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—Original Article—

Implications of the RhoA/Rho associated kinase pathway and leptin in primary uterine inertia in the dog

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Abstract. The underlying functional and molecular changes in canine primary uterine inertia (PUI) are still not clarified. Leptin (Lep) and obesity negatively affect uterine contractility in women, partly mediated by the RhoA/Rho associated kinase pathway, affecting myometrial calcium sensitization. We hypothesized that increased uterine Lep/Lep receptor (LepR) or decreased RhoA/Rho associated kinase expression contributes to PUI in dogs, independent of obesity. Dogs presented for dystocia were grouped into PUI (n = 11) or obstructive dystocia (OD, still showing strong labor contractions; n = 7). Interplacental full-thickness uterine biopsies were collected during Cesarean section for relative gene expression (RGE) of *RhoA*, its effector kinases (*ROCK1, ROCK2*), *Lep* and *LepR* by qPCR. Protein and/or mRNA expression and localization was evaluated by immunohistochemistry and *in situ* hybridization. RGE was compared between groups by one-way ANOVA using body weight as covariate with statistical significance at $P < 0.05$. Uterine *ROCK1* and *ROCK2* gene expression was significantly higher in PUI than OD, while *RhoA* and *Lep* did not differ. *LepR* RGE was below the detection limit in five PUI and all OD dogs. Litter size had no influence. Lep, LepR, RhoA, ROCK1, ROCK2 protein and/or mRNA were localized in the myometrium and endometrium. Uterine protein expression appeared similar between groups. *LepR* mRNA signals appeared stronger in PUI than OD. In conclusion, lasting, strong labor contractions in OD likely resulted in downregulation of uterine *ROCK1* and *ROCK2*, contrasting the higher expression in PUI dogs with insufficient contractions. The Lep-LepR system may affect uterine contractility in non-obese PUI dogs in a paracrine-autocrine manner.
Key words: Canine, Contractility, Dystocia, Parturition, Uterus

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Uterine inertia refers to the condition, when the bitch is unable to initiate and/or maintain contractions to progress through labor and deliver all puppies [1]. Uterine inertia may be idiopathic, when no apparent cause of myometrial contractile dysfunction is identified and is generally referred to as primary uterine inertia (PUI), although there are different views on its definition [2–8]. Uterine inertia can also develop due to obstruction of the birth canal or overstretching of the uterine musculature in bitches with large litter [2, 6, 7]. PUI is a frequent cause of canine dystocia with a reported incidence of up to 49% of all dystocia cases [3, 9]. Some breeds have been described to have a relatively high incidence of uterine inertia [2, 4, 6, 10]. Additionally, advanced age, obesity, very small or large litter, hormonal, electrolyte or metabolic imbalances may all be predisposing factors for uterine inertia [3–6, 9, 11–13]. Interestingly however, in our previous study, blood ionized calcium and glucose levels were not significantly different between PUI dogs and bitches with obstructive dystocia still showing strong contractions [12]. Recently, advances were made in identifying defects in the contractile properties of the uterus at the cellular and molecular level. Our group showed that bitches diagnosed with PUI had higher uterine mRNA expression levels of smooth muscle γ-actin and smooth muscle myosin compared to dogs in labor still showing strong contractions [8], while gene expression of members of the prostaglandin (PG) F2α and PGE pathway did not differ [14, 15]. Changes in uterine oxytocin receptor expression also did not seem to be implicated in the development of PUI [16]. Although these studies have provided detailed evidence of some of the molecular aspects of contractility, the pathophysiology of canine PUI is still far from being fully understood.

The small GTPase RhoA and its two effector kinase isoforms, the Rho associated coiled-coil containing protein kinase 1 (ROCK1 or

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p160ROCK) and 2 (ROCK2 or ROCKα) [17, 18] are involved in the regulation of smooth muscle contractions through the mechanism called calcium sensitization [19, 20]. Active GTP-bound RhoA activates ROCK, which, in turn, will inhibit myosin light chain phosphatase, so phosphorylation of myosin regulatory light chain is maintained, supporting sustained muscle contraction at a given intracellular ionized calcium (iCa) concentration [17, 21–23]. Additionally, ROCK can also phosphorylate myosin light chain, independent of the Ca-calmodulin activated myosin light chain kinase [24]. These two effects of ROCK on promoting calcium sensitization are highly correlated and functionally coupled with phasic myometrial contractions demonstrated *in vitro* [23], supporting the role of the RhoA pathway in uterine contractility. RhoA and the ROCKs are expressed in the myometrium of pregnant women [25–27], and in rats, gene or protein expression of RhoA or the Rho associated kinases were found to increase by late pregnancy or during labor [28, 29]. In rabbits, myometrial RhoA and ROCK1 expression is significantly higher in late pregnant animals, a finding that is accompanied by increased calcium sensitivity [30]. *In vitro* inhibition of the RhoA pathway in myometrial strips of term pregnant rats diminished the amplitude and frequency of oxytocin induced contractions [29, 31], and a potent cumulative relaxant effect on oxytocin induced human myometrial contractility was also demonstrated *in vitro* [25].

Obesity has been recognized as one of the risk factors for labor complications and dystocia in women [32–37] and in dogs [6, 38]. Obese and overweight women are more likely to have prolonged gestation, delayed onset of labor, and a higher rate of Cesarean section (CS) deliveries than women with normal body mass index [33]. Leptin (Lep) is primarily expressed and secreted by white adipose tissue [39], and its blood levels correlate positively with the amount of fat stores [40, 41]. Therefore Lep, directly or indirectly, may be involved in labor dysfunction in women as well as in dogs. *In vitro*, a cumulative inhibitory effect of Lep on the frequency and amplitude of spontaneous and oxytocin induced contractions was shown in myometrial strips collected from women undergoing elective CS [42, 43]. Lep's effects, at least in part, may be mediated through the RhoA pathway. Heterozygous leptin receptor (LepR) deficient non-pregnant mice had higher myometrial expression of ROCK1 and ROCK2 and greater Rho kinase-dependent myometrial contractions than wild type mice, however, pregnancy in these mice resulted in reduced Rho kinase-mediated contribution to tonic myometrial contractions compared with the non-pregnant state [44]. In obese women, myometrial expression of ROCK1 protein at term pregnancy was markedly downregulated compared to women with normal body mass index, however, Lep expression was not addressed in this study [45].

In dogs, uterine expression of members of the RhoA pathway has not been studied during normal pregnancy and parturition, or in PUI. Lep and LepR, on the other hand, were found in the uterus and placenta of pregnant bitches, and their gene expression showed distinct gestation stage-dependent changes [46]. This local tissue expression pattern as well as increased plasma Lep concentrations during canine gestation [47] may support a possible role of Lep during parturition in the dog, independent of obesity.

We hypothesized that RhoA, ROCK1, and ROCK2 expression is decreased, and Lep or LepR expression is increased in the uterus of non-obese bitches diagnosed with PUI compared to dogs with strong labor contractions. The goals of our study were 1) to describe RhoA, ROCK1 and ROCK2 gene and protein expression in the uterus of parturient bitches, and 2) to compare gene and protein expression and localization of Lep, LepR, RhoA and ROCKs between PUI dogs and dogs with strong labor contractions diagnosed with obstructive dystocia (OD). Additionally, as litter size, i.e. singleton *vs.* large litter, has been shown to predispose to uterine inertia, we investigated the influence of litter size relative to breed average on uterine expression of our candidate genes.

Materials and Methods

Animals and groups

Eighteen client owned dogs of different breeds admitted for CS due to dystocia and diagnosed with PUI (n = 11) or obstructive dystocia (OD, n = 7) were included. The population of dogs was the same as described in our previous study [8]. Medical and reproductive history was obtained from all dogs, and a complete physical and obstetrical examination was performed. Body weight, body condition score (BCS) and previous parturitions were recorded for all animals. Diagnostic imaging and bloodwork were performed in all cases. External tocodynamometry (Philips 50 XM M1350B, Böblingen, Germany) was carried out for 15–20 min. to monitor uterine contractions if the bitch was stable and fetal heart rates were not decreased.

The eleven bitches with PUI belonged to the following breeds: two of each Maltese and Maremma Sheepdog, and one of each Dachshund, German Shepherd, Labrador Retriever, Broholmer, Beagle, Boxer and French Bulldog. Grouping into PUI was done according to the following criteria and as we previously reported [8]: no puppy born yet, no or only very weak abdominal contractions on digital vaginal stimulation (feathering), and no or weak uterine contractions on tocodynamometry. Obstruction of the birth canal was excluded by digital vaginal examination and/or radiographs. In the history of the bitches, one of the following signs were present: prepartum temperature drop > 20 h, or first stage labor for ≥ 20 h, or normalization of the temperature without further progress into second stage labor; no or infrequent, weak abdominal contractions for > 4 h; passage of fetal fluids or the presence of green vulvar discharge for ≥ 2–3 h without visible abdominal contractions. The OD group had seven bitches from the following breeds: two Chihuahuas, and one of each West Highland White Terrier, Yorkshire Terrier, Cairn Terrier, Staffordshire Terrier, and a mixed breed dog. Bitches in the OD group failed to deliver due to obstruction of the birth canal, which was diagnosed by digital vaginal palpation and/or abdominal X-ray. Only bitches showing strong spontaneous and induced contractions by digital vaginal stimulation were included in this group. Two of the bitches from the OD group gave birth to two pups and one bitch to one pup before CS was performed. None of the participating dogs received any ecbolic or tocolytic medication, or had any signs of systemic illness (e.g. sepsis, metritis).

The study was approved by the Cantonal Veterinary Office Zurich (permit no. ZH086/15, Switzerland) and Dyreforsøgstilsynet Fødevarestyrelsen (permit no. 2015-15-0201-00513, Denmark). All participating owners signed an informed consent form.

Uterine tissue sample collection and preservation

Full thickness uterine biopsies at interplacental sites (IP), i.e, between two placentation sites, were collected at the time of CS at the uterine incision site after all puppies had been delivered, or at the end of surgery if concurrent ovariohysterectomy was performed. For gene expression, tissue samples were preserved in RNAlater^{TM} stabilization solution (Thermo Fisher Scientific, Waltham, MA, USA) for 24 hours at 4°C and stored at −80°C until analysis. For immunohistochemistry (IHC) and *in situ* hybridization (ISH), all tissues were preserved in 10% neutral phosphate buffered formalin at 4°C for 24 hours, washed every 24 h for 7 days in phosphate buffered saline, dehydrated in graded ethanol series and embedded in paraffin.

Homology cloning of canine-specific ROCK1 and ROCK2

As canine-specific sequences for *ROCK1* (GenBank accession no. XM_005623022.4) and *ROCK2* (GenBank accession no. XM_038690714.1) have not been characterized before and were available only as predicted sequences, we performed molecular cloning and sequenced the amplified partial coding sequences to confirm their identity. The primers used for cloning are listed in Table 1. After DNase treatment and reverse transcription of 200 ng total RNA from two IP uterus samples, a hot-start PCR was done using GeneAmp Gold RNA PCR Kit (Applied Biosystems, Foster City, CA, USA) following our protocol [46, 48]. For both *ROCK1* and *ROCK2*, the annealing temperature was set at 58°C. PCR fragments of 518 bp and 580 bp for *ROCK1* and *ROCK2*, respectively, were amplified. Autoclaved water and RT-minus control (no RT reaction carried out) were used as negative controls. PCR products were separated on a 2% ethidium bromide-stained agarose gel, extracted using Qiaex II gel extraction system (Qiagen GmbH), and ligated into

Table 1. List of canine-specific primers and TaqMan probe sequences used for homology cloning, semi-quantitative real-time (TaqMan) PCR, and *in situ* hybridization

Gene	Primers and TaqMan probe sequences	Amplicon length (bp)	GenBank accession number
Homology cloning			
ROCK1	Forward: 5'-AGC AAG AAA GCT GCT TCC AG-3'	518	
	Reverse: 5'-GCA CGC AGT TGC TCA ATA TC-3'		
ROCK2	Forward: 5'-ATT CCT TGG CTG CTC AAC TG-3'	580	
	Reverse: 5'-CGA ATC TGG CTC TCT TCA GC-3'		
Semi-quantitative real-time (TaqMan) PCR			
RhoA	Forward: 5'-GAC CCC AGA AGT CAA GCA CTT CT-3'	91	NM_001003273.3
	Reverse: 5'-CCG CCT TGT GTG CTC ATC A-3'		
	TaqMan probe: 5'-TCC CAA CGT GCC CAT CAT CCT GGT-3'		
ROCK1	Forward: 5'-CCA GAT GGT GGT GAA GCA TCA-3'	80	XM_005623022.4
	Reverse: 5'-TCT GAA GCT CAT TCT TAT GCG TAC A-3'		
	TaqMan probe: 5'-TGA ATG ACA TGC AAG CGC AAT TGG T-3'		
ROCK2	Forward: 5'-TGG AGC TTA AAT CTG AAC GTG AAA A-3'	86	XM_038690714.1
	Reverse: 5'-AGC TAT TTG AGC CTG CAT TTC ATT C-3'		
	TaqMan probe: 5'-CTG ACC CAG CAG ATG ATC AAG TAC CAG AAA G-3'		
Leptin	Forward: 5'-GGG TCG CTG GTC TGG ACT T-3'	86	NM_001003070.1
	Reverse: 5'-CTG TTG GTA GAT GGC CAA CGT-3'		
	TaqMan probe: 5'-TCC TGG GCT CCA ACC AGT CCT GAG T-3'		
Leptin receptor	Forward: 5'-CAT TTG CGG AGG GAT GGT T-3'	149	NM_001024634.1
	Reverse: 5'-AGC GGT TTC ACC ACG GAA T-3'		
	TaqMan probe: 5'-TTG ACT CTT CAC CAA CGT GTG TGG TTC C-3'		
GAPDH	Forward: 5'-GCT GCC AAA TAT GAC GAC ATC A-3'	75	AB028142.1
	Reverse: 5'-GTA GCC CAG GAT GCC TTT GAG-3'		
	TaqMan probe: 5'-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3'		
In situ hybridization			
RhoA	Forward: 5'- GGT AGA GTT GGC TTT GTG GGA-3'	280	
	Reverse: 5'-TCT GCC TTC TTC CGG TTT CA-3'		
ROCK1	Forward: 5'-CGG CTT GAA GAA TCG AAC AGC-3'	202	
	Reverse: 5'-CCA ACT TGT TAA CAG CCT GCG-3'		
ROCK2	Forward: 5'-TTC CTT GGC TGC TCA ACT GG-3'	304	
	Reverse: 5'-CTC CTC ATC CTT CAG CCG TG-3'		
Leptin receptor	Forward: 5'-CAT GGT GGG TGA CCG TGT TA-3'	232	
	Reverse: 5'-TCC CTC GAG TGA TTG GAT TGC-3'		

a pGEM-T vector (Promega, Dübendorf, Switzerland) according to our protocols [46, 49]. XL1 Blue competent cells were transformed using the pGEM-T vector containing our inset and amplified (Stratagene, La Jolla, CA, USA). Bacterial plasmids were purified using Pure Yield Plasmid MidiPrep System (Promega) and sequenced on both strands with T7 and Sp6 primers (Microsynth, Balgach, Switzerland). Results of the confirmed partial coding sequences were submitted to GenBank with the accession number MT636893 for *ROCK1* and MT636894 for *ROCK2*.

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

Total RNA isolation from IP uterine tissues was performed using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and as described previously [48, 50]. Total RNA concentration and quality was controlled on a NanoDrop 2000C® spectrophotometer (Thermo Fisher Scientific). Per sample, 200 ng total RNA was DNAse treated with RQ1 RNase-free DNase to remove genomic DNA, according to the manufacturer's instructions (Promega). Reverse transcription was performed using random hexamers as primers and reagents from Applied Biosystems as described previously [48]. Primers and probes for the semi-quantitative real-time (TaqMan) PCR were designed and labeled at the 5'-end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3'-end with 6-carboxytetramethyl-rhodamine (TAMRA) (Table 1). PCR reactions were run in duplicates using Fast Start Universal Probe Master (ROX) (Roche Diagnostics AG, Schweiz) in an automated fluorometer (ABI PRISM$_{TM}$ 7500Sequence Detections System, Applied Biosystems) following our protocol [48]. Canine *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*; Table 1) and canine-specific *cyclophyllin A* (Prod. No. Cf03986523-gH, Applied Biosystems) were used as reference genes. Negative control reactions were performed using autoclaved water. Efficiency of the PCR reactions was calculated using the CT slope method according to the manufacturer's instructions for the ABI PRISM$_{TM}$ 7500 Sequence Detection System and as previously described [50], and was ~100% for all genes. Selected PCR products were sent for sequencing (Microsynth). Relative gene expression (RGE) was calculated using the comparative CT method (ΔΔCT method), according to the manufacturer's protocol and as previously described [48]. The sample with the lowest detectable amount of transcript was used as the calibrator.

Immunohistochemistry

An indirect immunoperoxidase method was used [48] to detect protein expression and localization of Lep, RhoA, ROCK1 and ROCK2 in IP tissue samples. Due to the lack of availability of a specific primary antibody reactive with canine LepR, IHC was not carried out for this specific target. IP uterine tissues (2–3 μM thickness) were mounted on SuperFrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany), deparaffinized in xylene, rehydrated in graded ethanol series and rinsed briefly in tap water. For antigen retrieval, slides were incubated in 10 mM citrate buffer (pH 6.0, for RhoA, ROCK2 and Lep) or in EDTA (pH 9.0, for ROCK1) for 15 min at 100°C. Endogenous tissue peroxidase was quenched using 0.3% hydrogen peroxide in methanol. Non-specific binding sites were blocked with 10% goat serum (SeraCare Life Sciences, Milford, MA, USA), then slides were incubated with the respective primary antibodies (RhoA, Abcam, Cambridge, UK, polyclonal rabbit IgG, dilution 1:800; ROCK1, Bioss, Woburn, MA, USA, polyclonal rabbit IgG, dilution 1:500; ROCK2, Aviva Systems Biology, San Diego, CA, USA, polyclonal rabbit IgG, dilution 1:100; Lep, Aviva Systems Biology, polyclonal rabbit IgG, dilution 1:150) in a humid chamber overnight at 4°C. Incubation with pre-immune rabbit IgG instead of the primary antibody was used as the so-called isotype control for negative control for all antibodies, adjusted for the concentration of the primary antibody. After rinsing, a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) was added at a concentration of 1:100 to all slides and incubated for 30 min at room temperature. To amplify target signals, an avidin/ biotinylated peroxidase complex (Vectastain ABC Kit, Vector Laboratories) was applied, and then color reaction was achieved with 3,3'-diaminobenzidine as chromogen substrate (Liquid DAB + substrate Kit, Dako Schweiz AG, Baar, Switzerland). Slides where washed under running tap water and counterstained with Mayer's hematoxylin, dehydrated in graded ethanol series and protected with coverslips.

In situ hybridization

To confirm tissue localization of *LepR*, *RhoA*, *ROCK1* and *ROCK2* mRNA, ISH was carried out on representative paraffin embedded uterine IP samples from each group, i.e., from the three PUI subgroups according to litter size, i.e., small, breed average or large litter size (further specified below, please see Statistical analysis), and from the OD group. A previously described non-radioactive method was applied [51]. Primers used for synthesis of templates for cRNA probes are listed in Table 1. PCR products were separated by electrophoresis in 2% ethidium bromide-stained agarose gel, purified using the Qiaex II gel extraction system (Qiagen GmbH Hilden, Germany), ligated into a pGEM-T plasmid vector (Promega), and transformed and amplified in XL1 Blue competent cells (Stratagene). To obtain sense and antisense probes, the selected plasmid clones were digested with restriction enzymes *Not*I or *Nco*I (New England Biolabs, Frankfurt, Germany), and cRNA probes were labelled with digoxigenin (DIG-RNA labelling kit, Roche Diagnostics AG) following the manufacturer's instructions. For semi-quantitation of labeled cRNA concentration, dot-blot analysis was performed on a positively charged nylon membrane. Paraffin embedded IP uterine tissues were deparaffinized using xylene, rehydrated in graded ethanol series, digested with proteinase K (Sigma-Aldrich Chemie GmbH) and post-fixed with 4% paraformaldehyde. Hybridization was carried out overnight in a formamide chamber at 37°C. For the detection of digoxigenin-labeled cRNA probes, sheep alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche Diagnostics GmbH), diluted at 1:5000 in 10% goat serum, were used. Signals were visualized using the substrate 5-bromo-4-chloro- 3-indolyl phosphate and 4-nitro blue tetrazolium (NBT/BCIP; Roche Diagnostics).

Statistical analysis

Statistical analysis was performed using IBM SPSS® Statistics for Windows, version 24 (IBM Corp., Armonk, NY, USA). Data on age, body weight, body condition score and the number of current parturitions were compared between the PUI and OD groups with a t-test or a Mann-Whitney U test. Relative gene expression (RGE) in IP tissues was compared between the PUI and OD groups by one-way analysis of variance (ANOVA) on observed or logarithmically transformed data, where group was the fixed effect and body weight was the covariate. Inclusion of body weight in the analysis was performed to account for the significant body weight difference found between the PUI and OD groups (P = 0.017). To investigate if litter size has an effect on uterine physiology and the expression of our target genes, PUI bitches were further sub-grouped according to litter size using breed-specific data from Borge *et al*. [52], and as described in our previous study [8]. Small litter size (PUI-S) or large litter size (PUI-L) were defined as below the mean – 1 standard deviation (SD) or above the mean + 1 SD of the litter size for the breed, respectively. Average or normal litter size (PUI-N) was defined as within the mean ± 1 SD for the breed. Uterine RGE between PUI subgroups was compared using one-way ANOVA on observed or logarithmically transformed data with group as the fixed effect. Body weight was not included in this analysis, because it was not different between PUI subgroups (P = 0.639). The association between uterine *ROCK1* and *ROCK2* gene expression was analyzed by Pearson correlation. Results are presented as mean ± SD of observed data or geometric mean (X_g) ± deviation factor of logarithmically transformed data. Statistical significance was set at P < 0.05.

Results

Animals

Uterine gene and protein expression of RhoA, ROCK1, ROCK2, Lep and LepR was evaluated in 11 bitches diagnosed with PUI and in seven dogs with OD. Age (PUI: 4.45 ± 1.94 years, range 1.9–7.1 years; OD: 3.13 ± 2.43 years, range 0.9–8.1 years), litter size (PUI: 5.64 ± 3.07, range 2–9; OD: 4.29 ± 2.06, range 2–8), and current pregnancy number (PUI: 2.1 ± 0.99, range 1–4; OD: 1.57 ± 0.79, range 1–3) were not different between the two dystocia groups (P ≥ 0.220). None of the dogs in this study were obese; BCS ranged between 3/9 and 6/9, and was not different between groups (PUI: 4.43 ± 0.98, range 3–6; OD: 4.33 ± 0.82, range 3-5; P = 0.587). Body weight however was significantly higher in the PUI (28.26 ± 20.14 kg, range 5.6–70.1 kg) compared to the OD dogs (9.26 ± 6.67 kg, range 2.9–21.2 kg; P = 0.017).

RhoA, ROCK1 and ROCK2 in uterine IP tissue

Gene expression of RhoA, ROCK1 and ROCK2 by qPCR

RhoA, *ROCK1* and *ROCK2* gene expression was detected in all samples. IP mRNA concentration of *RhoA* was similar between the PUI and OD groups (P = 0.068; Fig. 1A). However, IP expression of *ROCK1* and *ROCK2* was higher in bitches in the PUI compared to the OD group (P = 0.010 and P = 0.039, respectively; Fig. 1B, C). Gene expression of *RhoA*, *ROCK1* and *ROCK2* was not different among PUI bitches carrying small, average or large litters (P ≥ 0.132; Figs. 2A–C). Analyzing all dogs together, IP mRNA expression of *ROCK1* and *ROCK2* was strongly positively correlated (r = 0.835, P < 0.0001; Fig. 3).

Protein and mRNA expression of RhoA, ROCK1, ROCK2 by immunohistochemistry and in situ hybridization

Protein expression of RhoA, ROCK1 and ROCK2 by IHC was

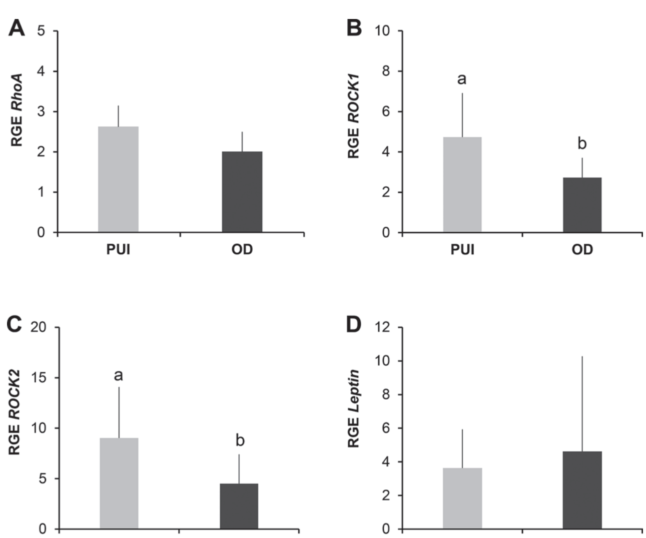

Fig. 1. Comparison of relative gene expression (RGE) of A) *RhoA*, B) *ROCK1*, C) *ROCK2*, and D) *Leptin* in inter-placental uterine tissue samples of dogs diagnosed with primary uterine inertia (PUI) or obstructive dystocia (OD). Bars represent the observed (*RhoA, ROCK1, ROCK2*) or geometric mean (*Leptin*), and whiskers the standard deviation or the deviation factor, respectively. Different superscripts denote significant difference between groups at P < 0.05.

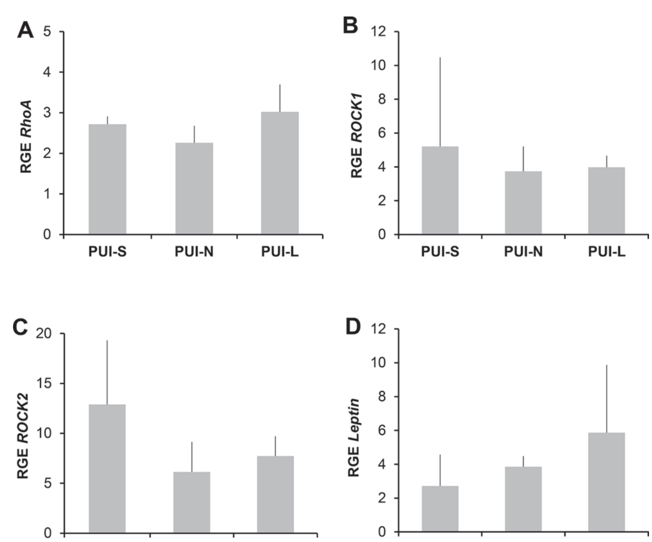

Fig. 2. Comparison of relative gene expression (RGE) of A) *RhoA*, B) *ROCK1*, C) *ROCK2*, and D) *Leptin* in inter-placental uterine tissue samples of dogs diagnosed with primary uterine inertia (PUI) carrying small (PUI-S), average (PUI-N) or large (PUI-L) litters relative to the breed average litter size. Bars represent the observed (*RhoA, ROCK2*) or geometric (*ROCK1*, *Leptin*) mean, and whiskers the standard deviation or the deviation factor, respectively. There was no significant difference in gene expression among groups.

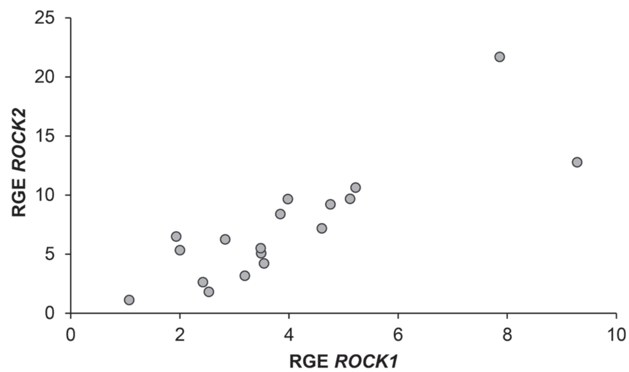

Fig. 3. Correlation of inter-placental relative gene expression (RGE) of *ROCK1* and *ROCK2* in the whole study population (n = 18; r = 0.835, P < 0.0001).

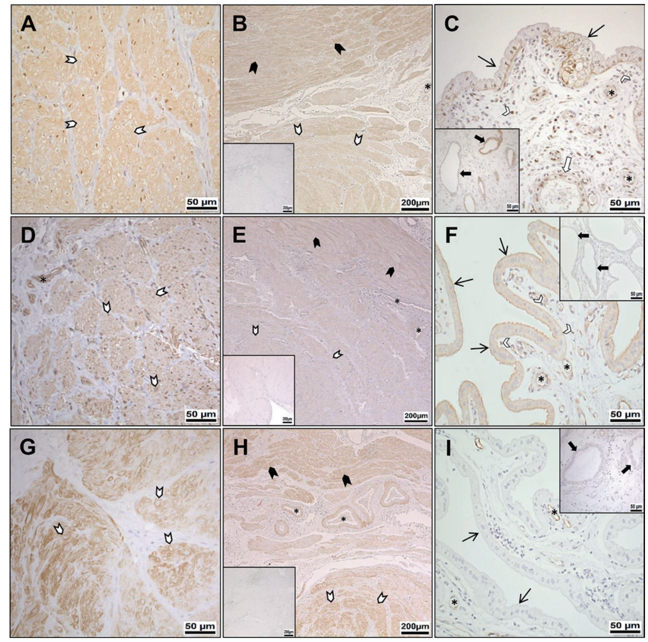

Fig. 4. Immunohistochemical detection of RhoA, ROCK1, and ROCK2 in uterine interplacental tissue in the PUI and OD groups. For RhoA (A–C), strong signals are visible in the smooth muscle cells of the myometrium (A; representative image from the PUI group). Both the circular (solid arrowheads) and longitudinal (open arrowheads) layer of the myometrium stained (B; representative image from the OD group). In the endometrium (C), the surface epithelium (thin black arrows), superficial (open arrow) and deep (solid arrows) uterine glands and stromal immune cells (small white arrowheads) showed positive immunoreactivity. Blood vessels (asterisks) also stained (B, C). No signals are visible in the isotype control for RhoA (lower inset in B). For ROCK1 (D–F), positive staining is seen in myocytes (D; representative image from the PUI group) of both the circular (solid arrowheads) and longitudinal (open arrowheads) uterine muscle layers (E; representative image from the OD group). The luminal epithelium (thin black arrows), superficial (not shown) and deep (solid arrows) uterine glands, and stromal immune cells (small white arrowheads) stained positive for ROCK1 (F). Blood vessels (asterisks) also stained (D–F). The isotype control is devoid of signals (lower inset in E). ROCK2 (G–I) signals are visible in smooth muscle cells (G; representative image from the OD group) of both myometrial layers, i.e., circular (solid arrowheads) and longitudinal (open arrowheads) (H; representative image from the PUI group), and strong immunostaining is also present in blood vessels (asterisks) (H, I). In the endometrium (I), no or occasional weak ROCK2 signals are present in the luminal epithelium (thin black arrows) and deep uterine glands (solid arrows). There is no staining in the isotype control (lower inset in H).

detected in the myometrium, both in the circular and longitudinal layers (Fig. 4). There were individual variations in myometrial protein signal intensity for RhoA, ROCK1 and ROCK2, but localization and immunosignal strength appeared similar between the PUI and OD groups, among the PUI subgroups and between myometrial layers (i.e., circular *vs*. longitudinal). Weak to strong immunostaining for RhoA was also present in the luminal epithelium and in the superficial and deep uterine glands (Fig. 4C). Weak signals were seen in the tunica intima and occasionally in the inner layers of the tunica media and basement membrane of blood vessels, and in immune cells of the stroma (Figs. 4B, C). Similarly, for ROCK1, weak positive immunoreaction was detected in the luminal and glandular epithelium (Fig. 4F). Staining was also visible in stromal immune cells and in the tunica intima and inner tunica media layers of blood vessels (Fig. 4F). ROCK2 immunostaining was weaker in the endometrium than in the myometrium, with no or occasional weak staining in the luminal epithelium, superficial and deep uterine glands (Figs. 4G–I) both in the PUI and OD groups. Strong signals for ROCK2 were seen in blood vessels (inner layers of the tunica media and tunica intima; Figs. 4H, I), which appeared stronger than for RhoA and ROCK1. Positive immunostaining for ROCK2 was also visible in immune cells of the stroma.

Localization pattern of *RhoA*, *ROCK1* and *ROCK2* mRNA by ISH (Fig. 5) was similar to that seen for protein expression by IHC for both dystocia groups. Signals were detected in the circular and longitudinal myometrial layers, luminal epithelium, superficial and deep glands of the endometrium, blood vessels and immune cells of the stroma in both PUI and OD samples. No difference in localization pattern was observed between the PUI and OD groups, or among PUI subgroups.

Lep and LepR in uterine IP tissue

Gene expression of Lep and LepR by qPCR

Lep gene expression in IP tissue was detected in nine out of 11 PUI dogs and in all seven OD dogs. *Lep* mRNA levels were similar between the PUI and OD groups (P = 0.823; Fig. 1D). Clear differences in *LepR* gene expression were detected between dystocia groups. *LepR* mRNA expression was under the detection limit of our assay in all OD bitches indicating low expression levels, while *LepR* gene expression was detected in six out of 11 PUI bitches. Litter size in the PUI group had no effect on *Lep* gene expression (P = 0.192; Fig. 2D).

Protein expression of Lep by immunohistochemistry and mRNA expression of LepR by in situ hybridization

Lep immunostaining was present in the smooth muscle cells of the circular and longitudinal myometrial layers (Figs. 6A, B). Staining

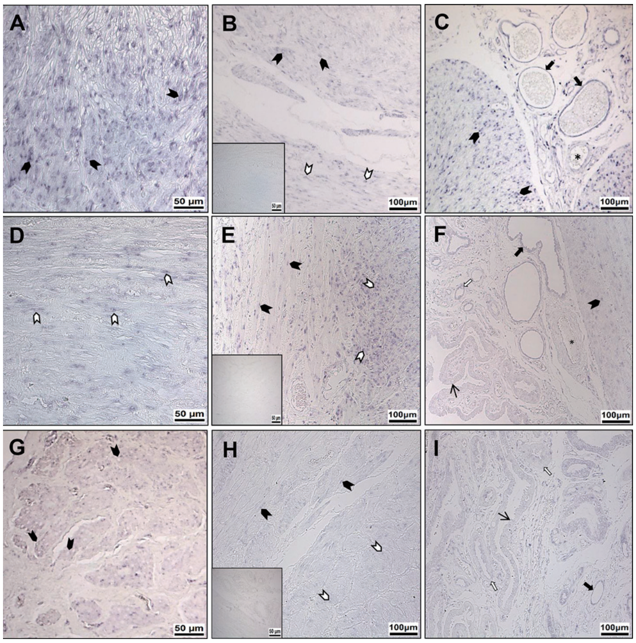

Fig. 5. Localization of *RhoA*, *ROCK1* and *ROCK2* mRNA in uterine interplacental tissue by *in situ* hybridization. For *RhoA*, positive signals are visible in myocytes (A) of the circular (solid arrowheads) and longitudinal (open arrowheads) myometrial layers (B, C). The same expression pattern is visible for *ROCK1* (D–F) and *ROCK2* (G, H). In the endometrium, positive signals are seen in the luminal epithelium (thin black arrows), superficial (open arrows) and deep (solid arrows) uterine glands for *RhoA* (C), *ROCK1* (F) and *ROCK2* (I). There was variable positive staining in blood vessels (asterisks) for *RhoA* and *ROCKs*. Negative controls for *RhoA*, *ROCK1* and *ROCK2* have no signals (lower insets in B, E, H, respectively).

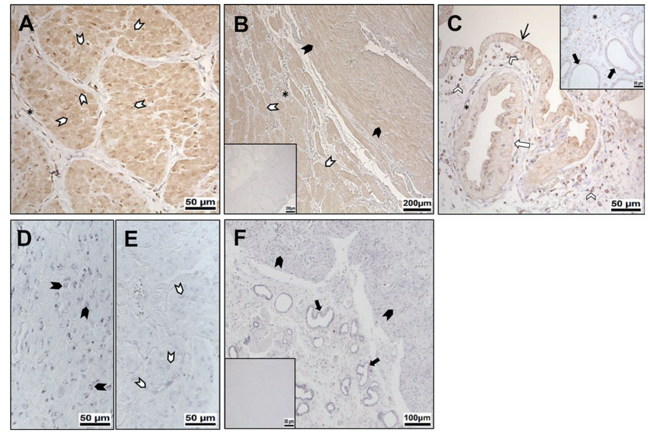

Fig. 6. Immunohistochemical detection of Lep, and *LepR* mRNA detection by *in situ* hybridization in uterine interplacental tissues in the PUI and OD groups. Positive immunostaining for Lep (A–C) is visible in the myocytes (A; representative image from the PUI group) of the circular (solid arrowheads) and longitudinal (open arrowheads) myometrial layers (B; representative image from the OD group). Weak positive signals for Lep are visible in the uterine luminal epithelium (thin black arrow), and in the superficial (open arrow) and deep glands (solid arrows) of the endometrium; stromal immune cells (small white arrowheads) also stained (C). Blood vessels showed positive immunosignals for Lep (asterisks). The isotype control is devoid of signals (lower inset in B). *LepR* mRNA (D–F) was localized in myocytes of the circular (solid arrowheads) and longitudinal (open arrowheads) myometrial layers. *LepR* mRNA signals were also found in the luminal (not shown) and glandular epithelial cells (deep glands shown with solid arrows in F). No signals are seen in the negative control (lower inset in F).

intensity in the myometrium varied individually. No difference in localization pattern or signal intensity could be noted between the PUI and OD groups, among PUI subgroups, or between myometrial layers (circular *vs*. longitudinal). Weak sporadic staining was observed in the luminal epithelium, superficial and deep glands of the endometrium in both dystocia groups (Fig. 6C). Staining was also noted in immune cells within the stroma, and in the tunica intima and media of small and large blood vessels (Figs. 6B, C).

For *LepR*, ISH revealed signals in the myocytes of the circular and longitudinal muscle layers (Figs. 6D–F). Positive signals were also observed in the luminal epithelium, superficial and deep glands (Fig. 6F) of the endometrium. Signals were inconsistent in blood vessel endothelial cells, blood vessel media and in the stroma. No difference in localization pattern between PUI and OD bitches or among PUI subgroups was seen. In these samples, overall uterine *LepR* signal intensity appeared stronger in PUI than in OD.

Discussion

Previous studies showed RhoA and the Rho associated kinases as important contributors to myometrial contractility through calcium sensitization [22, 28, 29], which prompted us to study their role in the development of PUI in dogs. We confirmed, for the first time, the identity of the predicted *ROCK1* and *ROCK2* sequences by molecular cloning, and the presence and localization of RhoA and the ROCKs in the canine uterus. As expected, mRNA (by ISH) and protein (by IHC) signals for RhoA and for its two effector kinases were found predominantly in the myometrium, pinpointing their role in uterine smooth muscle function in dogs. Signals were present in the luminal epithelium and blood vessels, and occasionally in uterine glands. The localization pattern of RhoA in the canine uterus was similar to the findings of Lartey *et al*. [26], who reported RhoA expression in muscular, glandular and stromal cells of the human uterus. Based on previous studies showing increased uterine mRNA and/or protein expression of Rho kinases with or without concomitant RhoA increases towards the end of gestation [29, 30], or during active labor after delivery of the first pup in rats [28], we expected to find lower expression of RhoA, ROCK1 and ROCK2 in PUI dogs, which had no or only very weak uterine contractions compared to OD dogs showing strong contractions. However, while mRNA expression of *RhoA* was similar between the two dystocia groups, *ROCK1* and *ROCK2* gene expression was higher in PUI bitches. Our results indicate that adequate labor contractions may cause down-regulation of gene expression of members of the RhoA pathway in the canine uterus as seen in the OD group. Similar changes in uterine mRNA expression of smooth muscle γ-actin and myosin between PUI and OD bitches

[8], or in oxytocin receptor gene expression between dogs in labor with dystocia and undergoing elective CS [16] were found before, but not for uterine expression of prostaglandin-endoperoxidase synthase 2 and the PGF2α pathway [14]. These differences seen here may be due to abnormal labor signaling, or protracted or inadequate uterine response in PUI bitches. Protein expression of RhoA and its kinases by IHC did not seem to differ between PUI and OD bitches. However, quantification of protein expression by Western blot, or investigation of the interplay between regulators of Rho protein activity and levels of the active, membrane-bound RhoA [22] could further clarify the role of RhoA and ROCKs in parturition dysfunction. In future studies, tissue-layer specific analysis of gene and/or protein expression i.e., endometrium and myometrium, may even more accurately address changes occurring in the uterus of PUI dogs.

According to previous reports showing negative effects of Lep on human and rat myometrial contractions *in vitro* [42, 43], we hypothesized that Lep and its receptor play a role in the development of canine PUI. However, we did not observe a difference in uterine Lep gene and protein expression between dogs with PUI or OD. Lep protein was present in the myometrium, and its localization pattern was in accordance with our previous findings during canine pregnancy and prepartum [46]. On the other hand, *LepR* mRNA levels were below the detection limit of our assay in all dogs in the OD group and were only detectable in about half of the dogs in the PUI group. Accordingly, in ISH, mRNA signal intensity in selected uterine samples also seemed to reflect this difference between groups. These changes in the sensitivity of the uterus to Lep despite similar Lep expression could be a mechanism by which the potentially negative effects of Lep on uterine contractility are mediated, at least in some of the PUI cases. Obesity is associated with high peripheral Lep levels in humans [40], and obese women have decreased myometrial ROCK1 expression [45]. We could not prove the negative influence of Lep on uterine RhoA and Rho associated kinase expression in canine PUI, and peripheral Lep levels were not measured. We analyzed uterine Lep and *LepR* expression, and thus our findings most likely reflect the autocrine-paracrine role of Lep on uterine responses in a study population of dogs with normal BCS, rather than the endocrine influence of high Lep concentrations associated with obesity.

We have previously shown that the number of puppies in utero below or above the average number for the breed affects uterine smooth muscle γ-actin gene expression in PUI dogs [8], probably through differences in stretch and/or hormonal signaling. However, it seems that litter size does not systematically affect all contractile and contractility-associated proteins studied so far in the canine uterus [8], including members of the RhoA pathway and Lep, as presented here. This further substantiates PUI as a multifactorial disease.

Similar to previous studies investigating canine PUI, we could include only a small number of bitches, which might be seen as a possible limitation of our study. However, we used strict inclusion criteria and also included body weight in the statistical analysis on gene expression to account for the significant weight difference between the PUI and OD groups.

In conclusion, our study confirmed the expression of RhoA, ROCK1 and ROCK2 in the canine uterus. In contrast to our hypothesis, uterine gene expression of *ROCK1* and *ROCK2* was higher in dogs with PUI compared to OD bitches which showed strong labor contractions. While Lep expression was not affected by PUI, *LepR* mRNA levels were generally low and undetectable in all OD dogs, while *LepR* gene expression was above the detection limit of our assay in about half of the PUI bitches. Therefore, the Rho associated kinases seem to be implicated in the etiology of PUI, and the finding of higher uterine mRNA levels may be the result of weak or absent uterine contractions in PUI bitches. Lasting, strong contractions in OD bitches likely resulted in downregulation of uterine *ROCK1* and *ROCK2* gene expression. Changes in the sensitivity of the uterus to Lep in non-obese dogs may be a mechanism by which Lep exerts its potentially negative effects on uterine contractility in a paracrine-autocrine manner, at least in some cases of PUI. Litter size did not seem to affect expression of members of the RhoA pathway and Lep. Our findings provide valuable information on the etiology of poor myometrial contractility in PUI, pinpointing its multifactorial etiology and emphasizing the need for further research in this area.

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References

1. **Johnson C.** False pregnancy, disorders of pregnancy and parturition, and mismating. In: Nelson RW, Couto CG (eds.), Small Animal Internal Medicine. St. Louis, MO: Mosby Elsevier; 2009: 931-935.
2. **Bennett D.** Canine dystocia—a review of the literature. *J Small Anim Pract* 1974; **15**: 101–117. [Medline] [CrossRef]
3. **Darvelid AW, Linde-Forsberg C.** Dystocia in the bitch: A retrospective study of 182 cases. *J Small Anim Pract* 1994; **35**: 402–407. [CrossRef]
4. **Linde Forsberg C, Persson G.** A survey of dystocia in the Boxer breed. *Acta Vet Scand* 2007; **49**: 8. [Medline] [CrossRef]
5. **Davidson AP.** Primary uterine inertia in four labrador bitches. *J Am Anim Hosp Assoc* 2011; **47**: 83–88. [Medline] [CrossRef]
6. **Johnston SD, Root Kustritz MV, Olson PNS.** Canine and Feline Theriogenology. Philadelphia, PA: Saunders; 2001.
7. **Feldman EC, Nelson RW.** Canine and Feline Endocrinology and Reproduction. St. Louis, MO: Saunders; 2004.
8. **Egloff S, Reichler IM, Kowalewski MP, Keller S, Goericke-Pesch S, Balogh O.** Uterine expression of smooth muscle alpha- and gamma-actin and smooth muscle myosin in bitches diagnosed with uterine inertia and obstructive dystocia. *Theriogenology* 2020; **156**: 162–170. [Medline] [CrossRef]
9. **Münnich A, Küchenmeister U.** Dystocia in numbers - evidence-based parameters for intervention in the dog: causes for dystocia and treatment recommendations. *Reprod Domest Anim* 2009; **44**(Suppl 2): 141–147. [Medline] [CrossRef]
10. **Gill MA.** Perinatal and late neonatal mortality in the dog. The University of Sidney: PhD Thesis; 2001.
11. **Hollinshead FK, Hanlon DW, Gilbert RO, Verstegen JP, Krekeler N, Volkmann DH.** Calcium, parathyroid hormone, oxytocin and pH profiles in the whelping bitch. *Theriogenology* 2010; **73**: 1276–1283. [Medline] [CrossRef]
12. **Frehner BL, Reichler IM, Keller S, Goericke-Pesch S, Balogh O.** Blood calcium, glucose and haematology profiles of parturient bitches diagnosed with uterine inertia or obstructive dystocia. *Reprod Domest Anim* 2018; **53**: 680–687. [Medline] [CrossRef]
13. **Bergström A, Fransson B, Lagerstedt AS, Olsson K.** Primary uterine inertia in 27 bitches: aetiology and treatment. *J Small Anim Pract* 2006; **47**: 456–460. [Medline] [CrossRef]
14. **Rempel LM, Lillevang KTA, Straten AT, Friðriksdóttir SB, Körber H, Wehrend A, Kowalewski MP, Reichler IM, Balogh O, Goericke-Pesch S.** Do uterine PTGS2, PGFS, and PTGFR expression play a role in canine uterine inertia? *Cell Tissue Res* 2021;

doi: 10.1007/s00441-021-03427-6. [Medline] [CrossRef]
15. **Rempel LM, Körber H, Reichler IM, Balogh O, Goericke-Pesch S.** Expression von PTGES, PTGER2 und PTGER4 im caninen Uterus von Hündinnen mit Dystokie. Congress Proceedings DVG-DGK Berlin 15.-17.10.2020, S. 279–280.
16. **Tamminen T, Sahlin L, Masironi-Malm B, Dahlbom M, Katila T, Taponen J, Laitinen-Vapaavuori O.** Expression of uterine oxytocin receptors and blood progesterone, 13,14-dihydro-15-Keto-Prostaglandin $F_{2\alpha}$ and ionized calcium levels in dystocic bitches. *Theriogenology* 2019; **135**: 38–45. [Medline] [CrossRef]
17. **Somlyo AP, Somlyo AV.** Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol* 2000; **522**: 177–185. [Medline] [CrossRef]
18. **Nakagawa O, Fujisawa K, Ishizaki T, Saito Y, Nakao K, Narumiya S.** ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett* 1996; **392**: 189–193. [Medline] [CrossRef]
19. **Julian L, Olson MF.** Rho-associated coiled-coil containing kinases (ROCK): structure, regulation, and functions. *Small GTPases* 2014; **5**: e29846. [Medline] [CrossRef]
20. **Thumkeo D, Watanabe S, Narumiya S.** Physiological roles of Rho and Rho effectors in mammals. *Eur J Cell Biol* 2013; **92**: 303–315. [Medline] [CrossRef]
21. **López Bernal A.** Mechanisms of labour—biochemical aspects. *BJOG* 2003; **110**(Suppl 20): 39–45. [Medline]
22. **Lartey J, López Bernal A.** RHO protein regulation of contraction in the human uterus. *Reproduction* 2009; **138**: 407–424. [Medline] [CrossRef]
23. **Hudson CA, Heesom KJ, López Bernal A.** Phasic contractions of isolated human myometrium are associated with Rho-kinase (ROCK)-dependent phosphorylation of myosin phosphatase-targeting subunit (MYPT1). *Mol Hum Reprod* 2012; **18**: 265–279. [Medline] [CrossRef]
24. **Kureishi Y, Kobayashi S, Amano M, Kimura K, Kanaide H, Nakano T, Kaibuchi K, Ito M.** Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J Biol Chem* 1997; **272**: 12257–12260. [Medline] [CrossRef]
25. **Moran CJ, Friel AM, Smith TJ, Cairns M, Morrison JJ.** Expression and modulation of Rho kinase in human pregnant myometrium. *Mol Hum Reprod* 2002; **8**: 196–200. [Medline] [CrossRef]
26. **Lartey J, Gampel A, Pawade J, Mellor H, Bernal AL.** Expression of RND proteins in human myometrium. *Biol Reprod* 2006; **75**: 452–461. [Medline] [CrossRef]
27. **Friel AM, Curley M, Ravikumar N, Smith TJ, Morrison JJ.** Rho A/Rho kinase mRNA and protein levels in human myometrium during pregnancy and labor. *J Soc Gynecol Investig* 2005; **12**: 20–27. [Medline] [CrossRef]
28. **Taggart MJ, Arthur P, Zielnik B, Mitchell BF.** Molecular pathways regulating contractility in rat uterus through late gestation and parturition. *Am J Obstet Gynecol* 2012; **207**: 76.e15–24. [Medline] [CrossRef]
29. **Tahara M, Morishige K, Sawada K, Ikebuchi Y, Kawagishi R, Tasaka K, Murata Y.** RhoA/Rho-kinase cascade is involved in oxytocin-induced rat uterine contraction. *Endocrinology* 2002; **143**: 920–929. [Medline] [CrossRef]
30. **Cario-Toumaniantz C, Reillaudoux G, Sauzeau V, Heutte F, Vaillant N, Finet M, Chardin P, Loirand G, Pacaud P.** Modulation of RhoA-Rho kinase-mediated Ca2+ sensitization of rabbit myometrium during pregnancy - role of Rnd3. *J Physiol* 2003; **552**: 403–413. [Medline] [CrossRef]
31. **Ergul M, Turgut NH, Sarac B, Altun A, Yildirim Ş, Bagcivan I.** Investigating the effects of the Rho-kinase enzyme inhibitors AS1892802 and fasudil hydrochloride on the contractions of isolated pregnant rat myometrium. *Eur J Obstet Gynecol Reprod Biol* 2016; **202**: 45–50. [Medline] [CrossRef]
32. **Weiss JL, Malone FD, Emig D, Ball RH, Nyberg DA, Comstock CH, Saade G, Eddleman K, Carter SM, Craigo SD, Carr SR, D'Alton ME, FASTER Research Consortium.** Obesity, obstetric complications and cesarean delivery rate—a population-based screening study. *Am J Obstet Gynecol* 2004; **190**: 1091–1097. [Medline] [CrossRef]
33. **Zhang J, Bricker L, Wray S, Quenby S.** Poor uterine contractility in obese women. *BJOG* 2007; **114**: 343–348. [Medline] [CrossRef]
34. **Stotland NE, Washington AE, Caughey AB.** Prepregnancy body mass index and the length of gestation at term. *Am J Obstet Gynecol* 2007; **197**: 378.e1–5. [Medline] [CrossRef]
35. **Norman SM, Tuuli MG, Odibo AO, Caughey AB, Roehl KA, Cahill AG.** The effects of obesity on the first stage of labor. *Obstet Gynecol* 2012; **120**: 130–135. [Medline] [CrossRef]
36. **Hautakangas T, Palomäki O, Eidstø K, Huhtala H, Uotila J.** Impact of obesity and other risk factors on labor dystocia in term primiparous women: a case control study. *BMC Pregnancy Childbirth* 2018; **18**: 304. [Medline] [CrossRef]
37. **Verdiales M, Pacheco C, Cohen WR.** The effect of maternal obesity on the course of labor. *J Perinat Med* 2009; **37**: 651–655. [Medline] [CrossRef]
38. **German AJ.** The growing problem of obesity in dogs and cats. *J Nutr* 2006; **136**(Suppl): 1940S–1946S. [Medline] [CrossRef]
39. **Park HK, Ahima RS.** Physiology of leptin: energy homeostasis, neuroendocrine function and metabolism. *Metabolism* 2015; **64**: 24–34. [Medline] [CrossRef]
40. **Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, *et al*** Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 1996; **334**: 292–295. [Medline] [CrossRef]
41. **Ishioka K, Hosoya K, Kitagawa H, Shibata H, Honjoh T, Kimura K, Saito M.** Plasma leptin concentration in dogs: effects of body condition score, age, gender and breeds. *Res Vet Sci* 2007; **82**: 11–15. [Medline] [CrossRef]
42. **Moynihan AT, Hehir MP, Glavey SV, Smith TJ, Morrison JJ.** Inhibitory effect of leptin on human uterine contractility in vitro. *Am J Obstet Gynecol* 2006; **195**: 504–509. [Medline] [CrossRef]
43. **Mumtaz S, AlSaif S, Wray S, Noble K.** Inhibitory effect of visfatin and leptin on human and rat myometrial contractility. *Life Sci* 2015; **125**: 57–62. [Medline] [CrossRef]
44. **Harrod JS, Rada CC, Pierce SL, England SK, Lamping KG.** Altered contribution of RhoA/Rho kinase signaling in contractile activity of myometrium in leptin receptor-deficient mice. *Am J Physiol Endocrinol Metab* 2011; **301**: E362–E369. [Medline] [CrossRef]
45. **O'Brien M, Carbin S, Morrison JJ, Smith TJ.** Decreased myometrial p160 ROCK-1 expression in obese women at term pregnancy. *Reprod Biol Endocrinol* 2013; **11**: 79. [Medline] [CrossRef]
46. **Balogh O, Staub LP, Gram A, Boos A, Kowalewski MP, Reichler IM.** Leptin in the canine uterus and placenta: possible implications in pregnancy. *Reprod Biol Endocrinol* 2015; **13**: 13. [Medline] [CrossRef]
47. **Cardinali L, Troisi A, Verstegen JP, Menchetti L, Elad Ngonput A, Boiti C, Canello S, Zelli R, Polisca A.** Serum concentration dynamic of energy homeostasis hormones, leptin, insulin, thyroid hormones, and cortisol throughout canine pregnancy and lactation. *Theriogenology* 2017; **97**: 154–158. [Medline] [CrossRef]
48. **Kowalewski MP, Schuler G, Taubert A, Engel E, Hoffmann B.** Expression of cyclooxygenase 1 and 2 in the canine corpus luteum during diestrus. *Theriogenology* 2006; **66**: 1423–1430. [Medline] [CrossRef]
49. **Kowalewski MP, Michel E, Gram A, Boos A, Guscetti F, Hoffmann B, Aslan S, Reichler I.** Luteal and placental function in the bitch: spatio-temporal changes in prolactin receptor (PRLr) expression at dioestrus, pregnancy and normal and induced parturition. *Reprod Biol Endocrinol* 2011; **9**: 109. [Medline] [CrossRef]
50. **Kowalewski MP, Meyer A, Hoffmann B, Aslan S, Boos A.** Expression and functional implications of peroxisome proliferator-activated receptor gamma (PPARγ) in canine reproductive tissues during normal pregnancy and parturition and at antiprogestin induced abortion. *Theriogenology* 2011; **75**: 877–886. [Medline] [CrossRef]
51. **Kowalewski MP, Mason JI, Howie AF, Morley SD, Schuler G, Hoffmann B.** Characterization of the canine 3beta-hydroxysteroid dehydrogenase and its expression in the corpus luteum during diestrus. *J Steroid Biochem Mol Biol* 2006; **101**: 254–262. [Medline] [CrossRef]
52. **Borge KS, Tønnessen R, Nødtvedt A, Indrebø A.** Litter size at birth in purebred dogs—a retrospective study of 224 breeds. *Theriogenology* 2011; **75**: 911–919. [Medline] [CrossRef]