ORIGINAL ARTICLE

Determination of progesterone concentration during the estrous cycle in dairy cows using a chemiluminescence assay

Determinação da concentração de progesterona durante o ciclo estral de vacas de leite utilizando a metodologia da quimioluminescência

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Abstract

The study aimed to determine the patterns of serum progesterone concentration in estrous cycle in dairy cows by a chemiluminescence assay (CLIA). Four non-lactating multiparous Jersey cows were used. Animals with a corpus luteum (CL) in any of the ovaries were induced into estrus. Day zero (d0) of the estrous cycle was defined as the day of visible estrus. Blood samples were collected and ultrasonography (US) of the ovaries were performed until a new manifestation of visible estrus was observed. The lengths of the estrous cycles (estrus to estrus) of the four cows were 20, 21, 22, and 23 days. The mean serum concentrations of P4 (x ± s) were 2.8 ± 1.4 ng/mL in proestrus, 2.4 ± 1.5 ng/mL in estrus, 2.0 ± 1.8 ng/mL in metestrus, and 11.9 ± 5.7 ng/mL in diestrus. The follicular and luteal phases of the estrous cycle were established based on P4 concentrations. P4 serum concentrations ≥5.48 ng/mL indicated the presence of functional CL, which was observed from d3 to d12 of the cycle. P4 concentrations decreased from d13 until next estrus. Thus, the previously mentioned P4 serum concentration was established as the limit for a predominantly functional CL. P4 concentrations < 5.48 ng/mL indicated a nonfunctional CL or CL that is not yet fully formed, which is observed in metestrus. A P4 standard curve, constructed based on the Lorentz distribution, was used to determine values of < 5.48 ng/mL of serum P4 concentration for the follicular phase and ≥ 5.48 ng/mL for the luteal phase. Data obtained from US examinations were consistent with P4 concentrations determined using CLIA. In conclusion, the automated CLIA was efficient in determining the P4 concentrations during the various stages of estrous cycles in dairy cows. The findings of the study will help researchers in the animal reproduction field. We recommend the use of CLIA because it´s available in many laboratories worldwide with the ability to process thousands of samples per day.

Keywords: Progesterone. Dairy cows. Hormonal profile. Estrus cycle. Chemiluminescence assay (CLIA).

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Resumo

O objetivo do estudo foi determinar o perfil da concentração da progesterona (P4) sérica durante o ciclo estral de vacas leiteiras pela metodologia da quimioluminesncência (CLIA). Foram utilizadas quatro vacas não lactantes da raça Jersey. O perfil padrão fisiológico da P4 compreendeu o período entre dois estros observados. O dia do estro visível foi definido como o dia zero (d0) do ciclo estral. Amostras de sangue foram colhidas e os ovários foram monitorados via ultrassonografia (US) até que um novo estro fosse observado. O comprimento do ciclo estral (de estro a estro) das quatro vacas foi de 20, 21, 22 e 23 dias. A concentração sérica média de P4 (x ± s) foi de 2,8 ± 1,4ng/mL no proestro, 2,4 ± 1,5ng/mL no estro, 2,0 ± 1,8 ng/mL no metaestro e 11,9 ± 5,7ng/mL no diestro. As fases folicular e luteal do ciclo estral foram estabelecidas baseadas nas concentrações de P4. A concentração de P4 ≥ 5,48 ng/mL indicou a presença de corpo lúteo funcional, observado do dia d3 ao d12 do ciclo estral. A concentração de P4 < 5,48 ng/mL indicou um corpo lúteo afuncional ou corpo lúteo ainda não totalmente formado, observado no metaestro. A curva padrão de P4 foi elaborada com base na distribuição de Lorentz, a qual determinou que valores < 5,48 ng/mL de P4 sérica definem a fase folicular e ≥ 5,48 ng/mL a fase luteal. Concluiu-se que a metodologia da CLIA foi eficiente ao determinar a concentração de P4 durante as fases do ciclo estral de vacas de leite. Os dados do estudo poderão auxiliar os pesquisadores na área da reprodução animal. Recomenda-se o uso da CLIA devido a sua disponibilidade em laboratórios no mundo todo, com a característica de processar milhares de amostras por dia.

Palavras-chave: *Progesterona. Vacas leiteiras. Perfil hormonal. Ciclo estral. Quimioluminêscencia (CLIA).*

Introduction

Assisted bovine reproductive techniques (such as artificial insemination, timed-artificial insemination, embryo transfer, fixed-time embryo transfer, and in vitro embryo production) have been increasingly used because of the need for reproductive efficiency. In order to make the aforementioned biotechnologies more efficient, it is necessary to provide laboratory support, based mainly on hormonal determinations of progesterone (P4). P4 concentration should be low before ovulation (Carvalho et al., 2016) or the animal should be cycling at the beginning of the breeding season.

Serum P4 profile can be determined using immunoassays such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), fluoroimmunoassay (FIA), or chemiluminescence immunoassay (CLIA). Immunoassays utilize the principle of binding of an antibody to its specific antigen to measure the concentration of either the antigen or the antibody. To detect and quantify the reaction, the antigen or the detection antibody is conjugated (labeled) with a signaling molecule. In theory, any marker that allows sensitive measurements can be used. One of the first markers developed was a radioactive isotope, which resulted in the development of RIA technique that is currently still in use. The measurement depends on the ability of the unlabeled (unknown) antigen to inhibit the binding of the radioactive antigen and the antibody, and thus it is considered a competitive immunoassay (Parker, 1981).

Skelley et al. (1973) reported that P4 could be determined using RIA in plasma and serum samples, with a sensitivity of 30.0 pg/mL. Karg (1976), and Hoffmann and Limburger (1973) established serum P4 patterns in the follicular and luteal phases of the estrous cycle in cows, and Kozicki et al. (1984) in the puerperium using RIA and ELISA. The limit of P4 serum concentration > 1.0 ng/mL represented the luteal phase with a functional corpus luteum (CL), and < 1.0 ng/mL represented the absence of CL function. The RIA has been widely used in determining P4 concentration. Its use was extended to measure concentrations of other reproductive hormones. However, the use of RIA is declining because of the risk associated with the handling of radioactive compounds and the generation of radioactive waste, which is harmful to the environment. In addition, the limited validity period of the major radioactive elements used in this technique results in reduced durability of the kits.

ELISA was developed in the 1970s (Houser, 2012), based on the antigen-antibody binding reaction with colorimetric enzymatic reactions and a spectrophotometer used for quantification

(Engvall and Perlmann, 1971). Kozicki et al. (1984) monitored dairy cows for 72 days postpartum to determine P4 concentrations using ELISA and RIA, and to compare the two methodologies. The study concluded that the two assays obtained high correlation rates between the concentrations of P4 from each method.

Concentrations of circulating P4 reflect its biosynthesis by CL and the rate of hepatic metabolism (Wiltbank et al., 2014). These concentrations are proportional to the dimensions of the CL (Sartori et al., 2004; Wolfenson et al., 2004). Reduced concentrations of P4 result in a chronic and sustained increase in LH frequency, and this prolongs the growth phase of the dominant follicle (Stevenson and Lamb, 2016).

One of the most recent techniques for the determination of serum P4 concentration is CLIA, that is based on the emission of light energy generated from a chemical reaction (Baeyens et al., 1998). Fantini Filho et al. (2004) used CLIA to measure P4 concentrations on days 7, 13, and 24 post-artificial insemination (AI) in pregnant cows, and recorded values of 1.56, 2.86, and 2.64 ng/mL, respectively. Volkmann (2006) and Tahir et al. (2013) determined P4 concentrations in canines using CLIA, and it proved to be an efficient method. Lv and Shi (2010) used CLIA to determine the P4 profile of *Meriones unguiculatus* (rodent) in the estrous cycle and in pregnancy. Except for the report by Fantini Filho et al. (2004), no other studies were found in cows during the physiological estrous cycle that used CLIA. Thus, there is no quantitative reference for serum P4 concentration (ng/mL) using CLIA to establish thresholds for when a cow is in the follicular phase or in the luteal phase of the estrous cycle. Some references of P4 determinations using CLIA in the estrous cycle and pregnancy are presented in Table 1.

Table 1 - Concentration of serum progesterone in some animal species, determined using chemiluminescence immunoassay (CLIA) or radioimmunassay (RIA), at different phases of the oestrous cycle and pregnancy

Author (metodology)	Species	Oestrus cycle (Phase)	Concentration (P4/ng/mL)	Oestrus cycle (Phase)	Concentration (P4/ng/mL)	Oestrus cycle/ parturition	Concentration (P4/ng/mL)
Wright (1990) (RIA)	Bitch	LH surge	(2.0 – 4.0)	ovulation	⩾ 5.5		
Badinand et al. (1993) (RIA)	Bitch	LH surge	> 1.0	ovulation	> 5.0		
Kutzler et al. (2003) (CLIA/RIA)	Bitch	Before LH surge	0.72 a 1.17	LH surge	2.02	ovulation	3.48 - 8.84
Tahir et al. (2013) (CLIA)	Bitch	Periovulatory phase	0.71 – 0.95				
Volkmann (2006) (RIA/CLIA)	Bitch	Preovulatory LH surge	1.5 – 2.2				
Xiao e Da Zhao (2010) (CLIA)	*Meriones unguiculatus* (virgin)	Proestrus Metaestrus Diestrus Estrus	3.08 ± 0.49 3.07 ± 0.55 2.92 ± 0.52 5.08 ± 0.87	d0 d6 d12 pregnancy	3.52 ± 0.66 20.8 ± 1.83 25.82 ± 1.95	Delivery day	6.79 ± 1.05
Xiao e Da Zhao (2010) (CLIA)	*Meriones unguiculatus* (multiparous)	Proestrus Metaestrus Diestrus Estrus	6.57 ± 1.35 5.41 ± 0.67 6.95 ± 1.12 12.08 ± 2.75	d0 d6 d12 pregnancy	5.05 ± 1.41 24.24 ± 3.19 12.49 ± 1.40	Delivery day	5.60 ± 0.88
Fantini Filho et al. (2004) (CLIA)	Beef cow	d7 post AI d13 post AI d24 post AI	1.56 2.86 2.64				

Note: d = day; AI = artificial insemination.

Our hypothesis was that CLIA can be used for the measurement of P4 concentrations. The objective of the study was to establish the physiological patterns of serum progesterone concentration in estrous cycle by chemiluminescence in dairy cows.

Material and methods

Animal management and treatment activities were performed in accordance with the internationally accepted ethical standards for animal welfare.

Animals

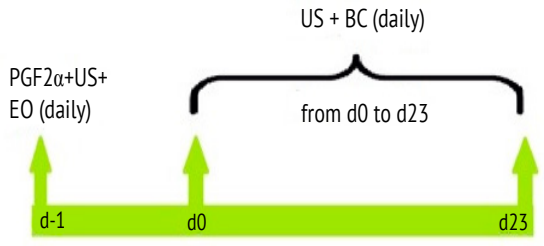
Four non-lactating multiparous Jersey dairy cows, with a mean age of 4.5 years (range 4 - 6 years), body condition score (BCS) of 3.0 (1 = very thin, 5 = fat) (Houghton et al., 1990), and mean weight of 448 kg (range 400 - 470 kg) were included in this study. At daytime, the animals grazed on paddocks with forage (Cynodon tifton), mineral salt (BellNutri 90, Bellman, São Paulo, Brazil), and water *ad libitum*.

Criteria for animal selection

From six available animals, four were selected. The cows that were included were free from gynecology problems, cycling (control of an estrous cycle prior to the start of the study and with CL in one of the ovaries), and out of the clinical puerperium period.

Ultrasonography and data collection

Animals with CL and ovarian follicles > 8.0 mm at the beginning of the study were selected. Estrus was induced by intramuscular injection of 500 mcg D-cloprostenol (Ciosin, Zoetis, São Paulo). Three daily observations (morning, afternoon, and evening) to detect estrus were made. Day zero (d0) of the cycle was defined by visible signs of estrus and presence of a follicle > 8.00 mm in diameter on ultrasonography. A complete estrous cycle was considered when the animals returned to a new manifestation of visible estrus and the presence of a follicle. Just after estrus observation (d0 = start of the study), the ovaries were scanned daily using ultrasound transducer (US) (Mindway, China, frequency bandwidth of 5.0) and the ovarian findings were recorded on ovarian maps. The ovaries were monitored for changes during the interval between two visible estrus phases. Below is the protocol diagram for ultrasonography and visualization of estrus (Figure 1).



Note: US = Ultrassongraphy; EO = Estrus observation; PGF2α= Prostaglandin F2α (D-Cloprostenol, 500 mcg, Zoetis, São Paulo); BC = Blood collection; d = day.

Figure 1 - Protocol diagram of induction and synchronization of estrus in Jersey cows, during the interval between two visible estrus phases.

Blood collections and P4 determinations

After scanning the ovaries, blood was drawn from *vena jugularis* or *v. caudalis* using vacuum tubes and Vacutainer® collection needles. After blood collection, the tubes were placed in a 37°C water bath for five minutes, centrifuged (2000 rpm) for 10 minutes to get serum, that was placed in labelled Eppendorf tubes and frozen at -20°C until measurement of P4 concentration.

All P4 levels were determined by immuno-chemiluminescence using a commercial kit (Access Progesterone Ref 33550; Beckman Coulter - Fullerton, CA, USA). The sensitivity for P4 ranged from 0.10 to 40.0 ng/mL.

The samples were placed in an automatic processing machine. The Access Progesterone test was used to measure the P4 concentrations. It is a competitive CLIA with paramagnetic particles for the quantitative detection of P4 levels in human serum using immunoassay systems.

Table 2 describes the cross-reactivity of the assay with substances that have similar structures as P4. Table 3 shows reference values established by the kit manufacturer using human serum samples.

Table 2 - Progesterone concentration measured by CLIA methodology and cross-reactivity according manufacturer specification (Beckmann, Immunassay Systems progesterone 33550, 2016)

Substance	Analyte (ng/mL)	Concentration (ng/mL)	Cross-reactivity (%)
17-α hidroxiprogesterone	50	1.18	2.36
Pregnenolone	200	0.73	0.36
DHEA Sulfate	4000	ND*	ND
5β-pregno-3α,20α-diol-3 glicuroníde	200	ND	ND
Cortisol	600	0.46	0.08
11-desoxicortisol	100	ND	ND
Corticosterone	15	0.91	6.08
Androstenediol	50	ND	ND
20-α diidroprogesterone	100	0.66	0.66
17-β estradiol	10	ND	ND
Estriol	10	ND	ND
Testosterone	10	ND	ND
Cortisone	100	ND	ND
Prednisolone	200	ND	ND
Medroxiprogesterone	100	1.38	1.38
Danazol	100	ND	ND

Note: ND = Not Detectable.

Table 3 - Reference values established by the kit manufacturer using human serum samples, indicating the mean P4 concentration as well as the range of observations

Reference group Women not pregnant.	n	Mean (ng/mL)	P4 (range) (ng/mL)
Middle follicular phase	14	0.69	0.31 – 1.52
Middle luteal phase	13	11.42	5.16 – 18.56
After menopause	49	0.25	< 0.08 – 0.78

Note: (Beckmann, Immunassay Systems progesterone 33550, 2016).

Criteria for the prediction of follicular and luteal phases

The follicular phase consists of proestrus (ovulatory follicle formation and estradiol [E2] secretion) plus estrus (sexual receptivity and peak E2); the luteal phase consists of the formation of CL + synthesis of P4 + luteolysis (Senger, 2012). The data on the hormonal measurements were adjusted, and then tested using R^2, Syx, Qui^2 and KS. The evaluation of the adjustment was performed, then it was found that the Lorentz distribution curve was the best (Figures 2 and 6), followed by the Weibull (Figure 3), Gauss (Figure 4) and Weber's distribution (Figure 5). Thus, in cows, serum P4 concentration ≥ 5.48 ng/mL was defined as the luteal phase of the estrous cycle and serum P4 concentration < 5.48 ng/mL was defined as the follicular phase.

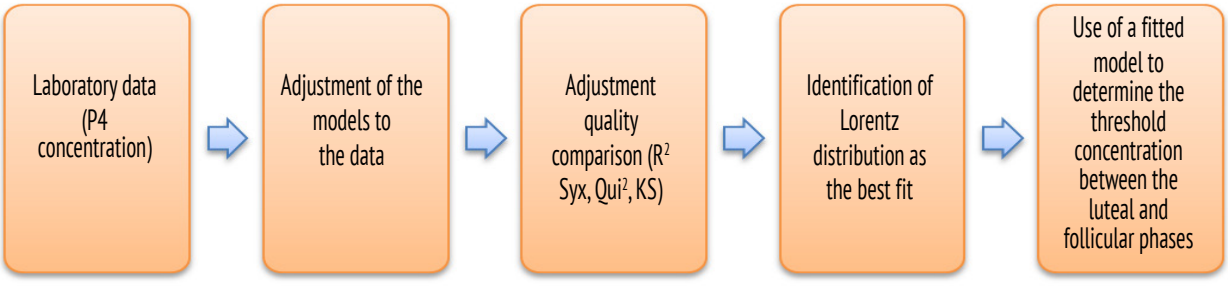


Figure 2 - Summary of the sequence for measurement of the threshold concentrations of P4 between the luteal and follicular phases in cows using CLIA.

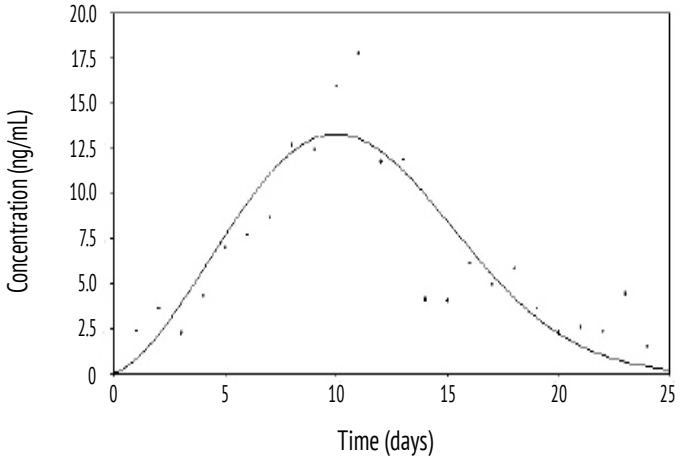


Figure 3 - Adjustment of the Weibull distribution based on serum progesterone concentrations of Jersey dairy cows over a complete estrous cycle.

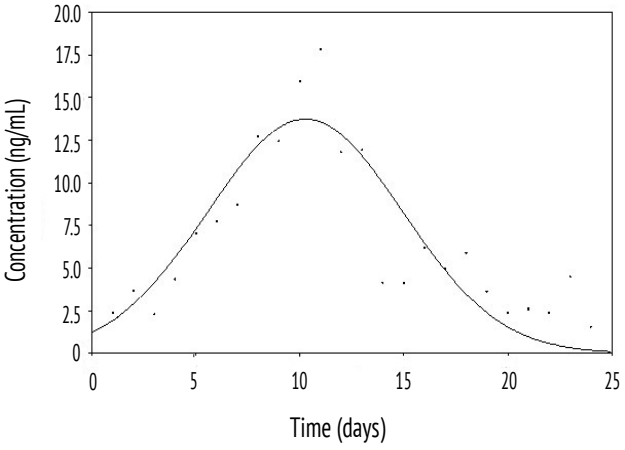


Figure 4 - Adjustment of the Gauss distribution based on serum progesterone concentrations of Jersey dairy cows over a complete estrous cycle.

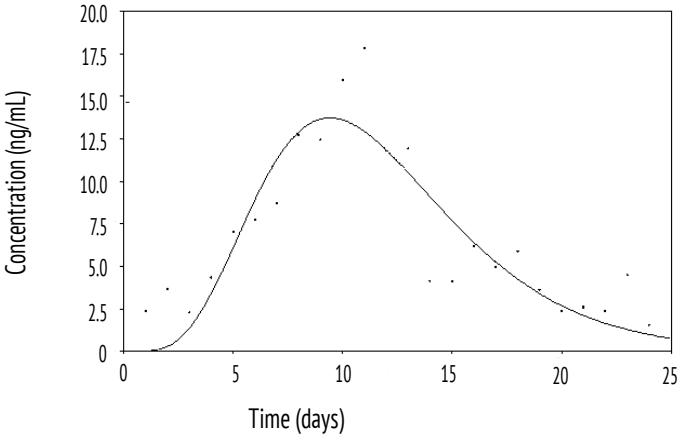


Figure 5 - Adjustment of Weber's distribution based on serum progesterone concentrations of Jersey cows over a complete estrous cycle.

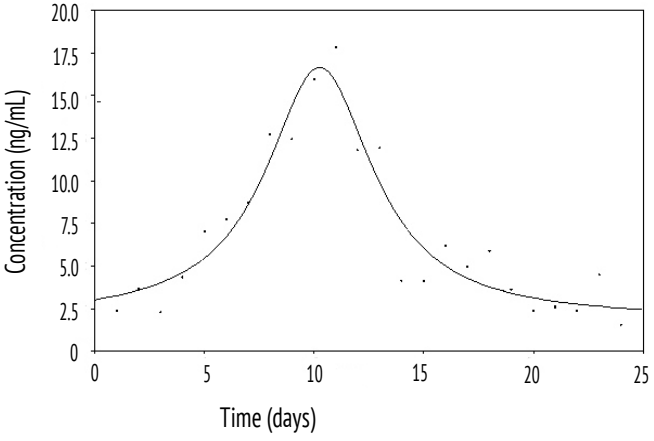


Figure 6 - Adjustment of Lorentz distribution based on serum progesterone concentrations of Jersey cows over a complete estrous cycle.

Most of the algorithms for estimating nonlinear parameters using the least square method focus on two approaches. In one approach, the model can be expanded as a Taylor series, and the corrections of the parameters are calculated for each interaction in the assumption of local linearities. In the other approach, several modifications of the gradient method were used. Marquadt (1963) uses the maximum likelihood method, performing an optimal interpolation between the Taylor series method and the gradient method. The interpolation is based on maximum likelihood, in which Taylor's truncated series provides an adequate representation of a nonlinear model (Marquadt, 1963).

In the study, 70 distributions were tested, among them the Weibull, Gauss, Weber, and Lorentz. The first two models were chosen for the test because they are classic, the third because it is highly flexible, and the fourth because of its trend characteristics.

The Weibull distribution is widely used for reliability (Johnson e Leone, 1964). This is one of the distributions developed from the concept of

exponentials and it is from this same family. It certainly is more flexible than the others, but it is still limited by its (exponential) variation factor.

The Gaussian Probability Density Function is of extreme importance in the field of probabilities, with applications in several areas of knowledge (Meyer, 1974).

The Weber distribution was originally developed to predict diameter probabilities in natural regenerations of *Ocotea odorifera* (Weber, 2009). Due to its flexibility, it has also been used in several fields of knowledge.

Distribution of Lorentz, also known as Cauchy or Breit-Wigner, is an absolutely continuous distribution class. A distributed random variable of Cauchy is a common example of a variable that has no expected value and variation.

The quality of adjustment was determined using the adjusted multiple coefficient (adjusted R^2), the standard error of the percentage estimate (Syx%), Kolmogorov-Smirnov (KS at 95% probability), Qui^2 P = 0.05, and graphical analysis of the residues. The Kolmogorov and Smirnov adherence test compares the maximum difference between the observed and the estimated frequencies, divided by the number of observations.

Results

After administration of D-cloprostenol for estrus induction, 100% of the cows showed signs of estrus within 21.25 ± 1.5 hours. The estrous cycles of the four cows lasted for 20, 21, 22, and 23 days. Two blood samples were removed because they were identified as outliers.

The results on Table 4 were obtained from the Lorentz distribution, since it showed the best adjustment, which is corroborated by the statistics shown on Table 5.

We tested 70 probabilistic models, some with better adjustment quality than the Lorentz model. However, these were not used because they did not present a biologically compatible tendency; they did not represent trends that occur naturally, as can be observed in figures 3 to 6, that show the graphical adjustments of the distributions, and 7 to 10, that show their respective residues in percentages.

Graphical residues are the qualitative analyses of the adjustment quality. They represent the error made by the adjusted model to the observed data. Based on the presented graphs, it is possible to verify that the Lorentz distribution showed the best adjustment, corroborating the information in Table 5. Thus, using the adjusted distribution, it was possible to establish the concentration limits of P4 obtained using CLIA. P4 concentrations were established in the follicular phase (< 5.48 ng/mL) and luteal phase (≥ 5.48 ng/mL) of the estrous cycle (Table 6).

Table 4 - Phases, lengths of the estrous cycle phases, dominant follicle (DF), and serum progesterone (P4) concentrations (ng/mL) in dairy cows determined using immunochemiluminescence

Estrous cycle (phases)	Lenght phases (days) (x ± s)	1st DF after estrus (days) (x ± s)	2st DF after estrus (days) (x ± s)	P4 concentration (ng/mL; x ± s) (range)
Proestrus	6.5 ± 3.5			2.90 ± 0.75 (0.22 – 4.90)
Estrus	1.0 ± 0.0			2.4 ± 1.5 (1.03 – 3.82)
Metaestrus	3.5 ± 1.7			3.0 ± 0.76 (0.63 – 4.93)
Diestrus	11.4 ± 5.7			11.2 ± 3.2 (5.49 – 28.61)
Days (total)		4.3 ± 0.5	9.2 ± 0.9	

Table 5 - Adjustment parameters of the adjusted Weibull, Gauss, Weber, and Lorentz curves, emphasizing the concentration of P4 on the 5th and 14th days of the estrous cycle in dairy cows

	Weibull	Gauss	Weber	Lorentz
Kscalc	0.37	0.42	0.50	0.15
Kstab	0.28	0.28	0.28	0.28
Qui^2calc	43.19	81.83	502.89	8.61
Qui^2tab	35.17	35.17	35.17	35.17
R^2adjusted	0.71	0.74	0.73	0.88
Syx	2.44	2.34	2.37	1.59
5th day of cycle (ng/mL)	7.69	7.30	6.12	5.48
14th day of cycle	9.96	9.97	9.09	7.60

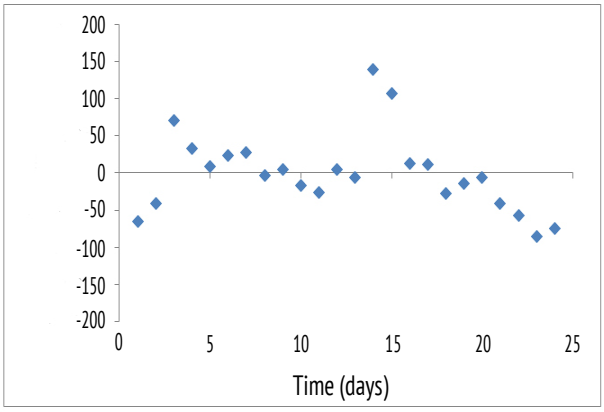


Figure 7 - Graphical distribution of residues by Weibull.

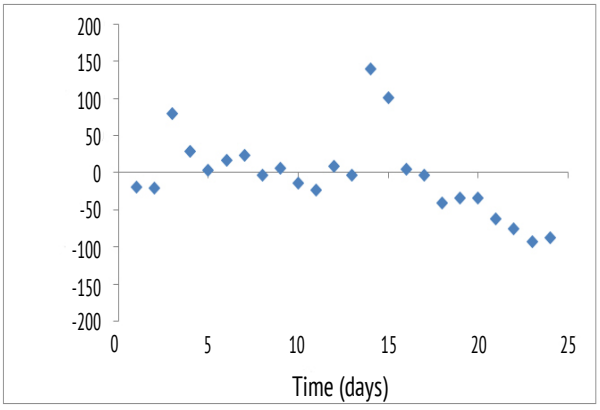


Figure 8 - Graphical distribution of residues by Gauss.

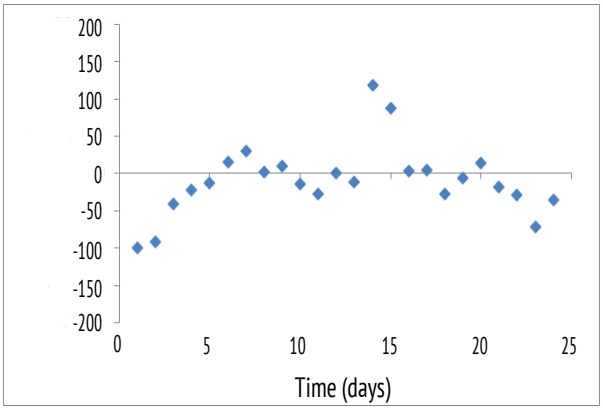


Figure 9 - Graphical distribution of residues by Weber.

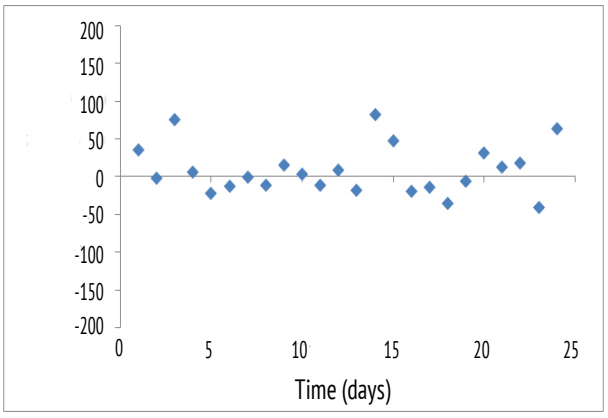


Figure 10 - Graphical distribution of residues by Lorentz.

Table 6 - Mean values of progesterone (P4) concentrations observed in bovine serum samples, as well as the range of observations in the follicular and luteal phases of the estrous cycle

Phases of estrous cycle	n	Mean (ng/mL)	P4 range (ng/mL)
Follicular phase (mean)	4	2.82	0.22 – 3.55
Luteal phase (mean)	4	11.42	5.49 – 28.61

Discussion

Progesterone is secreted by CL, that plays an important role in reproduction and endocrine regulation (Lv and Shi, 2010; Senger, 2012). Most animal reproduction biotechnologies are directly related to serum P4 concentrations and linked to reproductive efficiency.

During the past few decades, some methods to determine the blood levels of P4 have been developed, such as RIA. However, RIA has fallen into disuse due to the radioactive waste it generates. More recently, CLIA has been increasingly used because of its high efficiency and since it does not use radioisotopes. It is widely used and highly reliable in hormonal measurements in humans.

Our research group used CLIA to study the estrous cycle in cattle because the studies are scarce despite the usefulness of the method. Some studies using CLIA in animal breeding have been carried out mainly in canines (Kutzler et al., 2003; Luvoni

and Beccaglia, 2006; Volkmann et al., 2006) and in rodents (Lv and Shi, 2010).

A study was performed in beef cattle using Immulite (DPC, Los Angeles, CA) CLIA to verify the levels of P4 in d7, d13, and d24 after AI (Fantini Filho et al., 2004). They observed that the control group of beef cattle had P4 concentrations of 1.56 ng/mL at d7, 2.86 ng/mL at d13, and 2.64 ng/mL at d24 post-AI. Data from Fantini Filho et al. (2004) are quite different from the data obtained in the present research. Our results showed P4 concentrations of 5.49 to 28.6 ng/mL (mean, 11.2 ± 2.3 ng/mL) for the active CL (luteal phase), which are much higher than those reported by Fantini Filho et al. (2004). Such differences in P4 concentration could be explained by differences in kits, methodologies, or even accuracy of the tests. In their study, Fantini Filho et al. (2004) only reported the dosages of P4 but not the methodology, since their focus was not to verify the applicability of CLIA in the luteal phase.

The present study was based on P4 measurements of hundreds of samples and was confirmed with daily US findings. We studied a complete estrous cycle in dairy cows to observe the precision of the patterns of P4 concentrations using the CLIA methodology. P4 concentration (absolute quantification) standards were established for cows in proestrus, estrus, metestrus, and diestrus stages. The luteal and follicular phases were defined according to the P4 concentration in the blood serum and the US data. P4 concentration standards were established in cows in proestrus, estrus, metestrus, and diestrus. The luteal and follicular phases were defined according to the P4 concentrations in the blood serum, and the US data following previously reported parameters that were compatible with the phases of the estrous cycle and were previously defined using RIA (Wettemann et al., 1972; Karg 1976). We found that the mean concentrations of P4 reached 2.8 ng/mL in proestrus, decreased in estrous (2.4 ng/mL), decreased again in metestrus (2.0 ng/mL), and significantly increased in diestrus (11.9 ng/mL), which were compatible with an active CL.

The mean P4 concentration in canines on the day of LH peak (2.02 ng/mL), two and three days post-ovulation (6.44 and 8.84 ng/mL, respectively) were reported by Kutzler et al. (2003). Their findings are consistent with the concentrations observed in the present study, with observed values of 2.8 to 2.4 ng/mL in the preovulatory period (in proestrus and estrus, respectively) and with values > 5.48 ng/mL for the luteal phase.

Comparing our results with the standards defined for humans (Table 3), the values of P4 obtained in the present study were greater, with values of 0.31 to 1.52 ng/mL for the follicular phase (mean, 0.69 ng/mL) and 5.16 to 18.56 ng/mL (mean, 11.4 ng/mL) for the luteal phase. Our findings were validated by the Lorentz curve (Figure 6), when we set the limits for follicular and luteal phases in a physiological estrous cycle in dairy cows. The consistency of reference data already established for humans, along with those verified in cattle in the present study, can further support the use of CLIA for the measurement of P4 concentrations in animals. Our team will conduct new studies to also determine the P4 concentrations in beef cattle, horses, and other animal species using CLIA. Therefore the measurements of P4 concentration using CLIA, which is available at the Human Reproduction Clinic, may be applied to breeding cattle with financial costs similar to those of RIA (Kutzler et al., 2003). CLIA kits are easily available, allowing rapid analysis without the disadvantages of radioisotope immunoassays, which include radioactivity, radioactive waste storage, specific and complex environmental licenses, few laboratories, and short lifespan of the kit.

Conclusion

The CLIA was able to determine the hormonal profiles of P4 in the serum of cows during the physiological estrous cycle. It can be used as a substitute for traditional methods of hormone dosing, especially those using radioactive marker isotopes. Our findings can be used for further study of animal reproductive hormones.

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