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Canine corpus luteum regression: Apoptosis and caspase-3 activity

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Abstract

The present study evaluated the occurrence of apoptosis and caspase-3 activity in the canine corpus luteum during the period of luteal regression in eight pregnant and nine nonpregnant diestrus bitches. Intact luteal cells were obtained from corpora lutea in both peripartum pregnant bitches and nonpregnant diestrus bitches at approximately 65 d (range 63–68) after estrus, but not at days 75 and 85 in nonpregnant bitches. In all bitches, apoptotic cells were rarely detected and when present, those cells were more easily detected using the hematoxylin and eosin technique than using the critical electrolyte concentration technique. The luteal structures at 75 and 85 d of diestrus had histological characteristics similar to a corpus albicans. Caspase-3 activity was detected in morphologically normal corpora lutea from both pregnant and diestrus bitches around day 65, and also in the later structures considered corpus albicans tissue. These results suggested that apoptosis may not be the major mechanism involved in canine functional luteal regression, and that caspase-3 participated in both functional and morphological luteolysis and in the tissue reorganization involved in corpus albicans formation.
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Keywords: Bitch; Corpus luteum; Apoptosis; Caspase-3; Luteal regression

1. Introduction

Few studies have considered the morphological characteristics and mechanisms that regulate canine luteal regression during pregnancy and diestrus [1,2]. Ultra-structural analysis of the luteal cell compartmentalization has been previously described [1]. Morphological and histochemical aspects of luteal regression were reported from days 30 to 240 post-ovulation [2]. There appear to be no morphological differences observed during luteal formation and corpora lutea (CL) regression in pregnant and diestrus bitches, with the first signals of luteal regression observed at 28 d after the luteinizing hormone (LH) surge [1]. An ultrastructural study showed that canine functional luteolysis is completed at 120 d after the LH surge, and structural luteolysis by 240 d [2].

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Apoptosis is programmed cell death in the absence of inflammatory reaction. It is characterized by membrane blebbing, cellular retraction, DNA fragmentation and apoptotic body formation [3]. One of the initial reactions is the activation of calcium-dependent endonucleases, which leads to DNA cleavage in internucleosomal sites [4]. Caspase-3 is a protein found in vertebrates and homologous to the ones existent in the nematode Caenorhabditis elegans [3].

In several animal species and in humans, apoptosis is involved in the luteolysis phenomenon [5–8]. Caspase-3 family expression has been demonstrated during luteolysis in sheep, in part occurring because of protease expression [8]. Internucleosomal DNA fragmentation has been observed in spontaneous or PGF2α induced luteolysis of CLs in cows and hamsters, respectively [5,7]. Moreover, a significant increase in apoptotic DNA cleavage has been found in human corpora lutea from the middle to the end of luteal phase [6]. Caspase-3 activity is necessary for luteal apoptosis in normal or caspase-3 deficient mice [9]. Therefore, the occurrence of apoptosis and caspase-3 activity, a primary apoptotic executor [9], was examined in canine CL to gain insight into the process of spontaneous luteolysis in this species in which luteal function persists for 2 mo or longer after ovulation.

2. Materials and methods

2.1. Dogs

Seventeen adult cross-bred bitches, from 5 to 40 kg body weight, were maintained in individual cages, under natural light, fed a commercial diet twice a day, and given ad libitum access to water. There were eight pregnant bitches and nine nonpregnant diestrus bitches. The bitches had been previously identified as proestrus on the basis of vulvar swelling, serosanguineous vaginal discharge, or both. After the initial observations, daily vaginal cytology was performed until 80–90% superficial cells were detected [10,11]. Bitches were then artificially inseminated or naturally bred every 48 h until the end of estrus. Several male dogs were used for breeding. Pregnancy diagnosis was performed by ultrasonography (Pie Medical Scanner 480) at 25 d after the first breeding. The bitches intended for the nonpregnant group were not bred. Vaginal cytology was performed in all bitches during estrous cycle until the first day of cytological diestrus [10,11]. The day of the pre-ovulatory LH peak (day 0) was subjectively estimated as 8 d prior to the onset of diestrus vaginal smears [12].

Pregnant bitches were submitted to ovariohysterectomy at 63 d (P63, $n = 2$), 64 d (P64, $n = 3$) or immediately after natural parturition (postpartum, $n = 3$). Diestrus bitches were submitted to ovariohysterectomy at 65 d (D65, $n = 3$), 75 d (D75, $n = 3$) or 85 d (D85, $n = 3$) of the cycle. Ovaries were transported to the laboratory within 15 min of surgery.

2.2. Ovaries

The ovaries were dissected and the corpora lutea mechanically removed. The corpora lutea isolated for critical electrolyte concentration (CEC) and hematoxylin and eosin (H&E) analysis were cut into small sections of less than 0.5 cm fragments and fixed in formalin. The corpora lutea fragments for caspase-3 activity measurements were immediately frozen at −20 °C. The cytochemical analysis by the CEC technique was performed as previously described [13,14]. In brief, histological sections were stained with Toluidine Blue diluted in Mc Ilvaine buffer, pH 4.0, for 15 min. After staining, they were immediately immersed in magnesium chloride 0.5 M for 7 min, washed and dried overnight. The material was clarified in xylol. The microscopic analysis considered luteal cell morphology, presence of apoptotic cells (apoptotic bodies), and evidence of corpus luteum reorganization. Corpora lutea samples were used for immersion time standardization on magnesium chloride and Toluidine Blue staining. Similar evaluations were made of histological sections of corpora lutea that were stained with H&E solution. Measurement of caspase-3 activity was made on corpora lutea samples (150–170 mg) after thawing at room temperature for 15 min. Lysis buffer (430 μL) containing protease inhibitor was added and the material was dissociated into smaller fragments using surgical scissors. After centrifugation at $2000 \times g$ for 5 min the supernatant was re-centrifuged at $4500 \times g$ for 5 min, samples containing 10 μL of supernatant were diluted in 15 μL of ultra-pure water, and the Bradford method [15] was used for the protein quantification. Caspase-3 activity measurement was performed in the lysed sample after initial tests were conducted to determine the ideal sample dilution (from 1× to 100×); a 2× dilution was adequate in most cases. For the caspase-3 measurement, as previously described [16], 50 μL of the previously lysed sample was diluted at 1:1 in the caspase reaction buffer, in duplicate. Caspase activation was induced by homogenization and pre-incubation at 37 °C for 15 min. Caspase substrate ‘Abz-DEVDGVQ-EDD np’ (10 μL at 110 μM) was added, and the material was homogenized and

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