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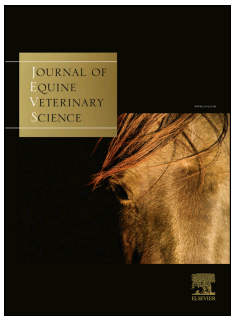

Corpus luteum vascularization and progesterone production in Autumn and Winter cycles of the mare: relationship between ultrasonographic characteristics of corpora lutea and plasma progesterone concentration in the last cycles before anestrus.

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PII: S0737-0806(17)30074-6

DOI: 10.1016/j.jevs.2017.05.001

Reference: YJEVS 2318

To appear in: *Journal of Equine Veterinary Science*

Received Date: 22 February 2017

Revised Date: 28 April 2017

Accepted Date: 1 May 2017

Please cite this article as: Panzani D, Di Vita M, Lainé AL, Guillaume D, Rota A, Tesi M, Vannozzi I, Camillo F, Corpus luteum vascularization and progesterone production in Autumn and Winter cycles of the mare: relationship between ultrasonographic characteristics of corpora lutea and plasma progesterone concentration in the last cycles before anestrus., *Journal of Equine Veterinary Science* (2017), doi: 10.1016/j.jevs.2017.05.001.

Corpus luteum vascularization and progesterone production in Autumn and Winter cycles of the mare: relationship between ultrasonographic characteristics of corpora lutea and plasma progesterone concentration in the last cycles before anestrus.

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Abstract:

In 20 estrus cycles of 15 mares, Color Doppler ultrasound of corpora lutea and plasma progesterone concentration ([P4]) were analyzed on days 6, 10, 14, 16 and 18 after ovulation. [P4] was positively correlated with corpora lutea cross sectional area (CSA), vascularized area (VA) and index of vascularization (IV=VA/CSA) (P<0.0001). CSA, VA and IV in corpora lutea of mares with [P4] < 1 ng/ml were significantly lower than in corpora lutea of mares with [P4] > 1 ng/ml. Mares with CSA <3473 pixels, VA<25.5 pixels and a IV <7.6% were prone to express [P4] <1 ng/ml 25.4, 7.9 and 7.6 times more than mares with higher values, respectively. CLs analyzed parameters differed significantly between last cycles of the breeding season and previous cycles until day 14 after ovulation (P<0.05). No significant differences were found in [P4] between last cycles and previous ones.

Keywords:

Mare; Corpus Luteum; Color Doppler Ultrasound; Progesterone; Fall Transition

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1. Introduction:

Mares' autumn transition into anestrus is a process still not completely explored. In the northern hemisphere temperate regions, although most mares stop cycling between November and January [1] anestrus can begin between August and February [1-3]. Factors analyzed to explain seasonality in the mare have been: latitude [4], age [4,5], recent reproductive history (foaled or barren) [5] and nutrition and fat reserves (leptin blood concentration) [6,7] In non-pregnant mares, corpus luteum (CL) progesterone production seems to decline significantly from one cycle to the subsequent at the end of the breeding season [1,3,8-10].

Also luteolysis appears to be negatively affected by fall transition, with an increase of the incidence of a persistent corpus luteum (25%) compared to the breeding season (8-10%) [11].

Color Doppler ultrasound has been applied for the evaluation of CL vascularization and perfusion in cattle and horses [12-19]. Corpora lutea maximum diameter [18,19] and vascularization [12,16,18,19] has been significantly correlated with plasma progesterone concentration during diestrus and pregnancy in the mare.

Diagnostic accuracy studies address how well a test identifies the target condition. Sensitivity, specificity and Likelihood Ratios (LRs) are all different ways of expressing test performances [20]. In the bovine species, to define CLs functionality, CL diameter [21], area, and vascularization area [22] cutoffs have been defined when plasma [P4] was above 1 ng/ml. Sensitivity and specificity (expressed in %) were defined as the probability to have a CL above a definite diameter [21] or area and vascularization [22] cutoffs when [P4] was above 1 or below 1 ng/ml, respectively.

Aims of this study were: to describe a cutoff to predict the CL functionality based on CL diameter and vascularization and to verify the existence of differences on CLs diameter, vascularization and P4 production between the last cycle before anestrus and previous cycles.

2. Materials and methods:

Animals, estrus cycles monitoring and blood sampling - Between October 2013 and January 2014, 15 barren, cyclic Standardbred mares aged 7 to 14 years, maintained in paddocks and fed with a balanced ratio of hay and commercial horse fodder, were studied for a total of 20 estrous cycles. Body condition score of the mares at the beginning of the study was 5-6 (where 1 is thin , 5 is moderate and 9 is obese) [23]. Ovarian activity was monitored twice a week for the whole period and daily during estrus until ovulation, by B-mode trans-rectal ultrasound (MyLab^{TM} 30 Gold machine equipped with a 5.0-7.5 MHz linear probe and a Color Doppler function; Esaote S.p.A, Florence, Italy). Mares were considered fallen into anestrus when no ovulations or CLs were evidenced by ultrasound for at least 4 weeks. On days 6, 10, 14, 16 and 18 after ovulation, CLs were scanned by Color Doppler (n= 20) and blood was collected by jugular venipuncture (n= 11). Blood was centrifuged, and plasma separated and stored at -20 °C until [P4] determination.

Color Doppler corpus luteum scanning -. For every CL, three images were taken in color flow mode at a standard depth of 10 cm, frequency of 5.0 MHz, 70% gain, and with a pulse repetition frequency (PRF) of 2.8, at maximum cross sectional diameter. Images taken were analyzed using ImageJ software (National Institutes of Health, Bethesda, USA). Cross sectional areas (CSA) of the CL were measured and any lacuna present was excluded. Images were cropped and colored zones selected (vascularized area, VA). The CSA and VA areas of the CL were measured and evaluated by counting the number of grey and color pixels, for each image taken respectively. The mean CSA and VA areas were calculated using the computer-assisted image analysis software, ImageJ (National Health Institutes, Bethesda, USA) and the mean ratio of VA to CSA was taken as an index of vascularization (IV) [18,19].

Plasma progesterone evaluation method – Plasma progesterone was analyzed with an ELISA assay adapted from Canepa et al 2008 [24]. To summarize, the monoclonal antibody anti-P4 was obtained from the mouse against P4 conjugated to bovine serum albumin. This antibody diluted at 1/160000 was aliquoted on 94 wells of a 96 wells plate (Immuno Maxisorp C96, Nunc) previously coated with an anti-mouse immunoglobulins G obtained in goat. After that, 10µl of each sample or of each

standard were added. After incubation, a solution of progesterone-11α-hemi-succinate-phosphatase alkaline conjugate was distributed in all the wells. After one hour, the plate was washed and a solution with p-nitrophenylphosphate and 100mM Diethanolamine 5mM MgCl2 was added. The plates were read at 405 nm in a photometer. For the validation of this method for equine plasma, a test of dilution was done. On this first test, the correlation coefficient was 0.992 between expected and observed data with a slope of 1.04. A test with an addition of P4 was done, and the correlation coefficient between expected and observed data was 0.914 with a slope of 0.97. The resulting coefficient of inter-assay variation was 10.3 and the intra-assay variation was 10.6. These coefficients were calculated on a reference which had an average of 1.0 ng/ml.

Progesterone concentration < 1 ng/ml was taken as the limit for a non-functional CL: mares showing P4 concentration < 1ng/ml were assumed not to have a functional CL at the time of sampling [25,26].

Statistical analysis - Statistical analysis was performed using GraphPad Prism version 6.00 for Mac Os X (GraphPad Software, La Jolla California USA, www.graphpad.com).

The normality of the distribution of populations studied was analyzed by Shapiro-Wilk test.

Pearson's correlation test was run to assess the relationship between [P4] and CSA, VA and IV, respectively.

Corpora lutea CSA and VA taken when [P4] <1 ng/ml and [P4] ≥1 ng/ml were compared using Unpaired Student’s t-tests.

Sensitivity (denoted in %) was defined as the number of mares correctly identified, by trans-rectal ultrasound (CSA, VA, and IV) as having a non-functional CL divided by the total number of mares with [P4] <1 ng/ml. Specificity was the number of mares identified by CSA, VA and IV as having a functional CL, divided by the total number of mares with [P4] ≥ 1 ng/ml. Positive Likelihood ratio was defined as the probability to have [P4] < 1 ng/ml if CSA, VA and IV were below the cutoff (sensitivity/1-specificity) [20].

The CLs CSA, VA and IV cutoffs sensitivity and specificity were defined at the highest Positive Likelihood ratio for the detection of a non-functional CL (P4 <1 ng/mL) and were evaluated using receiver operating characteristic (ROC) analysis. [20-22].

Differences between corpora lutea CSA, VA, IV (CL diameter and vascularization) and [P4] production between last cycles (=LC) before anestrus and previous cycles (=PC), on days 6, 10, 14, 16 and 18 after mares' ovulations were analyzed using unpaired Student's t-tests.

An area under the curve (AUC) has been calculated for each mare CSA, VA and [P4] between day 6 and 18 and data from PC and LC were compared using unpaired Student's t-tests.

Results were considered as statistically different with $P<0.05$.

3. Results:

Seven of the 15 mares went into anestrus during the period of the study: in 5 cases, corpora lutea progressively disappeared, at US examination, around day 18 after ovulation and no follicular growth and ovulation occurred anymore. These cycles were considered as last cycles (LC, N=5) before anestrus. In 2 cases, corpora lutea images persisted for 4 weeks after day 18 before progressively disappearing; no follicular growth and ovulation occurred thereafter. These cycles were considered as having a corpus luteum persistence before anestrus.

Eight of 15 mares did not enter into anestrus during the period of the study.

Consequently, the distribution of the 20 cycles studied and the data analyzed resulted as follows:

- 5 LC before anestrus that provided data on CSA, VA, IV (N=5) and [P4] levels (N=5) analyzed for relationship between CL diameter and vascularization and [P4] production and for differences between LC and PC on the same parameters;
- 2 cycles with persistent corpora lutea before anestrus that provided data on CSA, VA, IV (N=2) and [P4] levels (N= 2) analyzed for relationship between CL diameter and vascularization and [P4] production but not for differences between LC and PC,

- 13 PC that provided data on CSA, VA, IV (N=13) and [P4] levels (N= 4) analyzed for relationship between CL diameter and vascularization and [P4] production and for differences between LC and PC on the same parameters.

Consequently, relationship between CSA, VA, IV and [P4] were studied on 11 cycles (5 LC, 2 corpus luteum persistence and 4 PC) while differences on CSA, VA, IV between LC and PC were studied on 18 cycles (5 LC and 13 PC) and differences on [P4] levels between LC and PC were studied on 9 cycles (5 LC and 4 PC).

The data analyzed into this study (CSA, VA, IV, [P4]) were normally distributed as determined by Shapiro-Wilk test ($P>0.05$).

A strong positive correlation ($P<0.0001$) resulted for plasma [P4] between days 6 and 18 after ovulation and CSA ($r=0.527$), VA ($r=0.695$) and IV ($r=0.712$). CSA, VA and IV statistically explained 28%, 48% and 51% of the variability in [P4], respectively.

The differences between CLs diameter and vascularization taken when [P4] <1 ng/ml and [P4] ≥1 ng/ml are described in Table 1.

Table 1: Differences between corpora lutea CSA (pixels), VA (pixels) and IV (%), when [P4] < 1ng/ml or ≥ 1ng/ml (N= 11 cycles).

[P4] ng/ml (range)	**CSA (pixels)** **mean ± sd (range)**	**VA (pixels)** **mean ± sd (range)**	**IV (%)** **mean ± sd (range)**
[P4] <1 ng/ml (0-0.8)	2949.6 ± 1567.1 (1024-6044)[a]	19.9 ± 69.9 (0-287)[a]	0.4 ± 1.3 (0-11)[a]
[P4] ≥1 ng/ml (1.2-16.7)	9547.2 ± 4870.2 (3470-25592)[b]	1139.4 ± 796.4 (0-2384)[b]	11.7 ± 8.3 (0-38)[b]

Within columns $^{a\neq b}$: $P<0.05$

Area under the ROC curves for CSA, VA and IV were 0.95, 0.93 and 0.93 respectively with a $P<0.0001$. The CSA, VA and IV cutoffs (at the highest Positive Likelihood ratio) of a non-

functional ([P4] <1 ng/mL) corpus luteum, evaluated using a receiver operating characteristic curve analysis were described in Table 2.

Table 2: Highest CSA (pixels), VA (pixels) and IV (%) cutoffs (at the highest Positive Likelihood ratio) of a non-functional ([P4] <1 ng/mL) CL, evaluated using a receiver operating characteristic (ROC) curve analysis (N=11 cycles).

Parameter	**Value**	**Sensitivity**	**Specificity**	**Highest Positive Likelihood ratio**
CSA (pixels)	<3473	70.6%	97.2%	25.4
VA (pixels)	<25.5	88.2%	88.9%	7.9
IV (%)	<5.5	88.9%	88.2%	7.6

Considering the areas under the curve (AUCs) between diestrus days 6 and 18, PC and LC differed significantly for CSA (109438 ± 24488 vs 83045 ± 16893 pixels; P<0.05) and VA (18514 ± 8330 vs 8989 ± 4746 pixels; P<0.05), but not [P4] (80.7 ± 43.7 vs 73.7 ± 32.3 ng/ml; P>0.05).
The same occurred evaluating data of the single days: there were statistically significant differences for CSA (days 6 and 10) VA (days 6, 10 and 14), and IV (day 14) (P<0.05) but not for [P4] (Table 3).

Table 3: Mean ± sd CSA (pixels), VA (pixels), IV (%) and [P4] (ng/ml) in days 6, 10, 14, 16 and 18 post-ovulation of PC and LC.

Factor	**Cycle**	**Day 6** (mean ± sd)	**Day 10** (mean ± sd)	**Day 14** (mean ± sd)	**Day 16** (mean ± sd)	**Day 18** (mean ± sd)
CSA (pixels)	**PC (n=13)**	16713 ± 6245[a]	12127 ± 5817[a]	6277 ± 2690	4151 ± 1862	2819 ± 2082
	LC	10595 ± 2514[b]	8712 ± 636[b]	5935 ± 3700	3382 ± 2004	2981 ± 1230

	(n=5)					
VA (pixels)	**PC (n=13)**	2419 ± 880[a]	2325 ± 976[a]	1312 ± 968[a]	365 ± 650	103 ± 168
	LC (n=5)	1596 ± 668[b]	1144 ± 817[b]	544 ± 464[b]	64 ± 120	0 ± 0
IV (%)	**PC (n=13)**	17.6 ± 8.4	22.0 ± 10.0	16.6 ± 10.9[a]	6.1 ± 10.6	2.6 ± 4.4
	LC (n=5)	16.9 ± 10.1	12.8 ± 8.6	6.9 ± 4.8[b]	1.2 ± 2.3	0 ± 0
P4 (ng/ml)	**PC (n=4)**	8.3 ± 5.0	9.8 ± 4.7	5.7 ± 3.2	2.3 ± 2.7	0.1 ± 0.2
	LC (n=5)	9.1 ± 3.8	8.7 ± 2.9	4.0 ± 5.0	0.2 ± 0.4	0.0 ± 0.0

Within superscript $^{a \neq b}$: $P<0.05$

Discussion and Conclusions: It has been shown how luteal functionality, in terms of [P4] production, slowly decreases from summer cycles to the last cycle before anestrus [1,3,8,9]. A correlation between [P4] and CL vascularization was already found in mares [12,16] cattle [17,22] and woman [27,28]. In our study, although limited by the number of cycles analyzed, CSA, VA and especially IV of mares CLs had a strong correlation with [P4]. Crabtree and Wilsher also found a strong correlation of [P4] with CSA and VA but not with IV in pregnant mares [18]. IV is a parameter that changes when CSA and VA growth is divergent, and in that study, in the physiologic pregnant CLs, probably their growth was parallel making IV a constant. The areas under the ROC curves higher than 0.9 and their high significance ($P<0.0001$) for CSA, VA and IV showed that these parameters were able

to predict when [P4] was lower than 1 ng/ml. These results are consistent with the bovine species for CL dimensions and vascularization [21,22]. Corpora lutea producing progesterone, in the present study, had 3-4 folds larger CSAs and 10 folds larger VAs and IVs than not functional CLs ([P4] <1 ng/ml; $P<0.05$). Areas under the curves of CSA and VA showed to be significantly higher in previous cycles than during last cycles of the year, but [P4] did not, even though AUC for this hormone was numerically lower. CLs ultra-sonographic differences between PC and LC were shown clearly by the AUCs, but also evident between single examination days, up to the 14th day after ovulation. This trend was not found in [P4], may be also due to the smaller number of cycles evaluated. In this study 2/7 (28.6%) of the mares' last cycles developed in a persistent CL compared to 0/13 (0%) of the previous cycle. This is consistent with the 25% incidence of persistence of CL reported previously for the last cycle before vernal anestrus versus the 10% of normal cycles of the season [11]. In several livestock species has been reported that low level or absence of plasma [P4] resulted in compromised PGF2α response [11,29-31]. Although we were not able to show significant differences, previous cycles had a numerically higher [P4] on each sampling day, and this might have been the cause for such persistent CLs. In conclusion, CLs ultrasonographic characteristics seem to reflect their functionality, confirming previous studies performed in different species [14,16-19,21,22], and, on the group of animals studied, CLs of the last cycles of the year were smaller and less vascularized than those of the previous cycles.

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- Highlights
- Mares corpora lutea functionality was analysed by plasma progesterone concentration and Color Doppler ultrasound
- Last cycle corpora lutea functionality was compared with previous mares' cycles
- A positive correlation was found between plasma progesterone concentration and corpora lutea cross-sectional area and vascularization.
- ROC curve identified B-Mode and Color Doppler non-functional corpora lutea ultrasound characteristics.
- Color Doppler evidenced significant differences between last cycle and normal cycle's corpora lutea but not between the plasma progesterone concentrations, respectively.