ABSTRACTS

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Immunohistochemical localization of a proliferative marker (Ki-67) and VEGF in canine corpus luteum during early diestrus, early pregnancy and at cesarean moment

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OBJECTIVES AND METHODS: The aim of the present study was to immunolocalize the proliferative marker Ki-67 in bitches corpus luteum in the early cyclic diestrus and early pregnancy (approximately 25 days after LH surge) and at cesarean moment. For that, seven bitches were inseminated intravaginally at days 4 and 6 after the LH surge with fresh semen (Group I - GI). This group was submitted to ovarian hysterectomy (OHE) among 8 and 21 days after insemination. Another six (Ki-67) or eight (VEGF) bitches were not inseminated and were also submitted to OHE among 12 and 25 days after the LH surge (Group II - GII). Three others animals submitted to natural breeding had their ovary collected during cesarean (Group III - GIII).

The ovaries were isolated and a longitudinal section was made to count the number of corpus luteum; thereafter, the ovaries were placed in plastic cassettes for inclusion, fixed in 10% buffered formalin for 24h and stored in 70% ethanol until embedding in paraffin. Four micrometers tissue sections were mounted onto glass slides previously treated with Organosilane (sigma Chemical Co., USA). Section were deparaffinized with xylene and rehydrated in graded alcohol. The slides were then washed in distilled water ten times. For antigen retrieval, sections were put in a Pascal pressure chamber (Dako, USA) in sodium citrate solution 10 mM pH 6.0 (Ki-67) or maintain in a pre-heated water bath at 96°C in Tris-EDTA solution pH 9.0 for 20 minutes. After heating, slides were allowed to cool for 20 minutes and then washed ten times in tap water. Endogenous peroxidase activity was quenched with 8% peroxidase solution for 20 minutes (two times of 10 minutes) followed by 10 baths in tap water and incubated with a 3% milk solution for 1 hour at room temperature for blocking. Slides were then washed in TRIS buffered solution 10 mM (pH=7.4), encircled using DakoPen (Dako, USA) and incubated with the primary monoclonal mouse anti-human ki-67 antibody (Ki-67 Antigen, clone MIB-1, Dako, USA), diluted 1:50 in antibody diluent (Antibody Diluent with Background Reducing Components, Dako, USA) or with the primary monoclonal mouse anti-human vascular endothelial growth factor antibody (VEGF Antigen, clone VG1, Dako, USA), diluted 1:50 in antibody diliuents. Both were incubated in a humidified chamber for 18 h at 4°C. Then, slides were washed in TRIS buffered solution and incubated with the secondary antibody (Advance, Dako, USA) for Ki-67 immunostaining, or with the secondary biotinilated goat anti-mouse antibody (Dako, USA) and streptavidin-biotin (ABC Kit Vectastain, Vector, USA) for VEGF immunostaining, both in a humidified chamber and according to manufacturer instructions. Each tissue section was washed in TRIS buffered solution, and DAB chromogen (3,3’-diaminobenzidina, Dako, USA) was added as a chromogen staining substrate for 5 minutes. Reaction was stopped by rinsing in TRIS buffered solution. Tissue sections were counterstaining with Mayers hematoxylin for 1 minute, dehydrated and preserved using Permount (Fisher Scientific, USA) mounting medium. For negative controls, another section was incubated with mouse immunoglobulin (N-Universal Negative Control Mouse, Dako, USA) instead of primary antibody and for positive control a section of lymph node was used.

For the evaluation of immunoreactivity stained sections were observed at magnifications of 400x. For Ki-67 the number of positive nuclei was counted in five randomly selected microscopic fields and for VEGF the intensity of ten randomly selected microscopic fields was classified in weak (1), moderate (2) or strong (3). Data were analyzed by GraphPad Prism 5 (GraphPad Software Inc., USA) and are expressed as mean ± SD for Ki-67 or percentage of occurrence of each score for VEGF. The percentage of positive nuclei for Ki-67 that failed for normality (Shapiro Wilk test) was analyzed by nonparametric Kruskal-Wallis ANOVA followed by Dunn’s test. The score representative of VEGF among groups were compared by Qui-square test.

RESULTS: The immunostaining for Ki-67 was different among all groups (GI=5.6±2.7; GII=10.0±4.3; GIII=22.3±8.8; p<0.05; Figure 1). VEGF immunostaining intensity was similar between Group I and II (p=0.053), but both groups differs from Group III that had the major intensity (p<0.0001; Figure 1). Ours results of Ki-67, on all the evaluated periods it was possible to observed positive cells, which differs from the results of Srisuwatanasagul et al. (1) that report absence of positive cells during different stages of estrous cycle. According our results, an increase on proliferation index observed on late gestational diestrous could be related with a mechanism of self renewal attempting to survive. Regarding our VEGF results, the major immunostaining detection at late gestational diestrous (around day 60 after the ovulation), otherwise Mariani et al. (2) observed that cyclic diestrous corpus luteum obtained 70 days after the ovulation had a significant fall on VEGF immunostaining detection.
CONCLUSION. These observations could reinforce a different pattern of VEGF between the late cyclic and gestational diestrous.


Figure 1: Immunolocalization of Ki-67 (A, B, C) and VEGF (D, E, F) in bitch corpus luteum during early pregnancy (A, D), early diestrous (B, E) and caesarean moment (C, F). Negative and positive controls, inset in C and F. Immunohistochemistry, DAB, counterstaining with Mayer’s hematoxylin, magnification of 200X.