Luteal ANGPT-TIE system during selected stages of pregnancy, and normal and antigestagen-induced luteolysis in the dog

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Abstract

Rapid establishment of a vascular network is essential for normal functionality of the corpus luteum (CL). The early luteal phase is associated with increased expression of the VEGF system in canine CL. Acting in synchrony with angiopoietins (ANGPTs), VEGF system plays major roles in stabilization of blood vessels. However, the expression of the ANGPT system has not yet been investigated in the dog. Therefore, here, we investigated the luteal expression of ANGPT1, -2, and of their receptors TIE1 and -2, in pregnant dogs at selected time points during pregnancy and at normal and antigestagen-induced luteolysis. Additionally, luteal cells from early CL were incubated with PGE2 and its effects on the ANGPT system were assessed. Whereas the luteal *ANGPT1* was stable until mid-gestation, *TIE1* was elevated post-implantation, their expression decreased toward prepartum luteolysis. The *ANGPT2*- and *TIE2*-mRNA did not vary during pregnancy. The *ANGPT2/ANGPT1* ratio was elevated during prepartum luteolysis. PGE2 increased *ANGPT2*, but suppressed *ANGPT1* levels. None of the ANGPT-system members was affected by antigestagen treatment in mid-pregnancy. Localization of ANGPT1 was predominantly found in the *tunica intima* and *media* of vessels and ANGPT2 stained strongly in luteal cells. Both ANGPTs were localized in macrophages. TIE1 stained in the vascular *tunica media*, in luteal cells and macrophages, whereas TIE2 was colocalized with ANGPT1 in vascular components. In conclusion, high expression of *ANGPT1* during the increased presence of VEGFA in early canine CL implies its contribution to vascular network development. The upregulation of the *ANGPT2/ANGPT1* ratio during prepartum luteolysis indicates involvement of the ANGPT system in PGF2 α -mediated vascular destabilization.

Reproduction (2018) **156** 451–461

Introduction

The corpus luteum (CL) is a rapidly growing, transient endocrine gland, relatively small in the dog, that arises within the ovary following ovulation by differentiation of the remnants of granulosa and theca cells into luteal cells (Channing *et al.* 1980, Smith *et al.* 1994, Fraser & Wulff 2003, Kowalewski *et al.* 2015). During the early luteal phase, rapid angiogenesis and vasculogenesis are fundamental requirements for the proliferation, development and maintenance of CL (Stouffer & Hannebold 2015). An integral mediator of both processes is vascular endothelial growth factor A (VEGFA) acting through its respective receptors (VEGFR1/Flt-1, VEGFR2/Flk-1), which are referred to together as the VEGF system (Ferrara & Davis-Smyth 1997, Cebe-Suarez *et al.* 2006, Stuttfeld & Ballmer-Hofer 2009). The function of this system is complemented, and under certain conditions coordinated, by angiopoietins (ANGPTs) and their tyrosine kinase receptors, TIE1 and TIE2, respectively. ANGPTs affect development of vascular

networks by regulating the stability and/or disruption of endothelial cells, the growth of which is stimulated by VEGFs (Hanahan 1997, Brindle *et al.* 2006). In particular, ANGPT2 is a potent disruptor of endothelial cells (Brindle *et al.* 2006, Felcht *et al.* 2012) and allows VEGFA to induce proliferation, invasion and, thereby, formation of new blood vessels in developing CL during the early luteal phase (Fraser & Wulff 2003). At the mid-luteal phase, in the presence of VEGFA, ANGPT1 supports vessel integrity and inhibits vascular leakage, thereby promoting survival of blood vessels (Hazzard & Stouffer 2000, Wulff *et al.* 2000, Sugino *et al.* 2005). Finally, at the end of the CL life span, during the physiological processes of luteal regression or luteolysis, in the absence of angiogenic factors and in particular of VEGFA, the luteal microvasculature is disrupted by ANGPT2 (Hazzard & Stouffer 2000, Wulff *et al.* 2000, Sugino *et al.* 2005). Apparently, thus, establishment and maintenance of CL depend not only on vasculogenesis and angiogenesis, but also on vascular structural stability, being essential for an adequate supply of luteal

tissues with oxygen and nutrients, allowing the delivery of progesterone (P4), estrogens and other endocrine factors into the circulation.

In dogs, CLs exhibit an inherent life span, and there is no other major source of circulating P4 in this species during pregnancy and non-pregnant cycles (Concannon *et al.* 1989, Hoffmann *et al.* 1994, Nishiyama *et al.* 1999). This emphasizes the role of an adequate blood supply for the provision of circulating luteal steroids in this species. As in other mammalian species, the canine CL is formed immediately after ovulation, in a process which is associated with intense angiogenesis (Hoffmann *et al.* 2004a, Gram *et al.* 2015a). As recently reported from our research, a high vascularization rate and increased blood supply within canine CL during the early stages of its development are mirrored in increased availability of the VEGF system and vasodilatory endothelin receptor B (ETB) (Gram *et al.* 2015a,b). During early diestrus, prostaglandins (PG), in particular PGE2 acting as a luteotropic factor, appear to be involved in the establishment of canine CL function (Kowalewski *et al.* 2013, 2015, Janowski *et al.* 2014). Interestingly, PGE2 has been shown to increase expression of ETB in isolated canine primary luteal cells (Gram *et al.* 2015b). These findings show that PGs, in particular PGE2, participate in regulating luteal vascular function in the dog. As evidenced from investigations on the expression of type I membrane glycoprotein endoglin (END), the vascularization rate slows down in the mid-luteal phase in both CL of pregnancy and in non-pregnant dogs (Hoffmann *et al.* 2004a, Nowaczyk *et al.* 2017). Interestingly, in pregnant dogs, concomitantly with increasing circulating concentrations of luteolytic PGF2 α , an upregulation of the vasoconstrictive endothelin receptor ETA is observed during prepartum luteolysis (Gram *et al.* 2015b). This clearly indicates the involvement of vascular endothelial functionality at the time of cessation of luteal function during the onset of parturition in the dog. A possible contribution of the inflammatory and immune responses was recently indicated from our analysis of luteal transcripts during prepartum luteolysis (Zatta *et al.* 2017). Contrasting with the situation observed in pregnant animals, there is a slow luteal regression in the absence of pregnancy, obviously devoid of functional alteration of the vascular bed (Hoffmann *et al.* 2004b, Gram *et al.* 2015b).

Nevertheless, in contrast with other domestic animals, and despite the undisputed critical importance of the ANGPT system in regulating vascular stabilization and remodeling, until now its expression in the canine CL of pregnancy has not been investigated. Therefore, based on the hypothesis that the ANGPT system is involved in regulation of canine CL function, and in order to expand our understanding about the potential role of vascular growth factors in canine CL, here, the following experiments were performed: (1) a descriptive study addressing the protein and mRNA expression of

ANGPT1, -2 and their receptors TIE1 and TIE2 in canine CL at selected time points during pregnancy and at normal prepartum luteolysis; (2) an *in vivo* approach investigating P4-mediated effects in bitches, in which luteal function was interrupted during mid-gestation with the antigestagen aglepristone and (3) an *in vitro* functional approach investigating the role of PGE2 in regulating expression of the ANGPT system in canine primary luteal cells isolated from early CL.

Materials and methods

Experimental design, collection of luteal tissue and preservation

All luteal tissues were derived from pregnant, healthy, mixed breed dogs and were used in our previous studies (Kowalewski *et al.* 2013, Gram *et al.* 2015a,b). All experimental procedures were performed in accordance with animal welfare legislation and approved by the respective authorities of the Justus-Liebig University (permit no. II 25.3-19c20-15c GI 18/14 and VI 3-19c-20/15c GI 18,14), Giessen, Germany and the University of Ankara (permit no. Ankara 2006/06), Ankara, Turkey.

Briefly, CLs were collected following ovariectomy (OHE) at different stages of pregnancy: early luteal phase (pre-implantation, days 8–12, $n=5$, and post-implantation, days 18–25, $n=5$), mid-luteal phase (mid-gestation, days 35–40, $n=5$) and prepartum luteolysis ($n=3$). Additionally, aglepristone (Alizine, Virbac, Bad Oldesloe, Germany; 10 mg/kg bw; 2 $\times$ /24 h apart), P4 receptor (PGR) antagonist was used for termination of pregnancies in mid-pregnant dogs (days 40–45 of pregnancy; $n=10$) and OHE was performed 24 h ($n=5$) and 72 h ($n=5$) after the second treatment.

Dogs 2–8 years of age were mated 2 days after ovulation (determined as the day when P4 for the first time exceeded levels of at least 5 ng/mL in peripheral blood). The day of mating was considered as day 0 of pregnancy. The pre-implantation stage of pregnancy was detected by observation of embryos in uterine flushes. Prepartum luteolysis samples were obtained by assaying P4 concentration in blood serum starting on day 58 of pregnancy. Serum P4 levels were then measured every 6 h until a continuous decrease (below 2–3 ng/mL) was detected in 3 consecutive measurements, and then OHE was performed.

After collection, CLs were carefully dissected, cleared of surrounding tissues and fixed with 10% neutral PBS-buffered formalin for 24 h at +4°C, and then incubated in PBS for 1 week, dehydrated through an ethanol series and embedded in paraffin wax for immunohistochemistry (IHC). For total RNA extraction, tissue samples were incubated in RNAlater (Ambion Biotechnology GmbH, Wiesbaden, Germany) for 24 h at +4°C and then frozen at –80°C for later evaluation of gene expression.

Primary luteal cell cultures

Primary luteal cells were isolated from CLs of bitches ($n=15$) that underwent routine OHE in the Department of Small Animal Reproduction, Vetsuisse Faculty, University of Zurich. All dogs

were clinically healthy and were in the early diestrus stage of the reproductive cycle (7–14 days after clinical signs of estrus had ceased). All tissue materials were used in our previous study determining the role of PGE2 in regulating canine luteal steroid acute regulatory STAR protein expression and steroidogenesis (Kowalewski *et al.* 2013, Gram *et al.* 2015b). In those previous experiments, the steroidogenic capability of isolated cells and their responsiveness to stimulation with PGE2 were demonstrated. In the present study, using the available mRNA, the impact of PGE2 on gene expression of *ANGPT* system family members (i.e., *ANGPT1*, *ANGPT2*, *TIE1* and *TIE2*) was investigated in these cells. In brief, luteal cells were isolated following tissue dissociation with 0.15% collagenase (Sigma-Aldrich Chemie GmbH). Crude cell and remnant tissue suspension was filtered through a 75 µm cell strainer (BD Biosciences, Basel, Switzerland). This was followed by washing steps with PBS. Freshly isolated cells were then suspended in culture medium (DMEM/F12, pH 7.2–7.4, 10% heat-inactivated FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, 1% ITS (Insulin-Transferrin-Selenium); all from Chemie Brunschwig, AG, Basel, Switzerland), transferred into six-well plates and cultured in an incubator (37°C, 5% CO₂). Experiments were performed with cells that had reached 70–80% confluence. Importantly, non-trypsinized (i.e., passage 0) cells were used for *in vitro* experiments performed in serum-free medium. Cells were treated with 20 µM PGE2 (Sigma-Aldrich Chemie GmbH), which was determined to be an effective dosage in functional assays with canine proximal *STAR* promoter bearing intact cAMP/PKA-responsive elements (Kowalewski *et al.* 2013). Additionally, P4 concentrations were determined as a measure of steroidogenic activity in PGE2-treated cells (Kowalewski *et al.* 2013). Treatments were done for 6 h, a time course, which is needed to induce a full steroidogenic response at the level of STAR-dependent steroidogenesis (Kowalewski *et al.* 2013). With this approach, PGE2 was determined to be an activator of *STAR* gene expression in canine primary luteal cells at levels that are similar to those observed with dbcAMP treatment (Kowalewski *et al.* 2013).

RNA isolation, reverse transcription and semi-quantitative real-time (TaqMan) PCR

Total RNA was extracted from CL samples and luteal cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and our previously published protocols (Kowalewski *et al.* 2006b, 2011a). A NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific AG) was used to check the quality and quantity of isolated total RNA. Following DNase treatment with RQ1 RNase-free DNase (Promega, Dübendorf, Switzerland), for removal of genomic DNA, all RNA samples were reverse transcribed (RT) into cDNA using random hexamers as primers in the presence of other RT reagents (Applied Biosystems by Thermo Fisher). The ABI PRISM 7500 Sequence Detection System (Applied Biosystems) was used to perform semi-quantitative Real-Time (TaqMan) PCR. The amplification conditions were as follows: denaturation at 95°C for 10 min, followed by 40 cycles, 95°C for 15 s and 60°C for 60 s, each. As a control, all experiments were run with all necessary components of the

amplification reaction except for reverse transcriptase to check for any potential genomic DNA contamination (the so-called minus-RT control). Additionally, a non-template control was performed by using water instead of cDNA.

Self-designed primers and TaqMan Probes for *ANGPT* system members were selected using the Primer Express Software v 2.0 (Applied Biosystems) and were bought from Microsynth (Balgach, Switzerland). Primers and 6-carboxyfluorescein (6-FAM)- and 6-carboxytetramethylrhodamine (TAMRA)-labeled TaqMan probes are listed in Table 1. Amplifications of all genes were done in duplicates. Three different reference genes, *GAPDH*, *CYCLOPHILIN A* and β -*ACTIN* were used to normalize gene expression profiles.

For *CYCLOPHILIN A* (Prod. No. Cf03986523_gH) and β -*ACTIN* (Prod. No.184 Cf03023880_g1), commercially available canine-specific TaqMan Gene Expression Assays were used, purchased from Applied Biosystems. The comparative CT ($\Delta\Delta$ CT) method was used to calculate relative gene expression levels of each target gene according to the protocol of the manufacturer of the ABI Prism 7500 (Applied Biosystems). The sample with the lowest expression for each target gene was used as a calibrator. The amplification efficiency of the PCR assays was calculated by the CT slope method ensuring approximately 100% reaction efficiency.

Following quantification of *ANGPT1* and *ANGPT2* mRNA expression with the comparative CT method using three different reference genes (*GAPDH*, *CYCLOPHILIN A* and β -*ACTIN*) as normalizers, and applying the same Ct values and calibrators, relative gene expression values for both genes were divided by each other to calculate the *ANGPT2/ANGPT1* mRNA ratio.

Molecular cloning of canine *TIE1* and *TIE2* sequences

Canine-specific *TIE1* and *TIE2* (*TEK*) sequences have not been characterized previously, and were available from GenBank only as predicted sequences. In order to provide templates for the subsequent PCR reactions, molecular cloning and sequencing were performed following our previously published protocol (Kowalewski *et al.* 2006b, 2011b, Kautz *et al.* 2014). Therefore, using available predicted sequences, gene-specific primers were designed that allow us to amplify partial CDS of canine-specific *TIE1* and *TIE2* (*TEK*). Following DNase treatment (Promega) and RT reactions, a conventional hot start PCR reaction was done using the GeneAmp Gold RNA PCR Kit (Applied Biosystems) following the manufacturer's protocol. Primers, purchased from Microsynth, were as follows: *TIE1*, forward: 5'-AGA GCC GGG TCT TGG AGA-3', reverse: 5'-ATG CCC GCG TAA GTG AAG TT-3'; *TIE2* forward: 5'-AAT CAA CGG TGC CTA TTT CTG T-3', reverse: 5'-CAG ACC CTG CCA ACC TGT G-3'. PCR reactions were run with the annealing temperature set at 60°C for both *TIE1* and *TIE2*. Negative controls were as for TaqMan PCR. Specific amplicons of 601 bp and 604 bp for *TIE1* and *TIE2*, respectively, were generated, separated in 2% ethidium bromide-stained agarose gel and purified using the Qiaex II gel extraction system (Qiagen GmbH). Following subcloning of PCR products into the pGEM-T vector (Promega), they were transformed and amplified in XL1 Blue competent cells (Stratagene, La Jolla,

Table 1 List of self-designed primers used for Real-Time (TaqMan) PCR.

Gene	Primer sequence	Product length (bp)	Accession number
<i>ANGPT1</i>	F: 5'-AAG TCG GAG ATG GCC CAG ATA-3' R: 5'-CAG TCT GAG AGA GGA GGC TGG TT-3' T: 5'-CAC ACG GCC ACC ATG CTC GAG ATA G-3'	91	AB192412.2
<i>ANGPT2</i>	F: 5'-ACG AGG CGT ACT CGC TGT ATG-3' R: 5'-CCT GGT TGG CTT ATG CTG CTT-3' T: 5'-ATT CAC CTT AAA GGA CTT ACA GGG ACG GCC-3'	118	AB256019.1
<i>TIE1</i>	F: 5'-GAC CTG TGC TGA GCT CTA TGA GAA-3' R: 5'-ACG CAT CAG CTC GTA CAC TTC A-3' T: 5'-TAC CGC ATG GAA CAG CCT CGA AAC TG-3'	91	MG932084
<i>TIE2</i>	F: 5'-TCA GCC AGG TAC ATA GGA GGA AAC-3' R: 5'-GTT ACA TTC AGG TCC CCA CTT CTG-3' T: 5'-TCT TCA CCT CGG CCT TCA CCA GGC T-3'	96	MG932085
<i>GAPDH</i>	F: 5'-GCT GCC AAA TAT GAC GAC ATC A-3' R: 5'-GTA GCC CAG GAT GCC TTT GAG-3' T: 5'-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3'	75	AB028142.1

F, forward; R, reverse; T, TaqMan probe.

CA, USA). Plasmids were isolated and purified using Pure Yield Plasmid MidiPrep System (Promega) and were sent for commercial sequencing on both strands with T7 and Sp6 primers (Microsynth). Finally, the sequences obtained were submitted to GenBank with the following accession numbers: *TIE1* GenBank acc. Nr. MG932084, and *TIE2* (*TEK*) GenBank acc. Nr. MG932085. BLAST analysis revealed 93% identity with respective fragments of human and sheep *TIE1* sequences. There was a 92% identity with fragments of human and sheep for the *TIE2* sequence.

Immunohistochemistry

The indirect immunoperoxidase immunohistochemistry (IHC) assay was performed on tissue samples obtained from pregnant dogs using our previously published protocol (Kowalewski *et al.* 2006a,b). Briefly, formalin-fixed, paraffin-embedded CL samples were sectioned at 2–3 µm thickness and mounted onto SuperFrost microscope slides (Menzel-Glaeser, Braunschweig, Germany). Following deparaffinization with xylene and rehydration in a graded ethanol series, tissue sections were heated in a microwave oven at 560W for 15 min in 10mM citrate buffer pH 6.0 for antigen retrieval. Nonspecific antibody reactivity was blocked by incubating slides with 10% normal serum from the same species in which the secondary antibody was produced. Then, slides were incubated with primary antibodies overnight at 4°C. The dilutions of primary antibodies used in this study were 1:50 for canine-specific, rabbit polyclonal anti-ANGPT1 antibody (PAA008Ca01, Cloud-Clone Corp., TX, USA); 1:200 for rabbit anti-mouse ANGPT2 polyclonal antibody (TA343276, OriGene Technologies Inc., Rockville, MD, USA); 1:100 for rabbit anti-human TIE1 (H-180) polyclonal antibody (sc-9025, Santa Cruz Biotechnology Inc.) and 1:200 for goat anti-human TIE2 (A-14) polyclonal antibody (sc-31268, Santa Cruz Biotechnology). Macrophages were identified by using polyclonal rabbit anti-human MHCII antibody (orb101661, Biorbyt Limited, Cambridge, UK, dilution 1:200). As an isotype control, non-immune IgGs of the same species as the primary antibody were applied instead of primary antibodies, using the same protein concentration. Afterward, sections were rinsed

with IHC buffer containing 0.3% Triton X pH 7.2–7.4 (0.8mM Na₂HPO₄, 1.47mM KH₂PO₄, 2.68mM KCl, 137mM NaCl) and incubated with biotin-labeled secondary antibodies at 1:100 dilution each. These were horse anti-goat IgG BA-9500 and goat anti-rabbit IgG BA1000 (both purchased from Vector Laboratories Inc., Burlingame, CA, USA). Subsequently, sections were washed, incubated with a streptavidin-peroxidase Vectastain ABC kit (Vector Laboratories) for 30 min and then antigen-antibody complexes were visualized using the Liquid DAB+ substrate kit (Dako Schweiz AG). Counterstaining was performed using hematoxylin. Finally, sections were dehydrated in a graded ethanol series and xylene and mounted in Histokit 180 (Assistant, Osterode, Germany). Slides were assessed qualitatively.

Statistical analyses

Since the data obtained in our current study did not show a normal distribution, logarithmic transformations were performed for normalization. For statistical calculation of differences in the expression of *ANGPT*-system members between different stages of pregnancy (time-dependent effect), one-way ANOVA was performed. This, in case of $P < 0.05$, was followed by the Tukey–Kramer multiple comparisons test, in which all groups were compared with each other. In order to assess the effects of the P4 receptor antagonist (aglepristone) on expression of the *ANGPT* system, first a global comparison was performed by applying one-way ANOVA. Next, using the mid-pregnant group as a non-treated control, Dunnett's multiple comparison test was performed to show expression of the *ANGPT* system in CL of antigestagen-treated dogs. In this case, the results show the n -fold change in each target gene mRNA compared with its expression at mid-gestation in a non-treated control. The effect of PGE2 on expression of *ANGPT* system members in cell cultures was calculated using an unpaired two-tailed Student's t -test. For all analyses, numerical data are presented as geometric means $X_g \pm$ geometric standard deviation (s.d.). All statistical calculations were performed using the statistical software program GraphPad 3.06 (GraphPad Software). The level of significance was considered as $P < 0.05$.

Results

Quantification of gene expression

Expression of *ANGPT1*, -2 and their receptors *TIE1* and *TIE2* was detectable in all CL samples at every pregnancy stage investigated in the present study. The *ANGPT1* mRNA showed a time-dependent expression profile during pregnancy (ANOVA, $P < 0.0001$) (Fig. 1A). It was more or less constantly expressed from pre-implantation until mid-gestation ($P > 0.05$); however, it decreased significantly ($P < 0.01$) during prepartum luteolysis compared with all previous stages of pregnancy (Fig. 1A). The expression of *ANGPT2* mRNA did not differ significantly over time (ANOVA, $P = 0.3$) (Fig. 1B). The *ANGPT2/ANGPT1* ratio was also assessed and was time related (ANOVA, $P = 0.001$). Thus, whereas it was unaffected from early until mid-gestation ($P > 0.05$), it increased significantly ($P < 0.05$) during prepartum luteolysis compared with all previous stages (Fig. 1C).

As for *ANGPT* receptors, *TIE1* mRNA levels changed significantly over time (ANOVA, $P = 0.02$).

Figure 1 Expression of *ANGPT1*, *ANGPT2* and the *ANGPT2/ANGPT1* mRNA ratio as determined by Real-Time (TaqMan) PCR ($X \pm s.d.$) in canine CL at selected stages of pregnancy (A, B and C) and during antigestagen (aglepristone)-induced luteolysis in mid-pregnant dogs (D, E and F); compared with the mid-gestation group used as non-treated control). Pre-implant. = pre-implantation stage of pregnancy, Post-implant. = post-implantation stage of pregnancy, Mid-gest. = mid-gestation stage of pregnancy, Prep. Lut. = normal prepartum luteolysis.

Whereas its expression did not vary strongly from pre-implantation until mid-gestation, it was significantly lower at prepartum luteolysis compared with the post-implantation stage ($P < 0.05$) (Fig. 2A). Similarly, as for *ANGPT2*, the abundance of *TIE2* transcripts did not differ significantly over time (ANOVA, $P = 0.1$) (Fig. 2B).

With regard to the preterm luteolysis/abortion groups, the expression of all *ANGPT* family members and the *ANGPT2/ANGPT1* ratio remained unaffected by treatment with an antigestagen, both 24 h and 72 h after the second application of aglepristone, compared with the mid-gestation group (ANOVA: *ANGPT1*, $P = 0.3$; *ANGPT2*, $P = 0.7$; *ANGPT2/ANGPT1*, $P = 0.7$; *TIE1*, $P = 0.3$; *TIE2*, $P = 0.8$) (Figs 1D, E, F and 2C, D).

Immunolocalization of the ANGPT system in CL of pregnant dogs

Expression of *ANGPT1*, -2 and their receptors *TIE1* and *TIE2* was detectable at the protein level in the CL at all selected time points during canine pregnancy. Whereas *ANGPT1* was clearly detectable in vascular tunica media and tunica intima (endothelial cells) (Fig. 3A, B and C), only weak or no signals were observed in steroidogenic luteal cells (Fig. 3A, B, C and D). Additionally, some signals were localized in interstitial cells in proximity to luteal vessels, which were identified as immune system-derived macrophages (Nowaczyk et al. 2017). At mid-gestation and during prepartum luteolysis, signal intensities appeared weaker and remained primarily localized in

Figure 2 Expression of *TIE1* and *TIE2* as determined by Real-Time (TaqMan) PCR ($X \pm s.d.$) in canine CL at selected stages of pregnancy (A and B) and during antigestagen (aglepristone)-induced luteolysis in mid-pregnant dogs (C and D); compared with the mid-gestation group used as non-treated control). Bars with different letters differ at $P < 0.05$. Pre-implant. = pre-implantation stage of pregnancy, Post-implant. = post-implantation stage of pregnancy, Mid-gest. = mid-gestation stage of pregnancy, Prep. Lut. = normal prepartum luteolysis.

Figure 3 Immunohistochemical localization of ANGPT1 in canine CL at selected time points throughout pregnancy: pre-implantation (A), post-implantation (B), mid-gestation (C) and prepartum luteolysis (D). Strong ANGPT1 signals were detected in the *tunica media* of vessels (open arrows in A, B, C and D), endothelial cells (solid arrows in A, B, C and D) and in luteal macrophages (open arrowheads in B and C). Weaker or no signals are detected in luteal cells (solid arrowheads in A, B, C and D). No background staining is detected in the isotype control (inset in D).

vascular structures and immune cells (Fig. 3C and D). In particular, during prepartum luteolysis, luteal cells appeared negative for ANGPT1. Conversely, ANGPT2 was strongly represented throughout the CL life span. Signals were seen in luteal cells and macrophages. The staining intensity varied between cells within the same population (Fig. 4A, B, C and D). No staining was observed in the vascular bed (Fig. 4A, B, C and D). Detection of MHCII was performed in order to more easily differentiate cell types within canine CL (shown on consecutive sections in Fig. 4E and F). MHCII is a marker of antigen-presenting cells, shown recently to be predominantly localized in macrophages localized close to luteal blood vessels in canine CL (Nowaczyk *et al.* 2017). The distribution patterns of TIE1 were similar to those observed for ANGPT2. Thus, signals were present in luteal cells and macrophages (Fig. 5A, B, C and D). Those signals localized in luteal cells appeared, however, weaker than those observed for ANGPT2. Additionally, staining was observed in leukocytes localized in blood vessels (Fig. 5A and C). Relatively weak staining was noted for TIE1 in *tunica media* and *tunica intima* of vessels (endothelial cells) (Fig. 5A, B and D). Signals were present in all stages investigated, but intensities appeared weaker during prepartum luteolysis (Fig. 5D). As for TIE2 expression, it was predominantly localized in *tunica media* of larger vessels and *intima* of smaller vessels and capillaries (Fig. 6A, B, C and D). Sporadically, signals

Figure 4 Immunohistochemical localization of ANGPT2 in canine CL at selected time points throughout pregnancy: pre-implantation (A), post-implantation (B), mid-gestation (C) and prepartum luteolysis (D). Additionally, consecutive sections were stained against MHCII (E) and ANGPT2 (F, shown at mid-gestation). The detection of MHCII was performed in order to more easily differentiate cell types within canine CL. Solid arrowheads indicate luteal cells (A-F). ANGPT2 was localized in luteal cells and in interstitial cells, mostly those localized close to blood vessels (open arrowheads) expressing MHCII on consecutive sections, that were identified as macrophages (open arrowheads in E and F). No background staining is observed in the isotype control (inset in D).

were observed in luteal cells (Fig. 6A, B, C and D). Signals were detectable without striking differences throughout pregnancy, but appeared somewhat weaker at prepartum luteolysis (Fig. 6A, B, C and D).

PGE2-mediated effects on expression of the ANGPT-system in canine primary luteal cells from early diestrus

Whereas *ANGPT1* expression in luteal cells was significantly ($P < 0.0001$) decreased in response to the PGE2 treatment (Fig. 7A), expression of *ANGPT2* showed the opposite effect, being strongly upregulated ($P < 0.0001$) (Fig. 7B). The expression of *TIE2* did not change significantly ($P = 0.1$) (Fig. 7C); *TIE1* expression was generally low in isolated cells and remained below the detection limit in most of the isolates in response to treatment with PGE2.

Figure 5 Immunohistochemical localization of TIE1 in canine CL at selected time points throughout pregnancy: pre-implantation (A), post-implantation (B), mid-gestation (C) and prepartum luteolysis (D). TIE1 was expressed in the *tunica media* of the vessels (indicated as open arrows in A, B and D), endothelial cells (solid arrows in A and D), luteal cells (solid arrowheads in A, B, C and D) and in luteal macrophages (open arrowheads in A, B, C and D). Leukocytes stained with anti-TIE1 antibody are also localized in blood vessels (asterisks in A and C). No background staining is detected in the isotype control (insert to D).

Discussion

By interacting with other angiogenic and vasoactive factors, ANGPTs play crucial roles in balancing vascular maintenance and regression. Here, in addition to our previous studies showing time-dependent expression of VEGFA- and ET-family members, expression of the ANGPT-system was investigated in canine CL at selected time points during pregnancy from the early luteal phase until prepartum luteolysis. Distinct expression and localization patterns were found for ANGPTs and their receptors. Thus, whereas ANGPT1 was found predominantly in *tunica media* of large blood vessels, and its mRNA showed a time-related expression pattern with higher expression levels observed in early CL, ANGPT2 was predominantly localized in luteal cells, with no signals detectable in luteal vessels. Its signals were, however, clearly detectable throughout the CL life span. Interestingly, both factors, ANGPT1 and -2, were localized in interstitial cells found in close proximity to vessels, which were identified as macrophages (Nowaczyk *et al.* 2017). The increased availability of ANGPTs during the early luteal phase was associated with elevated levels of TIE1 receptor. TIE2 receptor was less strikingly regulated throughout the luteal phase, but appeared abundantly expressed, in particular in the vascular bed. The IHC signals appeared to be weaker during prepartum luteolysis, when TIE1 expression was

Figure 6 Localization of TIE2 in canine CL at selected time points throughout pregnancy: pre-implantation (A), post-implantation (B), mid-gestation (C) and prepartum luteolysis (D). TIE2 expression is observed in the *tunica media* of blood vessels (open arrows in A, B and C), endothelial cells (solid arrows in A, B, C and D) and weaker signals in luteal cells (solid arrowheads in A, B, C and D). No background staining is seen in the isotype control (insert to D).

also decreased. Whereas the localization pattern of TIE1 appeared similar to the distribution of ANGPT2, and targeted mainly to luteal and immune cells, TIE2 receptor seemed to mirror the localization of ANGPT1 and was predominantly found in luteal vessels. These distribution patterns of ANGPT family members clearly indicate possible interplay between different cellular components of the CL and, thereby, an involvement of ANGPTs in local autocrine and paracrine regulatory mechanisms. Thus, acting through its receptors, in particular TIE2, the clearly detectable ANGPT2, together with increased expression of VEGFA at the beginning of the luteal phase (Gram *et al.* 2015a), could contribute to development of the canine CL by stimulating sprouting of blood vessels. This would resemble the mechanism described before in humans (Wulff *et al.* 2000). Furthermore, the ANGPT1-mediated (Shirasuna *et al.* 2012) recruitment of periendothelial cells (i.e., pericytes) in the presence of VEGFA is a phenomenon described before for some species, e.g., for cattle (Schams & Berisha 2004), buffalo (Mishra *et al.* 2016) and rats (Matsuoka-Sakata *et al.* 2006). This could also apply in the canine species in which elevated VEGFA at post implantation (Gram *et al.* 2015a) coincides with the clearly detectable presence of ANGPT1 and TIE2 receptor in luteal vessels, possibly cumulatively contributing to structural and functional stabilization of a fully developed vascular bed. Later during pregnancy, at mid-gestation, a significant decrease in VEGFA expression (Gram *et al.* 2015a), at still relatively high availability of ANGPT1, could result in suppression of vascular function. Moreover, with

Figure 7 Effects of PGE2 on mRNA expression of *ANGPT1*, *ANGPT2* and *TIE2* in canine primary luteal cells obtained from non-pregnant dogs during the early luteal phase. Non-stimulated cells served as control. *ANGPT1* (A), *ANGPT2* (B) and *TIE2* (C) mRNA expression was determined by real-time (TaqMan) PCR ($X \pm s.d.$). Bars with different letters differ at $P < 0.0001$.

regard to vascular function, ANGPTs were described as being involved in the regulation of vascular tone and blood flow, acting on smooth muscle cells (Alfieri *et al.* 2014), in which canine ANGPT1 and TIE2 were also localized. Similarly, ANGPT-mediated nitric oxide (NO) release in arterioles was shown previously in mice (Alfieri *et al.* 2014). Nevertheless, the involvement of ANGPTs in regulating luteal vascular tone and blood flow in the dog remains to be elucidated. Furthermore, in particular, the localization of TIE1 and ANGPT2 in luteal cells, suggests involvement of ANGPTs in the regulation of luteal cell function. This seems to be an interesting finding; however, the exact function of ANGPTs in steroidogenic cells remains to be elucidated.

Although the physiological relevance of immune cells in regulating canine luteal function is not fully explored, the expression and relatively high abundance of the ANGPT system, mainly of ANGPT2 and TIE1, in immune cells attracted our attention. Based on our recent report, and as indicated elsewhere, these cells were identified as macrophages (Nowaczyk *et al.* 2017). In that previous research, the expression and localization of CD4-, CD8- and MHC II-positive cells were investigated in canine CL of pregnancy (Nowaczyk *et al.* 2017). While CD4- and CD8-positive macrophages were most abundantly represented during early luteal development, MHCII was elevated during luteolysis (Nowaczyk *et al.* 2017). The significance of immune cells, i.e., macrophages,

eosinophils and neutrophils in developing CL and their possible implication in vascular function, was shown in mice, in which depletion of luteal macrophages resulted in insufficient P4 production and disruption of the luteal microvascular network (Care *et al.* 2013). With regard to ANGPTs, there is evidence showing that ANGPT2 is capable of promoting leukocyte attachment to endothelial cells by activating expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) (Fiedler *et al.* 2006). Therefore, ANGPTs seem not only to exhibit direct angiogenic functions but also appear to be indirectly involved in luteal angiogenesis and vasculogenesis by regulating infiltration of immune cells (Fiedler *et al.* 2006). Whether this applies in the canine species, and the extent to which ANGPTs and their receptors could be involved in immune system-mediated regulation of canine luteal function, remains to be elucidated.

In developing canine CL, PGE2 seems to be among the important luteotrophic factors (Kowalewski *et al.* 2013). As shown in our previous study, the direct effects of PGE2 in regulating canine CL function relate to stimulation of steroidogenic acute regulatory (STAR) protein expression and steroid synthesis in luteal cells (Kowalewski *et al.* 2013). Even more peculiar appear to be the apparent indirect effects of PGE2 in the canine CL shown *in vitro* by our group in studies utilizing isolated luteal cells (Gram *et al.* 2015b, Kowalewski *et al.* 2015). Thus, PGE2 was shown to enhance the expression of prolactin receptor (*PRLR*) (Kowalewski *et al.* 2015) and *ETB* (Gram *et al.* 2015b) in these cells. Here, by applying the same *in vitro* approach, PGE2 was shown to be capable of modulating expression of the ANGPT system. Whereas *ANGPT1* mRNA was downregulated, the expression of *ANGPT2*, which was highly represented in canine luteal cells, was further potentiated by PGE2. In accordance with our IHC studies, showing relatively weak expression of *TIE1* and *TIE2* in canine luteal cells, the expression of both receptors either did not change (*TIE2*) in response to PGE2 or was very low or even below the detection limit (*TIE1*). Taking into account the luteotropic role of PGE2 during the early luteal phase in the dog, and the aforementioned concomitantly increased expression of VEGFA (Gram *et al.* 2015a), the role of PGE2 in promoting angiogenesis through upregulation of ANGPT2 expression is implied and certainly merits further research. Similarly, the interplay between luteal cell-derived ANGPTs and vascular function is worth more attention. Cumulatively, with regard to PGE2, our findings add a new aspect to the indirect regulatory functions of PGE2 in regulating canine CL, namely through induction of expression of vasoactive and angiogenic factors from the ANGPT family. The possible translational aspects of these findings should also be investigated in other species.

Figure 8 Cumulative schematic representation of expression profiles of angiogenic (VEGF system, endoglin, ANGPT system) and vasoactive (endothelin receptor B (ETB) and ETA) factors in the CL of pregnant and non-pregnant dogs. The expression of ANGPT system relates to the herein investigated CL of pregnant dogs. Arrows indicate the expression patterns of each factor: upregulation/increased expression ↑; downregulation/decreased expression ↓; or unchanged expression →. The early- and mid-luteal phases are characterized by increased blood supply and high vascularization rate as indicated by increased availability of the VEGF system and vasodilatory endothelin receptor B (ETB) and increased presence of endoglin (END). During luteal regression, vascularization of the CL is slowed down and does not change strongly during the extended luteal regression observed in non-pregnant cycles. Conversely, in pregnant dogs, an increased expression of the vasoconstrictive endothelin receptor (ETA) and elevated ANGPT2/ANGPT1 ratio are observed during prepartum luteolysis. No such increase in ETA is observed during late luteal regression in non-pregnant dogs. While blocking the progesterone receptor (PGR) does not affect the expression of ANGPT family members, the expression of ETA is upregulated, and luteal levels of ETB and the VEGF-system decrease.

Cessation of the luteal phase in pregnant dogs is associated with increased expression of vasoconstrictive ETA during active prepartum luteolysis, implying involvement of $\text{PGF}_2\alpha$ in this process (Nohr *et al.* 1993, Gram *et al.* 2015b). In this context, an additional regulatory aspect arises from assessing the *ANGPT2/ANGPT1* ratio in our study. As implied, e.g., in cattle (Goede *et al.* 1998) and mice (Maisonpierre *et al.* 1997), during luteolysis, the increased ratio of *ANGPT2/ANGPT1* seems to play an important role in vascular regression via inducing loosening of periendothelial support and initializing apoptosis of endothelial cells. Based on the observations from the present study, this may also apply in the canine species, in which upregulation of the *ANGPT2/ANGPT1* ratio during natural prepartum luteolysis in the absence of VEGFA implies destabilization of blood vessels.

Possible P4-mediated effects on ANGPT-system expression were investigated in experiments in which mid-pregnant dogs were treated with an antigestagen, aglepristone. This targeted suppression of P4 receptor (PGR) did not, however, affect the expression of ANGPT family members in our study. This contrasts with the effects aglepristone exerts on the expression of other angiogenic and vasoactive factors, which are strongly regulated following treatment. Thus, whereas

the expression of vasoconstrictive ETA was induced, the expression of ETB and VEGF family members (VEGF 164, VEGFR1 and VEGFR2), was significantly suppressed (Kowalewski 2014, Gram *et al.* 2015b). Therefore, it appears plausible that PGR is not involved in the angiogenic and vasoactive effects exerted by the ANGPT system in the canine CL.

Cumulatively, our study adds important information and complements our previous reports addressing the possible functions of the ET and VEGF systems in the dog. A summary of the most important findings from our current and previous studies, depicting the proposed model of involvement of the vascular system in regulating canine luteal function, is presented in Fig. 8.

With regard to the results presented herein, by combining a descriptive study with functional *in vivo* and *in vitro* approaches, the expression and potential roles of the ANGPT-system have been investigated in canine CL during pregnancy. Based on our results, ANGPTs appear to be involved in both vasoactive and angiogenic functions in the dog. Among the most important findings is the ubiquitous expression of ANGPTs in different cellular components of the canine CL throughout pregnancy. This includes luteal cells, the vascular bed and, importantly, immune system-derived cells. Whereas P4 is the most important luteal hormone

and its production depends on adequate vascularization of the CL, it does not seem to directly support the expression of ANGPTs. The modulatory effect that PGE2 exerts on ANGPTs expression in luteal cells is certainly a new aspect with considerable translational importance, worthy of more detailed studies in the future.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Author contribution statement

A G: Concept of the study, experimental design, generating data, analysis and interpretation of data and writing of the manuscript. M T P: Involved in the laboratory part of the project. A B, A T G-B, M P K: Knowledge transfer, critical discussion of data, editing of the manuscript.

Acknowledgements

Authors are thankful to Dr Barry Bavister for careful editing of the manuscript. They are also grateful to Prof. Dr Selim Aslan, University of Ankara, Turkey, and Prof. Dr Bernd Hoffmann, Justus-Liebig University, Giessen, and their teams for the provision of the tissue material. The technical expertise and contributions of Elisabeth Högger and Ricardo Fernandez Rubia are greatly appreciated. Part of the laboratory work was performed using the logistics at the Center for Clinical Studies, Vetsuisse Faculty, University of Zurich.

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Received 4 May 2018

First decision 15 June 2018

Revised manuscript received 11 July 2018

Accepted 2 August 2018