Correlation of Oxytocin (OTR) and Estrogen Receptor (ER) mRNA in the Canine Placenta with the Detected Circulating Levels of Oxytocin and Estrogen during Pregnancy and Parturition

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Abstract: Placenta is considered an important endocrine organ during gestation, as well as can also serve as a target organ for sex steroids and pituitary hormones, mainly for the cascade of events that trigger parturition. In order to initiate studies on the canine placental endocrinology regarding estrogen and oxytocin, the present study verified the correlation among the Estrogen Receptor alpha (ERα) and Oxytocin Receptor (OTR) gene expression in the placenta tissue with the serum concentrations of their respective hormones (estrogen and oxytocin) during canine pregnancy and parturition. Real-time PCR was performed to quantify the levels of ERα mRNA and OTR mRNA in the placenta tissue of bitches (n=46) during Early (up to 20 days of gestation; n=11), Mid (20 to 40 days; n=12) and Late Pregnancy (40 to 60 days; n=12) and Parturition (first stage of labor; n=11). Serum samples were collected for the evaluation of estradiol and oxytocin (OT) concentrations by radioimmunoassay. Placental ERα mRNA expression showed a progressive decrease along gestation towards parturition. Placental ERα mRNA expression was significantly higher until 40 days of pregnancy in comparison to Parturition. Regarding OTR mRNA expression, no statistical differences among groups were verified. The Parturition Group presented higher estrogen concentrations in relation to Early and Mid Pregnancy Groups. Serum oxytocin concentrations were not different among pregnancy and parturition. During late pregnancy, a positive correlation was observed between placental ERα mRNA and oxytocin serum concentration. In conclusion, ERα mRNA in the canine placental tissue varies in a time-dependent manner, especially during the period of placentation and placental growth. Conversely, serum estrogen concentrations do not regulate directly the changes in OT receptors expression in the canine placenta.

Keywords: Estrogen, Oxytocin, Receptor, Placenta, Canine

Introduction

Placenta is considered an important endocrine organ during gestation, as it plays a role in pregnancy maintenance, as well as in the initiation of the cascade of events that trigger parturition. However, the canine placenta has a unique characteristic, as it lacks steroidogenesis to support pregnancy, leaving to the corpus luteum the main function to produce progesterone and estrogen (Hoffmann et al., 1994; Concannon, 2009). In fact, Nishiyama et al. (1999) could not identify immunohistochemically the enzyme aromatase (estrogen convertase) and other steroidogenic enzymes in the canine placenta. Hence, circulating estrogen in pregnant dogs is apparently derived from the ovaries and is less dependent on the placenta. On the other hand, the canine
A closer look into the relationship among the circulating concentrations of E and OT and the expression of placental Estrogen Receptor (ER) and OTR has not been established, generating incoherent events of the canine reproductive physiology.

Hence, our purpose in the present study was to verify the correlation among the ER alpha and OTR gene expression in the placenta with the serum concentrations of their respective hormones (E and OT) during pregnancy and parturition in dogs.

Materials and Methods

Animals and Experimental Groups

The use of animals in the present study was approved by the Ethical Committee of the School of Veterinary Medicine and Animal Science-University of São Paulo.

Pregnant mongrel bitches (n=46), aged 2 to 6 years, were assigned to four groups according to gestational age as established by ultrasound, reproductive history and measure of the fetal crown-rump length (Evans and 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). Females of Early, Mid and Late Pregnancy Groups were subjected to ovariohysterectomy and bitches in Parturition Group underwent cesarean section followed by ovariohysterectomy.

Placental fragments (taken always from the central area of the placental girdle) were harvested during hysterotomy and, afterwards, maintained frozen at -196°C until processing.

Blood samples were taken by venipuncture (left or right jugular vein) during surgery, after food had been withheld from the bitches for 12 h. The blood samples were allowed to clot at room temperature into the evacuated tubes without anticoagulant and centrifuged for 10 min at 1500×g. The serum was drawn off, separated in 2 aliquots and stored at -20°C until analysis.

Real-Time PCR Amplification

The TRizol (Guanidinium thiocyanate-phenolchloroform extraction; Invitrogen®, Carlsbad, USA) reagent was utilized for total RNA extraction from placenta tissue samples. 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). The obtained cDNA was stored at -20°C.

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 in other canine tissue samples standardized and simultaneously employed for sequences previously deposited in GenBank (www.ncbi.nlm.nih.gov). In order to avoid false positive results, primers were constructed with the intron region of the amplicon.

The primer sequences were as follows: 18S, 5′- TGGTTGATCCTGCCAGTAGCA-3′ and 5′- ATGAGCATTGCAGTTTCCT-3′; RPS5, 5′- TCACCTGTTGARACCCT-3′ and 5′- CCTGATGGACCATACCGCAG-3′; ERα, 5′- TGGTTGATCCTGCCAGTAC-3′ and 5′- ATGAGCCATTCGCAGTTTCACT-3′; RPS5, 5′- CCTGATTCACACGGCGTAG-3′; OTR, 5′- GGACATATTCCTCACGCTCC-3′; OTR, 5′- GGTTCTTGGTGTTGGGTGTG-3′ and 5′- GAACATATCCCTACGCTCC-3′; OTR, 5′- GACAAAGGGATGAGTTGCTC-3′.

Real-time PCR was performed in an Eppendorf® Mastercycler Realplex using the Platinum® SYBR Green PCR Master Mixkit (Invitrogen®, Carlsbad, USA). Sample tubes without placenta 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 corpus luteum), which revealed positive amplifications (Veiga et al., 2014; 2015). All reactions were performed in a total volume of 25 µL and heated to 50°C for 2 min and then 95°C for 10 min followed by 45 cycles comprising denaturation at 95°C for 15 sec and then annealing for 60 sec at the following temperatures: 61°C for RPS5, 60°C for 18S and 59°C 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 randomized tests utilizing the Relative Expression Software Tool (Rest-384© -version 2; Munich, Germany), following the software’s instructions provided by (Pfaffl et al., 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/ΔΔCt. The relative amplification efficiencies for the analyzed genes were 1.98 (18S), 2.05 (RPS5), 2.08 (ERα) and 1.94 (OTR).

**Hormonal Assay**

Serum estradiol concentrations were measured by radioimmunoassay with 125I labeled using the commercial kit DSI-4400 (Diagnostic Systems Laboratories, Webster, Texas, USA), previously validated for canine samples. The sensitivity of the estradiol assay at 95% binding was 1.85 pg mL−1 and the low and high intra-assay coefficients of variation were 7.32 and 0.05%, respectively.

The oxytocin assay was based on the protocol proposed by Elias et al. (1997) in which 1 mL of canine serum was added to acetone and ether petroleum. The oxytocin concentrations were measured through radioimmunoassay with a sensitivity of 0.9 pg mL−1 and the low and high intra-assay coefficients of variation were 12.6 and 7.0%, respectively.

**Statistical Analysis**

All data were evaluated using SAS System for Windows (SAS Institute Inc., Cary, NC, USA). All variables were normally distribute and presented homogeneous variances. Therefore, the effect of gestational period was determined using parametric tests (One Way Anova-PROC GLM; LSD as post-hoc test). Pearson correlation was used to calculate the relationship between variables studied in each group.

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**

**Placenta Tissue ERα mRNA and OTR mRNA**

Placenta tissue ERα mRNA expression showed a progressive decrease along gestation towards parturition. The expression of ERα mRNA was significantly (p<0.05) higher until 40 days of pregnancy (Early and Mid Pregnancy) in comparison to Parturition (Fig. 1). Regarding OTR mRNA expression, no statistical differences (p>0.05) among groups were verified throughout pregnancy and parturition.

**Estrogen and Oxytocin Assay**

Parturition Group (2.14±0.05 pg mL−1) presented higher (p<0.05) estrogen concentrations in relation to Early (1.94±0.03 pg mL−1) and Mid (1.99±0.02 pg mL−1) Pregnancy groups (Fig. 2). However, no statistical differences (p>0.05) were verified among groups until 40 days of pregnancy, as well as between the Late Pregnancy Group (2.0±0.03 pg mL−1) and Parturition Group.

Serum oxytocin concentrations were not different (p>0.05) among pregnancy (18.13±4.6, 20.95±9.35, 15.29±5.93 pg mL−1, respectively) groups and during parturition (10.35±5.79 pg mL−1) (Fig. 2).
Correlation Analysis

Regardless of the stage of pregnancy or parturition, placental ERα mRNA positively correlated with OTR mRNA (r = 0.94, p<0.0001). During late pregnancy, a positive correlation (r = 0.73, p = 0.04) was observed between placental ERα mRNA and oxytocin serum concentration.

Discussion

The present work aimed to follow-up the placenta tissue expression of ERα and OTR mRNA in a temporal manner and to establish a relationship with the estrogen and oxytocin concentrations in the periparturient bitch.

The results of the current experiment reveal a progressive decrease in ERα mRNA expression in the
canine placenta tissue along gestation. Although we do not have data of the expression on the protein level, these findings suggest a higher responsiveness to estrogen during early pregnancy (up to 20 days), around the time embryonic implantation and placental development begins in dogs. In many mammalian species, estrogen is synthesized by the placenta and it is autocrinally responsible for placential growth and differentiation (Schuler et al., 2002). Therefore, we can speculate that estrogen plays also a role in the development and growth of the canine placenta, in a more likely endocrine manner. In fact, estrogen is recognized to be involved in cell division and hypertrophy, as well as in placentual vascular development (Tamada and Ichikawa, 1980). In dogs, Vermeirsch et al. (2000) observed immunohistochemical staining for ERα solely in the mesenchimal decidua cells, surrounding endothelial cells, between the maternal and fetal components of the placenta and at the hypertrophied basement membrane of the maternal capillaries of the placental labyrinth. Moreover, estrogen receptors and ERα mRNAs are present in the cells surrounding the myometrial blood vessels of ewes, indicating that estrogen is involved in blood flow during pregnancy (Wu et al., 1996). Furthermore, estrogen participates in the conceptus morphological changes, such as fetal elongation and acquisition of a cylindrical form in rats (Tamada and Ichikawa, 1980). In light of our results and others statements, we can hypothesize that the influence of estrogen on the canine placenta tissue is focused in a more hemodynamic and morphological action, particularly during the initial phases of fetal and placental development. Conversely, after 40 days of gestation until whelping, we observed a decreased expression of ERα mRNA in the placenta tissue, suggesting a less expressive influence of estrogen on placental activity and fetal development at this time point. Thus, we can infer that the canine placenta tissue responds to estrogenic stimuli in a temporal manner.

Interestingly, the expression of ERα mRNA in the canine placenta tissue positively correlated with the circulating concentrations of oxytocin exclusively in the Late Pregnancy Group. This data can allude to a cause-effect relation, i.e., changes in oxytocin concentrations at the end of gestation can alter the placenta responsiveness to estrogen. In other words, oxytocin can sensitize the placenta for estrogen stimulation only at final gestation, allowing for an exogenous interference to promote changes such as estrogen-induced vascular development. As a hint, we suggest that the administration of oxytocin in the bitch near term can have a beneficial effect in placental blood flow and oxygenation. In humans, plasma OT concentrations remain low throughout the pregnancy and birth. However, estradiol treatment results in increased circulating oxytocin levels (Mitchell et al., 1998; Amico et al., 1981). Klarenbeek et al. (2007) demonstrated in dogs that OT concentrations remain low at the end of pregnancy, increasing only during the expulsion stage of vaginal labor. Our results are consistent with the work of Klarenbeek et al. (2007), because the OT levels remained constant throughout the pregnancy and first stage of labor. These observations suggest that circulating OT in dogs minimally affects the induction of labor. Therefore, the activation of myometrial OT receptors may occur downstream of the paracrine activity of placental OT, rather than as an endocrine manner.

Regarding the endocrinology of late gestation and parturition, we observed a singular feature in dogs. While the increasing concentration of estrogen stimulates the rise in uterine oxytocin receptors towards the initiation of labor in women and other animal species (Blanks et al., 2003; Spencer et al., 2004), we could not find a correlation between estrogen concentration and the expression of placental OTR mRNA. The placenta is considered to be one of the OT-synthesizing organs responsible for stimulating endometrial PGF2α production, resulting in luteolysis at the beginning of labor (Ilidromiti et al., 2012). In dogs, we could not show a link among estrogen concentration, the increase in placental oxytocin receptors at term pregnancy and the initiating of labor. On the other hand, we showed a positive correlation between the OTR and ERα mRNA expressions in the placenta. Therefore, we should consider a possible stimulation mechanism that acts both on the expression of OTR and ERα mRNA in the canine placenta tissue. For this, studies should be conducted to verify the direct influence of the signaling by the fetal Hypothalamic-Pituitary-Adrenal (HPA) axis and the role of placental Corticotrophin Releasing Hormone (CRH) in the stimulation of oxytocin and prostaglandin synthesis by the canine placenta. Further studies on OTR sensitization should be performed in order to determine the total number of binding sites when exposed to endogenous oxytocin, as to consider a possible change in the binding affinity to oxytocin towards parturition. Moreover, the possibility that progesterone down regulates OTR mRNA should be further investigated using experiments on the hormonal control of pregnancy.

To our knowledge, there are few studies that demonstrate the simultaneous activity of estrogen and oxytocin in the placenta during pregnancy and parturition in dogs. Hence, this is the first research to show both the endocrine profile of estrogen and oxytocin and the hormonal receptors in the canine placenta throughout pregnancy. Although we haven’t obtained results on the estrogen and oxytocin expression at the protein level yet, our results highlight the possible application of a hormonal treatment in the periparturient bitch, with a focus on placenta as the target tissue.
Conclusion

As a descriptive study, we conclude that Estrogen Receptor (ER) mRNA in the canine placental tissue varies in a time-dependent manner. Placenta tissue ERα mRNA expression showed a progressive decrease along gestation towards parturition, while OTR mRNA expression remained unchanged. In addition, circulating levels of oxytocin positively correlate with placental ERα mRNA expression.

Author’s Contributions

Gisele Almeida Lima Veiga and Camila Infantosi Vannucchi: Conceptualized and supervised the research, collected samples, drafted the manuscript, ran all statistical tests and have read and approved the manuscript.

Marcella Pecora Milazzotto: Conceptualized and supervised the research and have read and approved the manuscript.

Marcilio Nichi: Ran all statistical tests and have read and approved the manuscript.

Cristina de Fatima Lucio and Liege Cristina Garcia Silva: Collected samples and have read and approved the manuscript.

Conflict of Interest

The researchers have no conflict of interest.

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