Estrogen and oxytocin receptors in the canine corpus luteum during pregnancy and parturition

Receptores de estrógeno e ocitocina no corpo lúteo durante a gestação e parto em cadelas

Gisele Almeida Lima VEIGA[1]; Marcella Pecora MILAZZOTTO[1]; Cristina Fátima LÚCIO[1]; Liege Cristina Garcia SILVA[1]; Camila Infantosi VANNUCCHI[1]

[1] Universidade de São Paulo, Faculdade de Medicina Veterinária e Zootecnia, Departamento de Reprodução Animal, São Paulo – SP, Brazil

Abstract

The expression of genes encoding the receptors for estrogen (ERαmRNA) and oxytocin (OTRmRNA) was studied in the corpus luteum during pregnancy and parturition in dogs. Real-time PCR was performed to quantify the levels of ERαmRNA and OTRmRNA in the corpus luteum of bitches during Early (up to 20 days of gestation), Mid (20 to 40 days) and Late Pregnancy (40 to 60 days), and Parturition (first stage of labor). The corpus luteum expressed mRNA for OTR, however ERα mRNA was not detected. There was a reduction of OTR mRNA expression in the corpus luteum from gestational Day 20 onward, which suggests an important role of OTR mRNA in the mechanism of pregnancy recognition in dogs. We concluded that the expression of OTR mRNA in canine corpus luteum vary over time, which support the idea that the sensitivity and response to hormone therapy can vary along the course of pregnancy and labor. Moreover, the canine CL lacks ERα mRNA expression during pregnancy.

Keywords: Canine. Estrogen. Oxytocin. Corpus luteum. Pregnancy. Parturition.

Resumo

A expressão dos genes que codificam os receptores de estrógeno (REα RNAm) e ocitocina (ROT RNAm) foi estudado no corpo lúteo de cadelas durante a gestação e parto. A técnica de PCR em tempo real foi realizada para quantificar a expressão do REα RNAm e ROT RNAm no corpo lúteo de cadelas durante o início (até 20 dias de gestação), meio (20 a 40 dias) e final da gestação (40 a 60 dias), e durante o parto (pródomos do parto). O corpo lúteo apresentou expressão do RNAm para o ROT, entretanto o RNAm para o REα não foi detectado. Houve redução na expressão do ROT RNAm no corpo lúteo a partir de 20 dias da gestação, indicando papel no mecanismo de reconhecimento gestacional em cadelas. Em conclusão, a expressão do ROT RNAm no corpo lúteo de cadelas apresentou variação ao longo do tempo de gestação, sugerindo que a resposta e sensibilidade à terapia hormonal pode variar conforme o momento da gestação e parto. Ademais, o corpo lúteo canino não expressa REα RNAm durante a gestação.

Palavras-chave: Cadelas. Estrógeno. Ocitocina. Corpo lúteo. Gestação. Parto.

Introduction

The canine corpus luteum (CL) is a temporary and essential endocrine gland during gestation, as it produces progesterone to ensure the maintenance of pregnancy, in spite of the lack of placental steroidogenesis. It has been shown that ovariectomy in pregnant bitches leads to embryo resorption or abortion, attesting the important role of the corpus luteum in providing the adequate hormonal support to pregnancy (CONCANNON, 2012). On the other hand, the participation of the canine corpus luteum in the important cross-talk among progesterone, estrogen and oxytocin along gestation and parturition is yet to be studied.

In dogs, estrogen secretion during pregnancy is mainly of ovarian origin and its profile depends on prolactin stimulation (CONCANNON, 2009). Serum concentration of estrogen increases progressively during days 45 and 60 of gestation, with a maximum concentration of 20-30 pg/mL before whelping

Correspondence to:
Camila Infantosi Vannucchi
Rua Prof. Orlando Marques de Paiva, 87 – Cidade Universitária
CEP 05508-270, São Paulo, SP, Brazil
e-mail: cacavann@usp.br

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(CONCANNON et al., 1975; ONCLIN; MURPHY; VERSTEGEN, 2001). Nevertheless, a sharp decrease is verified towards parturition, reaching levels similar to non-pregnant bitches immediately prior to birth (CONCANNON et al., 1975).

Estrogen controls the reproductive cycle through two receptor (ER) subtypes, namely, ERα and ERβ (SAUNDERS et al., 1997). In various species, ERα is differentially expressed during pregnancy and parturition in the corpus luteum (KUIPER et al., 1997; SAUNDERS et al., 1997; TESSIER et al., 2000). For example, in rats, the expression of ERα mRNA in several tissues is suppressed during pregnancy (MURATA et al., 2003). In dogs, recent studies have demonstrated increased ERα mRNA expression in the corpus luteum of nonpregnant bitches, suggesting that an autocrine and/or paracrine action of estrogen may participate on the endocrine control of the CL (PAPA; HOFFMANN, 2011). Moreover, for the pregnant bitch, the presence of ovarian ER during the period of high estrogen concentration indicates an autocrine action (HOFFMANN et al., 2004). However, the hormonal regulation of the corpus luteum during pregnancy is still a subject worthy of research, as any luteotrophic effect of estradiol has not been investigated in dogs (CONCANNON, 2009).

In several species, the prepartum luteolysis is considered a cascade of events culminating with the increased production of placental prostaglandin F2alpha (SENGER, 2005). Oxytocin triggers luteolysis by binding to its receptor at the uterine level and stimulates prostaglandin production by the placenta (GIBB; LYE; CHALLIS, 2006). Moreover, estrogen has a main role in this process, as it sensitizes uterine tissues for oxytocin action (GIBB; LYE; CHALLIS, 2006). Besides the endocrine function of oxytocin towards the transition from pregnancy to parturition, a paracrine or autocrine activity has also been suggested (MITCHELL FANG; WONG, 1997). At the beginning of the ovine pregnancy, the presence of the embryo blocks the increase of estrogen and oxytocin receptors in the uterus and the consequent release of prostaglandin F2alpha (WATHES; HANON, 1993). Unlike other species, there are no reports on any specific mechanism of canine embryo regulation of the uterine endocrinological profile. Therefore, the hormonal regulation of steroids and oxytocin during pregnancy needs to be clarified in canine endocrinology, as a manner to study endocrinological regulation without embryonic influence. Hence, we hypothesize that the canine CL can also regulate luteolysis itself by increasing the responsiveness to oxytocin by means of an estrogen stimulation of oxytocin receptor (OTR) synthesis.

In the present investigation it was characterized the expression of ERα and OTR in the canine corpus luteum during the various stages of pregnancy and parturition. These results will help to underline the specific hormonal sensitivity of the corpus luteum and, ultimately, contribute to elaborate hormone therapies during pregnancy and birth.

Materials and methods

Animals and experimental groups

The use of animals in the current study was approved by the Bioethics Committee of the College of Veterinary Medicine - University of São Paulo. Bitches were privately owned and reported as mismating and unwanted pregnancy. All owners were aware of the pregnancy interruption and agreed with the gonadectomy. Pregnant bitches were assigned to four groups according to gestational age as established by ultrasound, reproductive history, and measure of the fetal crown-rump length (EVANS; SACK, 1973): up to 20 days of gestation (Early Pregnancy Group, n = 11), 20 to 40 days of gestation (Mid Pregnancy Group, n = 12), 40 to 60 days of gestation (Late Pregnancy Group, n = 12), and first stage of labor (Parturition Group, n = 11). Clinical signs of the early phase of labor were vaginal elimination of the mucus plug, drop in body temperature, behavioral alterations such as isolation, restlessness, and lack of appetite. The pregnant

females were subjected to ovariohysterectomy, and the animals in Parturition Group underwent cesarean section followed by ovariohysterectomy.

After surgery, fragments of the CL were harvested. The corpus luteum was macroscopically isolated from other ovarian structures by gentle dissection. Fragments were washed with saline solution (0.9% NaCl), and stored at -196o C until further processing.

RNA isolation and cDNA synthesis

The Illustra RNAspin Mini RNA Isolation kit (GE Healthcare®, Freiburg, Germany) was used to extract the total RNA from the samples of corpus luteum, following the manufacturer's instructions. Total RNA was quantified after dilution in (RNase-free) water at a ratio of 1:100 in an Eppendorf® (model Vi 1.35) photometer. Subsequently, 1 µg of total RNA was used to synthesize the first strand of cDNA by reverse transcription using the Super Script® II reverse transcriptase (Invitrogen®, Carlsbad, USA) system in the presence of oligo (dT). Initially, total RNA was mixed with 1 µL oligo (dT), and diethylpyrocarbonate (DEPC)-treated water was added to a total volume of 12 µL; the reaction mixture remained at 70ºC for 10 min in a PTC-100® thermal cycler. The solution was cooled to -20o C for 1 min. Afterwards, 2 µL of First Strand Buffer, 2 µL of $MgCl_2$ 25 mM, 1 µL of deoxyribonucleotide triphosphate (dNTP), and 2 µL of 0.1 M dithiothreitol (DTT) were added followed by heating to 42 C for 5 min. Finally, 1 µL of SuperScript® II enzyme was added and the reaction was kept at 42ºC for 50 min and 70ºC for 15 min. The obtained cDNA was stored at -20ºC.

Real-time PCR amplification

To quantify their levels of genetic expression, comparative analysis of the target genes (ERα and OTR) and endogenous controls (18S and RPS5) was performed. The cDNAs were subjected to amplification of constitutive genes 18S and RPS5 as well as ERα and OTR with primers designed from sequences previously deposited in GenBank (www.ncbi.nml.nih.gov). In order to avoid false positive results, primers were constructed flanking the intron region of the amplicon.

The primer sequences were as follows: 18S, 5'-TGGTTGATCCTGCCAGTAGCA-3' and 5'-ATGAGCCATTCGCAGTTTCACT-3'; RPS5, 5'-TCACTGGTGARACCCCCT-3' and 5'-CCTGATTCACACGGCGTAG-3'; ERα, 5'-GGTCTTGGTGTTGGGTGTG-3' and 5'-GGACATATTCCTCACGCTCC-3'; OTR, 5'-GAACTTGTACAGCGCTTCCTC-3' and 5'-GACAAAGGTGGATGAGTTGCTC-3'.

Real-time PCR was performed in an Eppendorf® Mastercycler Realplex using the Platinum® SYBR Green PCR Master Mix kit (Invitrogen®, Carlsbad, CA, USA). Sample tubes without corpus luteum tissue were used for negative controls. No positive controls were adopted; however primers were previously standardized and simultaneously employed for amplification in other canine tissue samples (endometrium, myometrium and placenta), which revealed positive amplifications. All reactions were performed in a total volume of 25 µL and heated to 50ºC for 2 min and then 95ºC for 10 min followed by 45 cycles comprising denaturation at 95ºC for 15 seconds and then annealing for 60 seconds at the following temperatures: 61ºC for RPS5, 60ºC for 18S, and 59ºC for ERα and OTR. All reactions were performed as duplicates.

To calculate the relative expression levels of the target genes, Pfaffl's formula was applied to randomization tests utilizing the Relative Expression Software Tool (Rest-384© - version 2; Munich, Germany), following the software's instructions provided by Pfaffl, Horgan and Dempfle (2002). The efficiency of the amplification reactions for the different genes was established by amplifying serial dilutions of each sample. The relative efficiency (target gene/endogenous gene) was calculated from the slopes of product formation curves, where efficiency = $10^{-1/slope}$. The relative amplification efficiencies for the analyzed

genes were 1.98 (18S), 2.05 (RPS5), 2.08 (ERα), and 1.94 (OTR).

To verify the stability of the endogenous controls (RPS5 and 18S) in corpus luteum the variance of each was compared using the Brown-Forsythe test. No statistical difference was verified for the variance homogeneity of the endogenous controls (18S and RPS5) in the corpus luteum. Nevertheless, we choose to express data in relation to the RPS5 reference gene.

Statistical analysis

All data were evaluated using SAS System for Windows (SAS Institute Inc., Cary, NC, USA). The effect of gestational period (Early Pregnancy, Mid Pregnancy, Late Pregnancy and Parturition) was determined using parametric (One Way Anova –PROC GLM; LSD as post-hoc test) and non-parametric (Wilcoxon) tests, according to the residue normality (Gaussian distribution) and variance homogeneity of each variable. A probability value of $P < 0.05$ was considered statistically significant. Results are reported as untransformed means and standard error of the mean (SEM).

Results

With the present methodology, ERα mRNA was not detected in the corpus luteum throughout pregnancy and labor. However, OTR mRNA exhibited its highest expression ($P < 0.05$) before 20 days of gestation (Early Pregnancy Group), compared to Parturition Group (Figure 1). Hence, there was a decrease in OTR mRNA expression in the corpus luteum as gestation progressed.

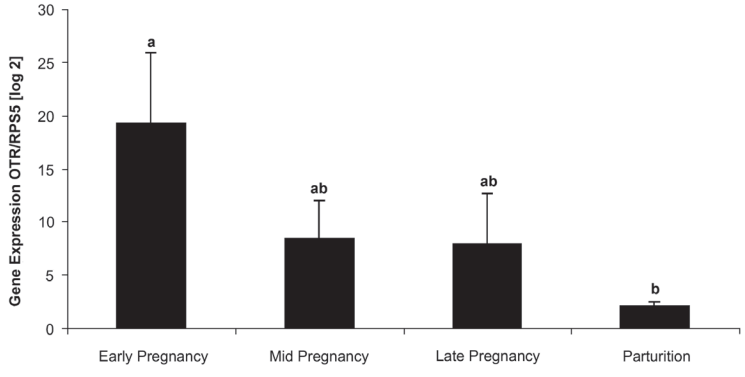

Figure 1 – OTR relative expression in the corpus luteum during Early, Mid and Late Pregnancy and Parturition. [a,b] values with different superscript letters differ significantly ($P < 0.05$)
Source: (VEIGA et al., 2014)

Discussion

In this study, it was examined the temporal expression of ERα mRNA and OTR mRNA simultaneously in the corpus luteum along gestation and parturition in dogs. The corpus luteum expressed mRNA for OTR, which suggests ability of the CL to respond to oxytocin in a time-dependent manner. On the other hand, it was not found the expression of ERα mRNA in the CL of periparturient bitches, in spite of increasing estrogen serum concentrations after 40 days of gestation until whelping (CONCANNON et al., 1975; ONCLIN; MURPHY; VERSTEGEN, 2001).

The existence of two estrogen receptor subtypes (ERα and ERβ) explains the differential action of estrogen in target tissues (KUIPER et al., 1997). For example, unlike the current study, only the expression of the subtype ERα is observed in the corpus luteum of sheep, while in bovines only ERβ is expressed (WALTHER et al., 1999; ZIĘBA; MURAWSKI; WIERZCHOŚ, 2000). Moreover, ERβ mRNA is the predominant subtype in the luteal cells of pregnant sows, whereas ERα mRNA is expressed only in the ovarian follicles, which suggests an autocrine/paracrine action for estrogen in the control of ovarian function during pregnancy (KNAPCZYK et al., 2008). Similarly, canine luteal and non-luteal cells show an equal pattern of ERβ expression, albeit there is a significant ERα expression on luteal cells after 65 days of ovulation in diestrous bitches (PAPA; HOFFMANN, 2011). Nevertheless, it was analysed pregnant and whelping bitches, which might be the reason for the distinct ER pattern in the corpus luteum previously verified by Papa and Hoffman

(2011). To this particular result we can infer the existence of an inhibitory action of progesterone during pregnancy, as in women it has been shown a decreased profile of ERα and increased expression of progesterone receptor in luteal cells (GREGORY et al., 2002; KLEIN; PAPA; HOFFMANN, 2001). In fact, the subtype ERα is related to specific gene and protein synthesis that has to be neutralized in order to favor embryonic growth and implantation process (CONCANNON, 1986). Additionally, ERβ is considered as a dominant-negative regulator of ERα and essential for differentiating effects of estrogen (BÖTTNER; THELEN; JARRY, 2014). Therefore, during canine CL life-span, the autocrine and/or paracrine action of estrogen could be primarily regulated by ERβ rather than ERα, which explains the lack of ERα mRNA expression in the present study.

The reduction of OTR mRNA expression in the corpus luteum from gestational Day 20 onward observed here may be associated with the mechanism of luteolysis inhibition during mid-gestation, relationship not yet established in dogs. The corpus luteum and the hypophysis store and release oxytocin; thus, the presence of OTR in the former denotes an autocrine function, as well as an endocrine action (SERNIA; GEMMELL; THOMAS, 1989). We believe that the establishment of pregnancy around day 20 may be a factor behind the decrease of OTR mRNA expression, thus inhibiting the possible autocrine or endocrine action of oxytocin and subsequent precocious luteolysis. Therefore, we speculate that embryos can participate on the recognition of pregnancy in dogs through the decrease in luteal sensitivity to the oxytocin mechanism of luteolysis. In fact, Hoffmann, Riesenbeck and Klein (1996) stated that paracrine and/or autocrine mechanisms are major factors involved in the control of luteal function in dogs, regardless of the endocrine profile. Therefore, the reduction OTR mRNA expression in the CL can contribute to avoiding possible stimulation of uterine luteolysine, which may threaten CL life-span. However, further studies on OTR expression in the canine corpus luteum during diestrus should be performed to better identify such characteristic of the canine reproductive physiology.

During parturition, we showed the lowest OTR mRNA expression in the CL. From this observation, our primary hypothesis could not be confirmed, as the prepartum CL is less responsive to oxytocin. During the late luteal phase in several animal species, oxytocin and PGF2alpha stimulate each other in a positive feed-back manner (SENGER, 2005). Therefore, oxytocin from the corpus luteum is one the pivotal mechanisms under prepartum luteolysis. However, in the pregnant bitch, Concannon et al. (1988) have shown that the immediate prepartum progesterone decrease coincides with an increase of PGF2alpha, suggesting that luteolysis is dependent mainly on prostaglandin. Also, Klarenbeek et al. (2007) showed in dogs that plasma oxytocin concentrations remain low in late pregnancy, increasing during the expulsive stage of parturition. In fact, luteolysis in the dog is independent of a uterine luteolysine (HOFFMANN et al., 1992). Regarding the physiology of gestation and labor, the canine placenta is responsible for the increasing production of prostaglandins (PGF2alpha and PGE2) as gestation progresses (KOWALEWSKI et al., 2010). Therefore, our results on decreased CL responsiveness to oxytocin, together with an unsynchronized hormonal profile between OT and PGF2alpha, suggest minor participation of oxytocin on prepartum luteolysis in dogs.

Under our methodology, it is possible to conclude that the canine CL does not express ERα mRNA during pregnancy. In addition, as decreased profile of OTR mRNA expression was verified in the present experiment. To our knowledge, this is the first research communication to assess simultaneously the OTR mRNA and ERα mRNA expression in the corpus luteum of pregnant and whelping bitches. However, our results highlight the need for further studies on OTR mRNA expression in CL of diestrous bitches, as well as ERβ mRNA in the corpus luteum of periparturient bitches.

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