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Subjective evaluation of luteal irrigation rate as diagnostic tool for pregnancy at 21 days on cows

Avaliação subjetiva da taxa de irrigação luteal como ferramenta diagnóstica para prenhez aos 21 dias em vacas

Evaluación subjetiva de la tasa de irrigación lútea como herramienta de diagnóstico de gestación a los 21 días en vacas

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Abstract
There is an increasing demand for validated techniques, with real time feedback, aiming to a higher reproductive efficiency on dairy herds. The objective of the current study was to evaluate the sensitivity, specificity and accuracy of early pregnancy diagnosis, on dairy cows, 21 days after AI, based on subjective evaluation of luteal irrigation rate. 316 ultrasound examinations were observed. 21 days after AI, the cows were assessed with color Doppler ultrasonography, performing a subjective classification of corpus luteum: poor irrigation, good irrigation or great irrigation. Cows with corpus luteum of poor irrigation were considered non-pregnant, and cows with good or great irrigation were considered as pregnant. The determination of sensitivity, specificity and accuracy was made by considering the definitive pregnancy diagnosis 27 days after AI. The sensitivity observed was 86,5%, specificity of 44,3% and accuracy of 69.8%. The early pregnancy diagnosis on dairy cows based on evaluation of luteal irrigation rate, 21 days after AI, could be useful on optimization of reproductive management of herds and should be considered as an option at the time of choice of the pregnancy diagnosis technique.
Keywords: Corpus luteum; Dairy cow; Pregnancy diagnosis; Doppler.

Resumo
Há uma crescente demanda por técnicas validadas, com resultados em tempo real, que visem a maior eficiência reprodutiva de rebanhos leiteiros. Objetivou-se neste estudo a avaliação da sensibilidade, especificidade e acurácia do diagnóstico precoce de gestação em vacas leiteiras, 21 dias após IA, baseado na avaliação subjetiva da taxa de irrigação do corpo lúteo. 316 exames ultrassonográficos foram avaliados. Após IA, os animais foram examinados por ultrassonografia no modo Doppler colorido aos 21 dias, realizando a classificação subjetiva do corpo lúteo em: pouca irrigação, boa irrigação e ótima irrigação. Sendo consideradas vacas com pouca irrigação como não prenhas e vacas com boa ou ótima irrigação como prenhas. A determinação da sensibilidade, especificidade e acurácia foi realizada com base no diagnóstico ultrassonográfico definitivo aos 27 dias após IA. A sensibilidade observada foi de 86,5%, especificidade de 44,3% e acurácia de 69,8%. O diagnóstico precoce de prenhez em vacas leiteiras com base na irrigação do corpo lúteo, aos 21 dias após inseminação artificial, pode ser útil na otimização do manejo reprodutivo de rebanhos, devendo ser considerado como opção no momento da escolha da técnica de detecção de prenhez.
Palavras-chave: Corpo lúteo; Vaca leiteira; Diagnóstico de prenhez; Doppler.

Resumen
Existe una creciente demanda de técnicas validadas, con resultados en tiempo real, que tengan como objetivo una mayor eficiencia reproductiva en los rebaños lecheros. El objetivo de este estudio es evaluar la sensibilidad, la especificidad y la precisión del diagnóstico precoz de la gestación en vacas de leche, 21 días después de la IA, basándose en la evaluación subjetiva de la tasa de irrigación del cuerpo lúteo. Se evaluaron 316 exámenes ultrasonográficos. Tras la IA, los animales fueron examinados mediante ecografía Doppler en color a los 21 días, realizando la clasificación subjetiva del cuerpo lúteo en: insuficiente irrigación, buena irrigación y óptima irrigación. Se considero que las vacas con insuficiente irrigación no estaban preñadas y que las vacas con buena o óptima irrigación estaban preñadas. La sensibilidad, la especificidad y la precisión se determinaron a partir del diagnóstico ecográfico definitivo a los 27 días de la IA. La sensibilidad fue del 86,5%, la especificidad del 44,3% y la precisión del 69,8%. El diagnóstico precoz de gestación en vacas lecheras basado en la irrigación del cuerpo lúteo a los 21 días de la inseminación artificial puede ser útil en la optimización de la gestión reproductiva de los rebaños, y debe considerarse como una opción en el momento de la elección de la técnica de detección de gestación.
Palabras clave: Cuerpo lúteo; Ganado vacuno de leche; Diagnóstico de preñez; Doppler.

1. Introduction

Currently there is a lot of advances on animal reproduction studies due to developments of reproductive organs ultrasonography (Pugliesi et al., 2017), there is a demand for reduction of calving interval, leading to an increased productivity, and consequently, increased lucrativity for the dairy activity (Silva et al., 2018). When, for countless motives, the pregnancy rate and calving interval are harmed, the expenses with maintenance of dry cows raises, as well the animals culling rate (Barbosa et al., 2011).

Between the pregnancy diagnosis methods, routinely used on bovines, there is the ultrasound on bright mode (gray scale), to morphological evaluation of reproductive organs, mostly on females, being of grand importance for timed artificial insemination and embryo transfer protocols (Pugliesi et al., 2017), granting the advantage of the simultaneous and immediate result when used on the field (Varuguese et al., 2017). Usually it is used for pregnancy diagnosis at 28 days of gestation, by observing the gestational vesicle, to accompany the reproductive cycle and to diagnose diseases of the reproductive system (Guimarães et al., 2015; Pugliesi et al., 2017). But, beyond that, the Doppler mode ultrasonography can also be used for evaluation of blood irrigation in an organ, being an indicator of its functionality (Pugliese et al., 2017).

The corpus luteum is a highly vascularized structure, essential to the estrous cycle phases and gestation, due to its production of progesterone (Guimarães et al., 2015), with positive correlations being observed between the levels of plasmatic progesterone and the pregnancy of a cow (Herzog et al., 2011). Therefore, the evaluation of CL by doppler ultrasonography can help to estimate its functionality and, consequently, contribute to the selection of embryo recipients and early pregnancy diagnosis in cows, buffaloes and sheep (Pugliesi et al., 2017; Samir & Kandiel, 2019; Santos et al., 2020). In beginning stages of pregnancy in cows (between 15 and 18 days after artificial insemination (AI)) alterations on luteal perfusion rates can be already observed, presenting an increase, when compared with non-pregnant cows (Herzog et al., 2011). Holstein dairy cows present an increase of CL perfusion rate, with dominant presence of medium and large diameter blood vessels, when observed on day 12 to 21 after AI (Varughese et al., 2017).

The early detection of pregnancy can present economic advantages, once it raises productivity, decrease calving interval, leading to a resynchronization of the non-pregnant cows of the herd on a fastest way and an increase in the proportion of pregnant cows in a shorter period of time, thus increasing the reproductive efficiency on the herd (Hassan et al., 2019; Pinaffi et al., 2015; Silva et al., 2018; Samir & Kandiel, 2019).

Using early pregnancy diagnosis techniques based on the corpus luteum irrigation is possible to realize a second insemination within 14 days after the first in timed-AI, representing 16 days in advance on the protocol, when compared to the conventional in timed-AI system (Pugliesi et al., 2017). On embryo recipient cows handling, the early diagnosis, at 21 days, can represent 9 to 14 days of advance for resynchronization on the embryo transference protocol (Guimarães et al., 2015).

There are some studies that evaluated the CL perfusion rates for early pregnancy diagnosis on farm animals (Barbosa et al., 2011; Guimarães et al., 2015; Hassan et al., 2019; Herzog et al., 2011; Pinaffi et al., 2015; Samir & Kandiel, 2019; Santos et al., 2020; Silva et al., 2018; Varughese et al., 2017). However, the necessity of recording the ultrasonography images and subsequent computational analysis, represents a limiting factor for the use of such techniques on the field, in addition, it does not fill in the real time results existent demand (Samir & Kandiel, 2019).

The proportion differences of vascularized areas evaluated by ultrasonography can be useful for early pregnancy diagnosis, on which, the subjective classification in categories as high, medium and low vascularization can lead to a higher test sensibility and specificity (Silva et al., 2018).

There is a growing demand for techniques that aim at a higher reproductive efficiency on dairy herds and the lack of studies about the early pregnancy diagnosis applied at field, on nonexperimental conditions. Therefore, the objective of this study was to assess the sensibility, specificity and accuracy of early pregnancy diagnosis, 21 days after AI, on dairy cows, based on the subjective classification of corpus luteum irrigation rate, carried out on a high-performing dairy farm.

2. Methodology

This research was approved by the Ethics Committee on the Use of Animals (UFAC), under protocol 58/2018.

The study was realized on a dairy farm in the municipality of Arapoti - Paraná, with latitude of -24.1548, longitude of -49.8285 and altitude of 860 meters. The region has a humid subtropical climate, according to Köppen-Geiger climate classification, with average annual pluviosity of 1137 mm.

253 Holstein breed dairy cows were evaluated, with average milk production of 36,0 l/cow/day, average age of 3,2 years (cows with age between 13 months and 5,5 years), belonging to the primiparous and multiparous categories. They received diet based on corn silage, pre-dried of Umbu wheat, oat hay, ration, mycotoxin adsorbent, cottonseed, soybean hull, *ad libitum water*, mineral supplementation, with buffered mineral salt, and anionic salt for the dry cows. The animals remained on *free stall* installations and were milked twice a day.

Were analyzed 316 rectal palpations and ultrasonography evaluations from December 2019 to November 2020. It was developed a quantitative field research, followed by descriptive statistical analysis of data from reproductive monitoring activities realized on the accompanied farm.

All of the animals were previously assessed and in case of estrous signs it was realized the artificial insemination with frozen semen. Twenty one days after AI (D21), all cows were examined through manual rectal palpation and ultrasonography analysis of the corpus luteum degree of vascularization, when this structure was present. The chronology used for pregnancy diagnosis was adapted from Guimarães et al., 2015. All of the animals in reproduction of the herd were evaluated on the study, including animals on natural estrus, animals under resynchronization protocol and animals with previous uterine disorders.

The ultrasound exams were realized using the colorful Doppler mode on the ultrasound equipment Honda 1600V, with Doppler function, on frequency of 7,5 Hz, with linear transrectal transductor. The same evaluator, Veterinary Doctor, performed all of the exams, between 6 am to 10 am.

The evaluation of vascularization degree was realized on a subjective manner, adapted from Santos et al. (2020), classifying the corpus luteum according to following scale: poor irrigation (range from 0 to 25% of vascularized area), good irrigation (range from 26 to 75% of vascularized area), great irrigation (range from 76 to 100% of vascularized area). Whereas, cows with poor irrigation were considered non-pregnant; Cows with good or great irrigation were considered pregnant.

At 27 days after artificial insemination (D27), ultrasonography evaluations were realized on the same animals previously assessed, to obtain the definitive pregnancy diagnosis, by gestational vesicle visualization with the presence of a viable fetus.

The determination of sensibility, specificity and accuracy of early pregnancy diagnosis at 21 days after AI, was based on the definitive diagnosis at 27 days after AI, classifying the results as: true positives (a), false positives (b), true negatives (c) and false negatives (d). The sensibility was determined by Se= a/(a+d)x100, specificity by Sp= c/(c+b)x100. The predictives values, positive and negative, were determined respectively by, PPV= a/(a+b)x100 and NPV= c/(c+d)x100 (Martin et al., 1987). The accuracy was quantified by the formula: Ac=[(a+c/(a+b+c+d)]x100.

3. Results

Among the 316 ultrasonography exams executed, eight embryonic losses were observed, not being such results considered for the sensibility, specificity and accuracy determination. The results analyses are shown on Table 1.

Table 1 - Positive and negative diagnosis, positive and negative predictive values, sensibility, specificity and accuracy, on ultrasonography evaluation, at 21 and 27 days after AI.

Day	True positives	False positives	True negatives	False negatives	Positive Predictive Values (%)	Negative Predictive Values (%)	Sensibility (%)	Specificity (%)	Accuracy (%)
21	161	68	54	25	70,3	68,3	86,5	44,3	69,8
27	186	0	122	0	100	100	100	100	100

Source: Authors.

One hundred and sixty-one positive pregnancy predictions at 21 days show correspondent correlation with the definitive diagnosis at 27 days after AI. A total of 68 diagnoses were considered as false positives. Therefore, the positive predictive value found was 70,3%. Were observed 54 true negatives and 25 false negatives, consequently the negative predictive value was 68,3%. Thus, the sensibility observed was 86,5%, specificity of 44,3% and general accuracy of 69,8%.

4. Discussion

The rectal palpation and progesterone detection on blood or milk are options of methods for pregnancy diagnosis in bovines (Samir & Kandiel, 2019). However, ultrasonography is considered the “gold standard” for evaluation of the reproductive system of cows under reproductive protocols (Pugliesi et al., 2017).

Use of evaluation of corpus luteum perfusion rate has its start as an adjuvant to the assessment by rectal palpation in bovines, which was traditionally realized also by the size of corpus luteum at ultrasonography (Herzog et al., 2010; Pugliesi et al., 2017), since that latter has strong correlation with different phases of estrous cycle (Herzog et al., 2010).

The CL size evaluation, solely, is considered a good indicator to early pregnancy diagnosis at 21 days (Guimarães et al., 2015), and there may be a directly proportional correlation between corpus luteum size and pregnancy, on the diagnosis made at 18 days (Herzog et al., 2011). It is considered as a functional corpus luteum, in taurine dairy cows, that structure bigger or equal to 4,5 cm^2, during evaluation based on corpus luteum diameter assessed by ultrasonography (Herzog et al., 2010).

There is no consensus among authors about comparison between the use of indicators as, CL size, CL blood flow and P4 concentrations. Herzog et al. (2010) observed a delay in the decline of irrigation levels in relation with levels of

progesterone. However, the irrigation rate and progesterone concentration, present high contiguity of oscillations, mostly during the static phase of the corpus luteum (Herzog et al., 2010).

In a study of Hassan et al. (2019), the perfusion rates and CL size presented changes earlier (before day 19 after AI), on pregnant cows, when compared to alterations on serum concentration of P4 on the same *Bos indicus* cows.

On findings of Herzog et al. (2010) major correlation between luteal blood flow and progesterone serum concentrations of more than 1.0. ng/mL were observed, when compared to correlation of corpus luteum size and serum progesterone. Were described 68% and 100% of sensibility and specificity, to correlation blood flow/progesterone concentration. While were observed 41% and 100% to sensibility and specificity, respectively, on the correlation between corpus luteum size/progesterone concentration.

These factors then make the pregnancy diagnosis based only on corpus luteum size subject to high risk rates of false results (Herzog et al., 2010).

Apparently the levels of progesterone serum concentration has a directly positive correlation with the CL vascularization, where females with low vascularization shown low rates of progesterone and cows with medium or high vascularization shown higher progesterone concentrations (Hassen et al., 2019; Silva et al., 2018). Similar correlations were also observed on dairy buffalos (Samir & Kandiel, 2019). And consequently animals with higher progesterone concentrations show higher fertility rates (Pinaffi et al., 2015).

In this study numerically high rate of sensibility (86,5%) was observed on early pregnancy diagnosis, based on CL irrigation, at 21 days after AI. The present sensibility is lower than found by Guimarães et al. (2015), on cows pregnancy diagnosis at 14 days after embryo transference, based on CL blood flow, where a100% of sensibility for detection of pregnancy on embryo recipients was reported. Corroborating these results, a study of Silva et al. (2018), which analyzed early pregnancy diagnosis at 20 days and definitive diagnosis at 35 days, on crossbred cows under in timed-AI, observed 100% of sensibility; Similarly, a rate of 100% to sensibility on evaluation of diagnosis at 21 days after in timed-AI was observed, on dairy buffalos (Samir & Kandiel, 2019). Thus, comparing with the literature, the value of sensibility reported in this study was lower than expected.

With 54 true negatives and 68 false positives, the specificity found was 44,26%. This result can be considered significantly low, when compared to similar woks, as the study of Silva et al. (2018), with specificity of 83,33% on crossbred cows, Samir and Kandiel (2019) with 90,9% of specificity rate on dairy buffalos and rate of 79,3% of specificity on the diagnosis of non-pregnant cows under embryo transference protocol, on Guimarães et al. (2015) study. The high number of false positive reported could have occurred due to late ovulation in response to in timed-AI protocol, longer estrous cycle, and mostly, embryonic loss between early diagnosis day and the pregnancy reconfirmation day (Pugliesi et al., 2017; Samir & Kandiel, 2019).

The general accuracy rates reported on latest studies, about early pregnancy diagnosis on cows, buffalos and ovines (Guimarães et al., 2015; Samir & Kandiel, 2019; Santos et al., 2020; Silva et al., 2018) ranged from 88,3% to 96,4%. In this study was observed an accuracy of 69,8%, being therefore comparatively low. Corroborating with this result, on Pinaffi et al. (2015) study there was no correlation between the various rates of CL perfusion, 11 days after artificial insemination in beef cattle, and pregnancy rates.

However, the choice of the moment that the evaluation of CL irrigation should be made, for correctly pregnancy diagnosis, is of prime importance. It should be realized on days close to the corpus luteum regression or close to the fetal recognition by the cow (Pugliesi et al., 2017). On evaluation of CL irrigation rate, 15 to 18 days after artificial insemination it is already possible to observe variation on the corpus luteum irrigation, whereas that the decrease of irrigation can be an indicative of luteolysis on non-pregnant cows, that can serve as an indicator to classify pregnant and non-pregnant animals,

once that the blood supply can function as a limiting factor to the progesterone production by the luteal cells (Herzog et al., 2010; Herzog et al., 2011; Guimarães et al., 2015; Silva et al., 2018).

In a study of Samir and Kandiel (2019), which assessed the early detection of pregnancy in dairy buffalos, higher accuracy, specificity and minor incorrect positives or negatives results were observed as day 21 postpartum neared, presenting better technique adequation, the closer to the third week of pregnancy. Similar data were reported by Varughese et al. (2017), which observed greater correlations and distinction capacity, based on luteal blood flow, among others indices, of pregnant cows, non-pregnant cows and complicated pregnancies, during evaluation at 21 days after AI, when compared with evaluation at 12 days after insemination. In a study of Pinaffi et al. (2015), the assessment was made 11 days after AI, so such factor might be a justificative of the non correlation observed. But, in the present study, the evaluations were realized on day 21 after AI, according with other authors recommendations. For this reason, probably such results can not be justified by the moment of choice to realize the pregnancy diagnosis.

The positive and negative predictive values were 70,3% and 68,3%, respectively. Among the factors that can affect the power of pregnancy detection during ultrasound use to assess irrigation rate, it can be cited equipment configuration, possible energy oscillation on the workplace, animal moving and the experience of the professional, since the quantity of color signs depends directly on the manner and way that the image is used and interpreted on the equipment (Guimarães et al., 2015; Pugliesi et al., 2017). Additionally, other variations caused by the different corpus luteum portions and respective irrigation oscillations can be observed, leading to under or overestimated results of blood irrigation rate (Guimarães et al., 2015). As the results analyzed in this study are from approximately one year of reproductive monitoring, with a large number of animals and palpations, on a field situation of work, many can be the interferences in the results, such as on the PPV and NPV values.

The equipment configuration surely remained the same in all days of the experiment. Nevertheless, variables such as the animal moving, the evaluator experience and oscillations of vascularization on different portions of the CL, indeed are uncontrollable factors, that can be pointed out as probable justification for the observed results. The use of the early pregnancy detection technique by evaluation of CL irrigation rate can be used as complementary to the CL size evaluation technique, reducing the false negative diagnosis rate, because the animal should present both, good irrigation rate and large CL size, to be considered pregnant (Pugliesi et al., 2017).

Furthermore, high rates of individual variation within the cows can be observed, so the establishment of a clear cutoff point between the irrigation rates that will be designated as pregnancy indicative or otherwise, can be difficult, not being considered as most advantageous when compared with the early pregnancy diagnosis based on the uterus, by some authors (Herzog et al., 2011). The present findings corroborated with the aforementioned, once the higher error rate on the diagnose were observed on those cows classified as with CL of good irrigation (52 false positives), when compared with all irrigation grades, as for example those classified as CL with poor irrigation (25 false negatives), demonstrating certain difficult to distinguish between rates of irrigation of 0 to 25% of vascularized area (poor irrigation) and of 26 to 75% of vascularized area (good irrigation). Likewise, Silva et al. (2018) observed significant differences on the CL diameters among groups of low and high vascularization, not being noticed such difference on the group classified as medium vascularization, on crossbred cows evaluation, 21 days after in timed-AI. In work of Guimarães et al. (2015), the evaluation by irrigation rate detected only two levels of progesterone serum concentration, meanwhile, those concentrations, when measured on plasma, presented three levels of significant differences. Such findings, corroborate with the present study in relation to the difficulty to realize the correct irrigation score grading, when assessed by ultrasound.

The higher absolute error rate on early diagnosis observed on animals with good and great irrigation (68 false positives) can be justified by the possible increase of blood flow with turbulence observed on vessels of corpus luteum in luteolysis process, 21 days after AI, followed of posterior definitive negative diagnosis, as reported on work of Varughese et al.

(2017). These changes present on corpus luteum in luteolysis can lead to false positives, compromising the early diagnosis technique efficacy.

Luteal perfusion rate assessment provides more information about luteal activity than the corpus luteum size evaluation (Herzog et al., 2010). However, when used in isolated ways, without corpus luteum size assess, is considered insufficient for the diagnosis by some authors (Guimarães et al., 2015).

Based on the present and similar studies, there are a need of joint evaluation between perfusion rate and blood flow turbulence, and P4 serum concentration, for distinguish, at 21 days after AI, among pregnant cows, non-pregnant and complicated pregnancy (Varughese et al., 2017).

In a study of Herzog et al. (2011), cases of apparent embryo loss, 15 days after AI, presented luteal perfusion rate numerically lower than those found on pregnant cows. Such results differ from those found in this study, where eight embryo deaths were noted at 27 days after AI, of which, seven were accompanied by corpus luteum classified as CL with good irrigation and only one CL with poor irrigation, during evaluation at 21 days. However, as aforementioned, CL in process of luteolysis can present high rates of blood irrigation (Varughese et al., 2017), therefore being able to observe good luteal vascularization rates on the same animal that posteriorly would suffer an embryo loss.

Despite the relatively low rates of sensibility, specificity and accuracy observed on the present study, it must be consider that this evaluations were realized on a high dairy production herd, including animals under different conditions and reproductive protocols, under evaluation at field conditions, additionally, the study embrace a large sample of ultrasound examinations, thus might have as consequences, the variations noted on the present study and similar studies. Possibly, the results observed reflected the rates that can be found when the early pregnancy detection on dairy cows technique is applied at field conditions.

5. Conclusion

The early pregnancy diagnosis on dairy cows based on corpus luteum irrigation, at 21 days after artificial insemination, present high sensibility (86,5%), being useful in optimizing the reproductive management of herds. The influence of external and not controllable factors on false positives and negatives rates, at field conditions, must be considered in the choosing technique moment.

Other studies aiming to applicate this technique on routinely activities should be developed in order to observe the applicability of this tool on herds with different characteristics and reproductive performance, as well executed by different professionals.

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