than the cows in the Con group when the mean of two waves (12.8 ± 0.5 vs. 10.6 ± 0.6 mm, P = 0.01) and the first wave (13.3 ± 0.6 vs. 10.7 ± 0.7 mm, P = 0.009) was evaluated, with a tendency towards this effect in the second wave after treatment (11.8 ± 0.4 vs. 10.4 ± 0.6 mm, P = 0.06).

Although the cows in the P4i group had a larger first wave DF diameter, there were no significant differences on the daily DF growth rate between groups (Table 1), due to the longer follicular wave growth period in the P4i group (9.2 ± 0.6 vs. 7.4 ± 0.5 d; P = 0.03). That is, a longer interval between the emergence of the first wave and the time when the DF reached a developmental size plateau or atresia. Besides, cows in the P4i group (n = 10) had a longer (P = 0.02) interval between emergence of the first and second wave than cows from the Con group (n = 10; 9.4 ± 1.0 vs. 6.5 ± 0.7 d).

Considering the cows were on random days of the estrous cycle on D0, no difference was detected in relation to the day of emergence of the first wave after treatment for groups P4i and Con (3.77 ± 1.13 and 3.69 ± 0.70 d, respectively; P = 0.9). However, cows in the P4i group had a lesser (P = 0.03) number of waves emerging during the experimental period than cows in the Con group (2.00 ± 0.20 vs. 2.54 ± 0.14 waves).

In this sense, dynamics data from two cows were used as example for the most frequent patterns observed in P4i and Con groups, respectively. P4i cows often had longer follicular waves with larger DF (Fig. 3A), whereas Con cows had shorter waves and smaller DF (Fig. 3B).

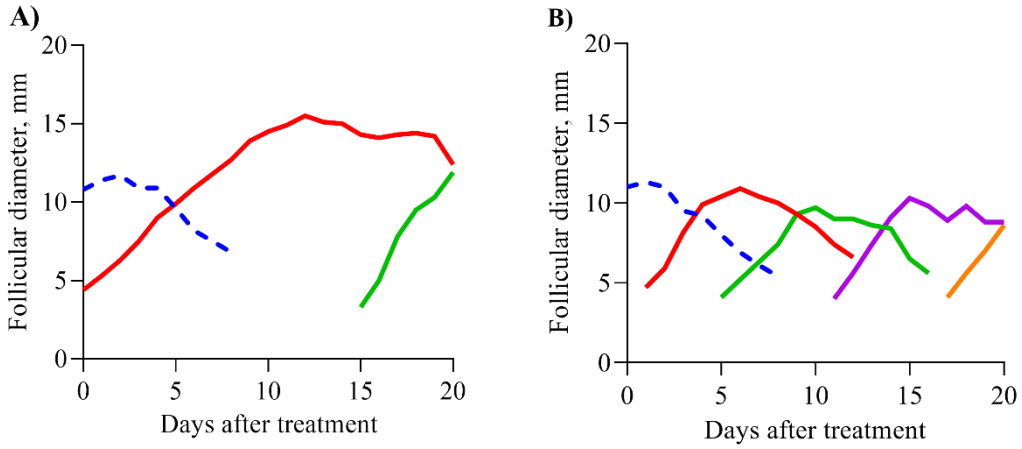

Figure 3. Patterns of development of the dominant follicle in each wave of two representative cows with the most frequent observed for P4i (A) and Con (B) groups.

Using a diameter of 8 mm as the cutoff point [25], cows in the P4i group had more (P = 0.005) days with a dominant follicle > 8 mm present in the ovaries than cows in the Con group (12.5 ± 0.5 vs. 8.3 ± 1.3 d) during the 21-d period of ultrasound evaluation (Fig. 4).

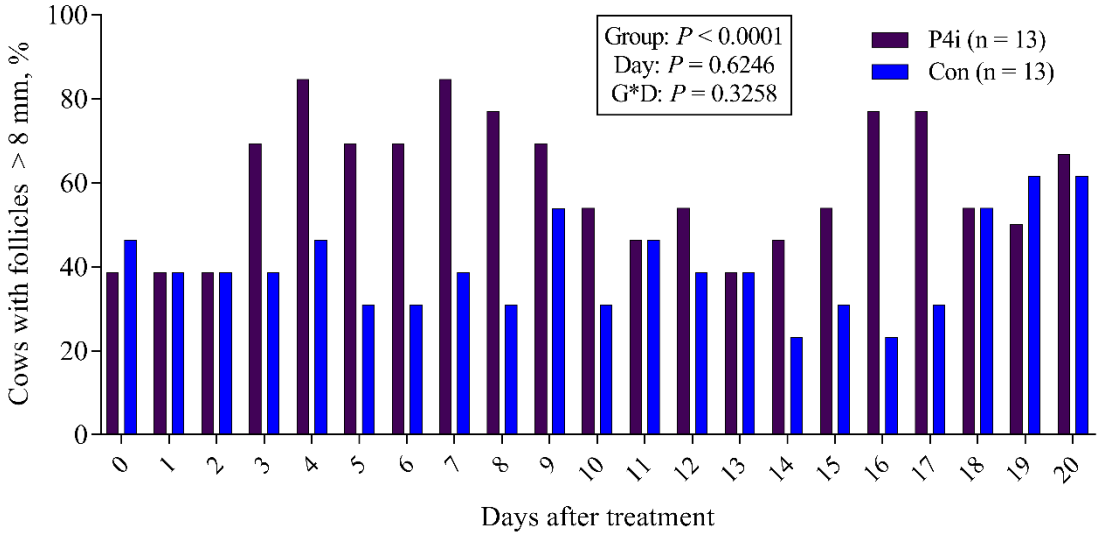

Figure 4. Percentage of cows presenting follicles with ovulatory capacity on each day during the 21d period of ultrasound evaluation after treatment with (P4i) or without (Con) 150 mg of long-acting injectable progesterone (P4i) in oily vehicle.

Table 1. Ovarian dynamics of anestrous Nelore (*Bos indicus*) primiparous cows receiving or not (Con) 150 mg of long-acting injectable progesterone (P4i).

Variable	Con	P4i	*P*-value
DF growth rate, mm/d (n)	0.92±0.09 (13)	1.08±0.05 (13)	0.18
Maximum ∅ of DF, mm (n)	10.6±0.6 (13)	12.8±0.5 (13)	0.02
Interval between 1st and 2nd wave, d (n)[a]	6.5±0.7 (13)	9.4±1.0 (10)	0.02
First wave			
DF growth rate, mm/d (n)	0.85±0.08 (13)	1.00±0.05 (13)	0.10
Plateau, d (n)	11.1±0.8 (13)	12.9±1.0 (13)	0.15
Maximum ∅ of DF, mm (n)	10.7±0.7 (13)	13.3±0.6 (13)	0.01
Interval between emergence and plateau, d (n)	7.4±0.5 (13)	9.2±0.6 (13)	0.03
Second wave			
DF growth rate, mm/d (n)	1.01±0.1 (13)	1.15±0.09 (10)	0.38
Maximum Ø of DF, mm (n)	10.4±0.6 (13)	11.8±0.4 (10)	0.06
Interval between emergence and plateau, d (n)[b]	7.0±0.6 (13)	7.7±0.7 (10)	0.45

[a] Only cows with two completely evaluated waves were included in this analysis
[b] For cows that did not have two waves completely evaluated, the interval between emergence and the end of the study was considered.
Ø = diameter.

3.4. Discussion

This study provides important and unprecedented data regarding the ovarian dynamics of postpartum primiparous beef cows treated with long-acting P4i. However, refuting the first hypothesis initially proposed for the study, none of the cows expressed signs of estrus or returned to cyclicity within a 20-d period after treatment.

The second hypothesis was also rejected as the mean P4 concentration in the P4i group remained above 1 ng/mL for only 3 d after treatment. In contrast with our data, it has been reported that in prepubertal Nelore heifers, treatment with P4i was able to promote an increase in P4 concentration that remained above 1 ng/mL (1.20 ± 0.5 ng/mL) for at least 11 d [31]. This difference may be due to the use of different assays for P4 dosage or even greater steroid catabolism in lactating beef cows than in heifers. However, there are no studies that directly compare both categories of female *Bos indicus* in this regard.

Also, at 1 d after treatment, there was a peak of circulating P4 of 3.0 ng/mL, similar to data from Simões [23], that found a peak of 3.8 ± 0.6 ng/mL on D1 after treatment in *Bos indicus* cows. However, in our study, circulating concentrations of P4 dropped rapidly, remaining below 0.5 ng/mL after D7. In this sense, longer exposure to intermediary concentrations of P4 may be necessary to induce a sufficient increase in LH pulse-frequency to result in ovulation. According to Robertson et al. [37], after 5 d of permanence of intravaginal devices, cows exposed to subluteal concentrations of P4 (0.5 PRID; mean 2.1 ng/mL) had a higher frequency of LH pulses (0.89 vs. 0.61 pulses/h) than cows with luteal concentrations (2 PRIDs; mean 6.2 ng/mL). Furthermore, the preovulatory peak occurred earlier in cows in the subluteal group after device removal (34.3 ± 2.0 vs. 51.4 ± 2.2 h).

The exposure of anovular cows [13,26] and prepubertal heifers [15–17,27,28] to P4 has been reported as one of the main strategies for inducing cyclicity and to improve fertility. However, as previously reviewed [12], the response to treatments is variable, depending on several factors such as the interval between parturition and treatment, BCS, genetic group and parity. It is known that primiparous cows suffer more from postpartum anestrus due to the high energy requirement for their own development and nursing the offspring [2]. In the present study, we challenged *Bos indicus*, lactating, primiparous cows, submitted to daily management stress. These factors may be the cause for the lack of response to treatment with P4i.

Treatment with subluteal doses of progestins increases pulse-frequency of LH [29,30] as a result of reduced E2 negative feedback over GnRH secretion. The results obtained in our study indicate that the treatment with P4i probably increased LH pulsatility not sufficiently to induce ovulation but resulting in a larger diameter of the first wave DF as previously reported in prepubertal Nelore heifers and in lactating *Bos indicus* and *Bos taurus* cows [23,31,32]. In this sense, association with an ovulation inducer (e. g., E2 esters or GnRH) could be useful.

The increase in DF diameter was not observed for the second wave probably because the study ended before the DF of this wave reached its maximum diameter, primarily in the P4i group, which had fewer cows emerging three waves during the evaluation period. Furthermore, an increase in the follicular GR was not observed, but in the duration of the follicular wave, probably due to a greater gonadotropic support that allowed the follicle to continue to grow for a longer period of time.

Occurrence of ovulation in response to a treatment with GnRH depends on the existence of responsive follicles in the ovaries, the concentration of P4 at the time of

treatment [33], and the dose and the analogue used [34]. Recently, Madureira et al. [35] reported 52.8% of ovulation after treatment with a double dose of GnRH (Buserelin acetate, 16.8 µg) at the beginning of an ovulation synchronization protocol in Zebu females on random days of the estrous cycle. In this way, considering that in our study cows treated with P4i had more days with a responsive follicle in the ovaries, it is possible to speculate that the use of this hormone prior to a protocol starting with GnRH could be beneficial, increasing ovulation responses and overall synchronization.

From the 15th d after treatment on, circulating concentrations of P4 did not differ between the P4i and Con groups, remaining below 0.4 ng/mL. Several studies show that, in dairy cows, circulating P4 concentrations above 0.4 ng/mL at the time of ovulation inducer administration resulted in lower ovulation incidence and lower P/AI [34,38,39]. Thus, pretreatment with long-acting P4i 7 d before the start of a TAI protocol with 7 d of implant permanence would not present risks, since on the day of AI (D9) circulating concentrations of P4 had already returned to baseline levels.

When administered 10 d before the start of an E2/P4-based TAI protocol, P4i promoted improvements in fertility of Nelore cows in anestrus [23,32]. Such results may be related to increased LH pulse-frequency and E2 release from the DF. Furthermore, P4 exposure may have reduced the occurrence of luteolysis before pregnancy recognition and, consequently, early embryonic loss [36].

In conclusion, although treatment with 150 mg of P4i was not able to induce the return cyclicity in primiparous cows in the early postpartum period, this treatment induced changes in ovarian dynamics such as increase in DF diameter and length of the follicular wave. In addition, the daily plasma P4 profile after P4i administration is now revealed for the first time in suckled Nelore cows during early postpartum. Studies that determine the amplitude and frequency of LH pulses after this treatment should be carried out for further clarification. Moreover, this study provides complementary information to studies that already used P4i prior to TAI protocols and opens up possibilities for the strategic use of this pharmaceutical product.

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4. FINAL CONSIDERATIONS

The first work described in this study robustly demonstrates the effects on the reproductive physiology of postpartum anestrus cows after treatment with long-acting injectable progesterone (P4i). Noteworthy is the increase in the diameter of the dominant follicle and the interval between waves, which results in more days with follicles with ovulatory capacity present in the ovaries of cows treated with P4i. Thus, there are possibilities to study new strategies for the use of this drug, as a pre-treatment to a protocol started with GnRH. The results of the second work represent the beginning of a process of validation and establishment of Immulite 1000 Progesterone (LKPW1) as a routine analytical protocol for P4 measurement in pharmacology and pathophysiology studies of bovine reproduction. Considering its precision and accuracy over a wide range of interest, the next steps involve conducting concordance studies with other analytical methods, as well as analyzing a vast number of samples obtained from animals with physiological or pharmacological concentrations of circulating P4, characterizing the results obtained in various situations.