Original Article

mRNA and Protein Expressions of Prostaglandin E_2 Receptors (EP_2 and EP_4), Cyclooxygenase-2 and Prostaglandin E Synthase in the Cervix of Cyclic Bitches and those with Pyometra

Pichanun Linharattanaruksa[1] Theerawat Swangchan-Uthai[1] Sayamon Srisuwatanasagul[2] Muhammad Khalid[3] Kaywalee Chatdarong[1*]

Abstract

Prostaglandins play a vital role in regulation of cervical patency. Prostaglandin E_2 (PGE_2) synthesis is regulated by cyclooxygenase (COX) and prostaglandin E synthase (PGES). PGE_2 acts through prostaglandin E receptors (EP), EP_2 and EP_4, to stimulate muscle relaxation. The aim of this study was to investigate mRNA expressions (EP_2, EP_4, COX-2, and PGES) and protein expressions (EP_2, EP_4, and COX-2) in the bitch cervix. Two groups of bitches, normal cyclic bitches and bitches with pyometra, were studied. Cyclic bitches were categorized into anestrus (n = 10), estrus (n = 7), and diestrus (n = 11), whereas bitches with pyometra were defined as open- (n = 18) or closed-cervix pyometra (n = 8) depending on the presence or absence of vaginal discharge, respectively. Cervices from the internal to external os were collected immediately after ovariohysterectomy. RNA extraction from cervical tissue was determined for levels of EP_2, EP_4, COX-2, and PGES mRNA using real-time qPCR. Western blot was performed to evaluate the protein expressions of EP_2, EP_4, and COX-2. There were no differences of mRNA and protein expressions in the bitch cervix among the stages of the estrous cycle. However, the expressions of PGES mRNA was higher in the cervix of bitches with open-cervix than closed-cervix pyometra ($p < 0.05$). The differences of protein expressions of EP_2, EP_4, and COX-2 were not observed. Our findings suggest that mRNA and protein expressions of the enzymes involved in PGE_2 synthesis and PGE_2 receptors are not influenced by hormonal status during the estrous cycle whereas PGES mRNA expression is likely associated with cervical relaxation in the bitches with pyometra.

Keywords: cervical patency, dog, prostaglandins, transcription

[1] *Department of Obstetrics, Gynaecology and Reproduction,*
[2] *Department of Anatomy, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand*
[3] *Department of Veterinary Clinical Sciences, the Royal Veterinary College, University of London, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire, AL9 7TA, UK.*
***Corresponding author: E-mail:** kaywalee.c@chula.ac.th*

บทคัดย่อ

การแสดงออกของเมสเซนเจอร์อาร์เอ็นเอของตัวรับพรอสตาแกลนดินอี2(ซับไทป์ 2 และ 4) ไซโคลออกซีจิเนส-2 และเอนไซม์พรอสตาแกลนดินอีซินเทส ในคอมดลูกสุนัขที่อยู่ในวงรอบการเป็นสัดและในสุนัขเป็นมดลูกอักเสบแบบมีหนอง

พิชนันท์ ลีฬหรัตนรักษ์ [1] ธีรวัฒน์ สว่างจันทร์อุทัย [1] ศยามณ ศรีสุวัฒนาสกุล [2] มูฮัมหมัด คาลิด [3] เกวลี ฉัตรดรงค์ [1]*

พรอสตาแกลนดินมีความสำคัญในการควบคุมการเปิดปิดของคอมดลูก การผลิตพรอสตาแกลนดินอี$_2$ (PGE_2) ควบคุมโดยเอนไซม์ไซโคลออกซีจิเนส (COX) และพรอสตาแกลนดินอีซินเทส (PGES) พรอสตาแกลนดินอี$_2$ทำงานผ่านการจับกับตัวรับฮอร์โมนพรอสตาแกลนดินอี$_2$ (EP) ซับไทป์ EP_2และ EP_4 ทำให้เกิดการคลายตัวของกล้ามเนื้อ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจหาระดับการแสดงออกของเมสเซนเจอร์อาร์เอ็นเอ คือ EP_2 EP_4 COX-2 และ PGES ในคอมดลูกของสุนัขระยะต่างๆ ของวงรอบการเป็นสัด และในสุนัขที่มีปัญหามดลูกอักเสบแบบมีหนอง ทำการศึกษาในสุนัขปกติจำนวนทั้งหมด 28 ตัว โดยแบ่งออกเป็น 3 กลุ่ม คือ แอนเอสตรัส (10 ตัว) เอสตรัส (7 ตัว) และไดเอสตรัส (11 ตัว) ส่วนสุนัขกลุ่มที่มีปัญหามดลูกเป็นหนองจำนวน 26 ตัว แบ่งออกเป็น 2 กลุ่ม คือ มดลูกเป็นหนองแบบคอมดลูกเปิด (18 ตัว) และแบบคอมดลูกปิด (8 ตัว) โดยดูจากการพบหรือไม่พบของเหลวไหลจากช่องคลอด เก็บคอมดลูกจากช่องเปิดด้านในถึงช่องเปิดด้านนอกหลังการทำหมันโดยตัดรังไข่และมดลูกออก สกัดอาร์เอ็นเอจากตัวอย่างคอมดลูกแล้วนำมาตรวจหาระดับการแสดงออกของ EP_2 EP_4 COX-2 และ PGES ด้วยวิธี quantification real-time PCR (qPCR) จากการศึกษาพบว่า การแสดงออกของเมสเซนเจอร์อาร์เอ็นเอที่สนใจไม่มีความแตกต่างกันในสุนัขในแต่ละระยะของวงรอบการเป็นสัด แต่การแสดงออกของเมสเซนเจอร์อาร์เอ็นเอของPGES ในกลุ่มสุนัขที่มีปัญหามดลูกเป็นหนองแบบคอมดลูกเปิดมีค่ามากกว่าในกลุ่มคอมดลูกปิด ($p < 0.05$) ดังนั้น ระดับฮอร์โมนที่เปลี่ยนแปลงในแต่ละระยะของวงรอบการเป็นสัด น่าจะไม่มีผลต่อการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างพรอสตาแกลนดินอี2และตัวรับฮอร์โมนพรอสตาแกลนดินอี$_2$ ซับไทป์ 2 และ 4 แต่ PGES น่าจะมีส่วนเกี่ยวข้องกับกลไกการเปิดของคอมดลูกในสุนัขที่มีปัญหามดลูกเป็นหนองแบบคอมดลูกเปิด

คำสำคัญ: ภาวะเปิดปิดของคอมดลูก สุนัข พรอสตาแกลนดิน ทรานสคริปชั่น

[1] *ภาควิชาสูติศาสตร์ เธนุเวชวิทยา และวิทยาการสืบพันธุ์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330*
[2] *ภาควิชากายวิภาคศาสตร์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330*
[3] *Department of Veterinary Clinical Sciences, the Royal Veterinary College, University of London, UK*
***ผู้รับผิดชอบบทความ E-mail:** kaywalee.c@chula.ac.th*

Introduction

Relaxation of the cervix is regulated by complex mechanism. Current knowledge about mechanism of cervical relaxation arises from the studies of pregnancy and parturition in humans (Timmons et al., 2010), rats (Chien and Macgregor, 2003), rabbits (Fukuda et al., 2007) and sheep (Kershaw-Young et al., 2009). In women, understanding of the control of cervical relaxation is crucial for the management of labor including its induction in case of prolonged pregnancy and treatment of undesired preterm births. In sheep, cervical relaxation may facilitate transcervical intrauterine artificial insemination (Kershaw et al., 2007). The cervical relaxation is regulated by serum hormonal concentrations, prostaglandin synthesis, and extracellular matrix remodeling (Kershaw-Young et al., 2009). In bitches, relationship between hormonal status and cervical patency is partly known. In general, progesterone induces closure of the cervical canal and estrogen is capable of opening the canal during estrus which is important in fertilization process (Silva et al., 1995). Moreover, prostaglandin E_2 (PGE_2) was shown to induce cervical ripening in human when administered intravaginally (Feltovich et al., 2005). Biosynthesis of PGE_2 is regulated mainly by two enzymes, cyclooxygenase (COX) which converts arachidonic acid to prostaglandin H_2 (PGH_2), and prostaglandin E synthase (PGES) which converts PGH_2 to PGE_2. There are two forms of COX, COX-1 and COX-2. COX-1 is a constitutive enzyme, whereas COX-2 is specific to inflammatory process (Tamada et al., 2012) and can be regulated by hormonal changes (Kowalewski et al., 2006). Studies in baboons and sheep revealed that cervical glandular epithelial cells were the major source of COX-2 mRNA (Wu et al., 2004; Wu et al., 2005). Moreover, a previous study in sheep showed that COX-2 mRNA was highly expressed in the cervix at estrus when serum estradiol

concentrations were high, indicating a relationship between PGE_2 and estradiol (Kershaw et al., 2007). Prostaglandin E_2 exerts its role by coupling to prostaglandin E receptors (EP); EP_1, EP_2, EP_3 and EP_4. EP_1 and EP_3 are involved in the contraction of smooth muscle, whereas EP_2 and EP_4 function to induce smooth muscle relaxation (Narumiya et al., 1999). EP_4 mRNA expression in the rat cervix was shown to be involved in cervical remodeling at the term time (Feltovich et al., 2005).

In bitches, pyometra is a common uterine disease which affects 25% of adult animals before they reach 10 years of age (Verstegen et al., 2008).The disease is classified into two categories; open- and closed-cervix pyometra. Clinical signs of pyometra depend primarily on whether the cervix is patent enough to allow the drainage of purulent fluid in uterus (Pretzer, 2008). Bitches with closed-cervix pyometra suffer from enlarged uteri and risk of uterine rupture. Cervical patency is a key to decide whether medical treatment is possible for pyometra bitches. To release pus from the uterus, aglepristone, an antiprogestin along with prostaglandins are reported to successfully induce cervical relaxation (Fieni et al., 2001). However, the exact mechanism of the induction of cervical relaxation in the bitch is unknown. In our previous study, the number of neutrophils was significantly higher in the bitches with open-cervix compared to that with closed-cervix pyometra (Kunkitti et al., 2011). Moreover, induction of cervical relaxation by neutrophil infiltration was also demonstrated in rabbits at the term time and was reported to be mediated by EP_4 (Fukuda et al., 2007). Taken together, we hypothesized that there were associations between PGE_2 [via its receptors (EP_2 and EP_4)], enzymes involved in PGE_2 synthesis (COX-2 and PGES), and various stages of the estrous cycle which were influenced by sex steroid hormones. Moreover, the corresponding relationships might exist also in bitches affected with open- and closed-cervix pyometra. The present study aimed to investigate mRNA expressions (EP_2, EP_4, COX-2 and PGES) and protein expressions (EP_2, EP_4, and COX-2) in normal cyclic bitches during different stages of the estrous cycle and those with pyometra.

Materials and Methods

Animals: Twenty eight bitches of various breeds with an average age of 2.1±0.8 years (ranging from 1-4 years) subjected to routine spaying were divided into 3 stages of estrous cycle; anestrus (n = 10), estrus (n = 7), and diestrus (n = 11), on the basis of presence or absence of large follicles or corpora lutea on the ovaries, serum progesterone concentrations and vaginal cytology (Table 1). Another group of 28 bitches diagnosed as having uterine content by ultrasonography were divided into open-cervix (n = 18) and closed-cervix pyometra (n = 10) according to presence or absence of vaginal discharge, respectively. The bitches affected with pyometra had an average age of 7.0±3.9 years (ranging from 2-15 years).

Cervical tissue collection: Cervical tissue from each animal was collected immediately after ovariohysterectomy and prepared as previously described (Kunkitti et al., 2011). In brief, after collection the cervices were longitudinally cut into 2 parts. The cervical tissue was then cut into 0.1 g pieces and frozen in liquid nitrogen immediately and stored at-80^{0}C until used for RNA and protein extraction.

RNA extraction, reverse transcription and real-time qPCR: Cervical tissues were pulverized with sterile mortar and pestle. Total RNA was extracted from frozen cervical tissue using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) at room temperature following the manufacturer's instructions. The RNA concentration and purity were accessed using spectrophotometer at 260 and 280 nm (Nanadrop ND-2000, Wilmington, Delaware, USA). After extraction RNA was stored at -80^{0}C. To ensure complete removal of any trace amounts of genomic DNA, DNA digestion was performed with RNase-free DNase set (1 U/µg RNA, Promega, Madison, USA) at 37^{0}C for 30 min. Subsequently, single-stranded complementary DNA (cDNA) synthesis was performed using Omniscript First-Strand cDNA Synthesis Kit according to the guidelines supplied by the manufacturer (Invitrogen, Carlsbad, CA, USA). Ten µl of extracted RNA was incubated with 2 µl of random primer (100 µM), 2 µl of 5 mM deoxynucleotide triphosphates (dNTP) mix, 2 µl of 10x Buffer RT, 0.25 µl of RNase inhibitor (40 units/µl), 1 µl of Omniscript RT and 2.75 µl of RNase free water at 37^{0}C for 60 min. Selected negative control samples were prepared by including all reagents as above, minus the reverse transcriptase. A master mix of RT reagent was prepared once to minimize potential variation.

For quantification of mRNA expression of EP2, EP4, COX-2, and PGES, the quantitative real-time PCR (qPCR) standard curve method was employed as previously described (Swangchan-Uthai et al., 2011). To standardize the quantification method, an endogenous RN18S1 was chosen as reference gene. The primer pairs of interested genes were inferred from published canine sequences as shown in Table 2. The primers were then tested by

Table 1 Criteria used to determine the stage of estrous cycle, ovarian structure, appearance of vaginal cytology, serum progesterone level, and number of healthy cyclic bitches.

	Anestrus	Estrus	Diestrus
Dominant ovarian structure	Absence of ovarian activity	Follicles	Corpora lutea
Majority of cells in the vaginal cytology	Basal cells	Cornified cells	Non-cornified cells
Serum progesterone (ng/ml)	< 1	2-15	> 15
(n)	10	7	11

n indicates the number of animals within each group

conventional PCR amplification using Platinum PCR supermix (22 U/ml complexed recombinant Taq DNA polymerase with Platinum® Taq Antibody, 22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM $MgCl_2$, 220 µM dGTP, 220 µM dATP, 220 µM dTTP, 220 µM dCTP, and stabilizers) containing Tag polymerase (Invitrogen; Invitrogen Ltd. UK), 50 ng cDNA and 20 µM primers. The hot-start PCR reaction was run for 2 min at 95^{0}C (activation of Taq polymerase), followed by 38 cycles, each one comprising a denaturing step at 95^{0}C for 30 sec, annealing at 62.5^{0}C (for EP_2 and EP_4) or 64.1^{0}C (for COX-2) or 62.5^{0}C (for PGES) for 30 sec, extension at 72^{0}C for 20 sec and final elongation at 72^{0}C for 5 min. The amplification products were separated by electrophoresis through a 2% (w/v) agarose gel (Bio-Rad, CA, USA). The desired PCR products of all interested genes (EP_2, EP_4, COX-2, PGES and RN18S1) were visualized as a single band. The identity of the PCR products was confirmed by DNA sequencing (GATC Biothech, London, UK).

In order to optimize real-time qPCR assay, serial dilutions of external standards and sample to be used in melting curve and annealing temperature analysis were prepared. The purified PCR product of each gene was produced from a conventional PCR amplification and then purified by QIAquick PCR Purification kits in accordance with guidelines supplied by Qiagen (QIAGEN, Valencia, CA, USA).The concentration of purified PCR product was determined by NanoDrop ND-1000 spectrophotometer.In the following stage, the optimal annealing temperature and melting curve analysis were assessed using a temperature gradient feature ranging from 50-65^{0}C.

The transcripts of each gene were determined by an optimized qPCR procedure with a single-plex SYBR Green I assay (CFX 96 Real-Time PCR Detection System, Bio-Rad Lab, Inc, CA, USA). The amplification mixes contained 10 µl of KAPA SYBR FAST qPCR Kits (GRI, Braintree, Essex, UK), 0.5 µl of 20 µM forward and reverse primers mix, 4.5 µl of nuclease free water and 5 µl of unknown sample (50 ng of cDNA).The qPCR reactions for both standards and samples were performed in duplicate in PCR plate. Thermal cycling conditions applied to each assay consisted of an initial Taq activation step at 95^{0}C for 5 min followed by 38 cycles of denaturation at 95^{0}C for 15 sec, annealing temperature as shown in Table 2, extension at 72^{0}C for 20 sec followed by amplicon-specific fluorescence acquisition reading (range 74-84^{0}C).Absolute concentrations of the PCR product were calculated by comparing the Cq values of the unknown samples to a standard curve using CFX ManagerTM Software Version 1.0.1035.131 (Bio-Rad Lab, Inc) and expressed as fg/µg reverse-transcribed RNA.

Western Blot analysis: Frozen cervical tissues were ground to powder with sterile mortar and pestle and treated with lysis buffer containing Tris-Glycine-SDS buffer, sodium, orthovanadate (200mM), and protease inhibitor cocktail set 1 (Calbiochem, Germany). The sample was vortexed and centrifuged at 13,000 rpm at 4^{0}C for 10 min, supernatant were collected and the sample was boiled at 100^{0}C for 5 min. Two µl of cervical lysate solution were used for determination of the protein concentration by spectrophotometry using Protein A280 technique (NanoDrop® ND1000 spectrophotometer, NanoDrop Technologies Inc, Wilmington, Delaware, USA). The rest of lysate was mixed with 5% β-mercaptoethanol (Sigma-Aldrich GmbH, Steinheim, Germany) and 0.02% bromophenol blue (Sigma-Aldrich GmbH, Steinheim, Germany) and stored at -20^{0}C. Protein extracts (30 µg) were resolved on a 12% SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel) with a 4% stack. Proteins were transferred to a polyvinylidine difluoride membrane (BioTraceTM PVDF transfer membrane, MI, USA) and blocked with 5% BSA in TBS-T at room temperature for 1 hour. Immunoblots were performed by using goat polyclonal antihuman EP_2 (sc-22196), goat polyclonal antihuman EP_4 (sc-16022) (Santa Cruz, California, USA), rabbit polyclonal antimurine COX-2 (catalogue 16016 Cayman Chemecal, Ann Arbor, Mich), and mouse monoclonal beta actin (AC-15, ab6276, abcam®, Abcam plc, San Francisco, USA) at dilution of 1 : 250, 1 : 250, 1 : 250 and 1 : 5,000, respectively, in clocker solution and incubated at 4^{0}C overnight. Horseradish peroxidase (HRP-)-conjugated horse anti-goat IgG (PI-9500, Vector Laboratories, California, USA) and polyclonal rabbit anti-mouse immunoglobulin (DAKO, Glostrup, Denmark) were used as the secondary antibodies at dilution of 1 : 500 and 1 : 1.000 in TBS-T, respectively, and incubated at room temperature for 5 hours with intensive washing in TBS-T between each step.

Table 2 Primer sequences for EP_2, EP_4, Cox-2 and PGES, expected product's length, and specific annealing temperature used for real-time PCR

Primer	Accession number	Primer sequence	Annealing	Product-length (bp)
EP_2	NM_001003170.1	F: 5′-TTC TCC TGG CTA TTA TGA CC-3′	62.5	273
		R: 5′-ATC TAC TGG CGT TTG ACT G-3′		
EP_4	NM_001003054.1	5′F: -GGT ACG GGT GTT CAT CAA C-3′	62.5	323
		5′AGA R: AGA GGA GGG TCT GAG ATG TG-3′		
COX -2	NM_001003354.1	5′F: -ACA GGA GAG AAG GAA ATG GC-3′	64.1	250
		5′R: -GGA TTG AGG CAG TGT TGA TG-3′		
PGES	NM_001122854.1	5′F: -ACC ATC TAC CCC TTC CTG T-3′	62.5	214
		5′R: -CTG CTT CCC AGA CGA TCT-3′		

Bound antibodies were developed with diaminobenzidine (DAB) (ImmPACTTM DAB Peroxidase Substrate, Vector Laboratories, California, USA) for 5 min until the brown color of protein band developed. Membrane was scanned with HP scanner (HP Scanjet 2400c, HP, USA) to get the image. The optical density of EP_2, EP_4, and COX-2 bands were measured by using Quantity One Software Program version 4.4.0 (Bio-Rad Laboratories). The specific density of each sample was calculated by subtraction of the background density and divided by the specific density of quality control band.

Statistical analyses: Statistical test of analysis of variance (ANOVA) using SPSS for Microsoft Windows software (version 19; SPSS Inc., Chicago, IL, USA)was performed to analyze the differences of mRNA and protein expression in bitches during different stages of the estrous cycle (anestrus, estrus and diestrus). Data were tested for homogeneity of variance using Levene's test. Post hoc comparisons were performed using Bonferroni correction to test for significances. All data are expressed as mean±SEM. For pyometra groups, t-test was used to analyze differences of mRNA expression between open-cervix and closed-cervix pyometra groups. Values were considered to be statistically significant at $p \leq 0.05$.

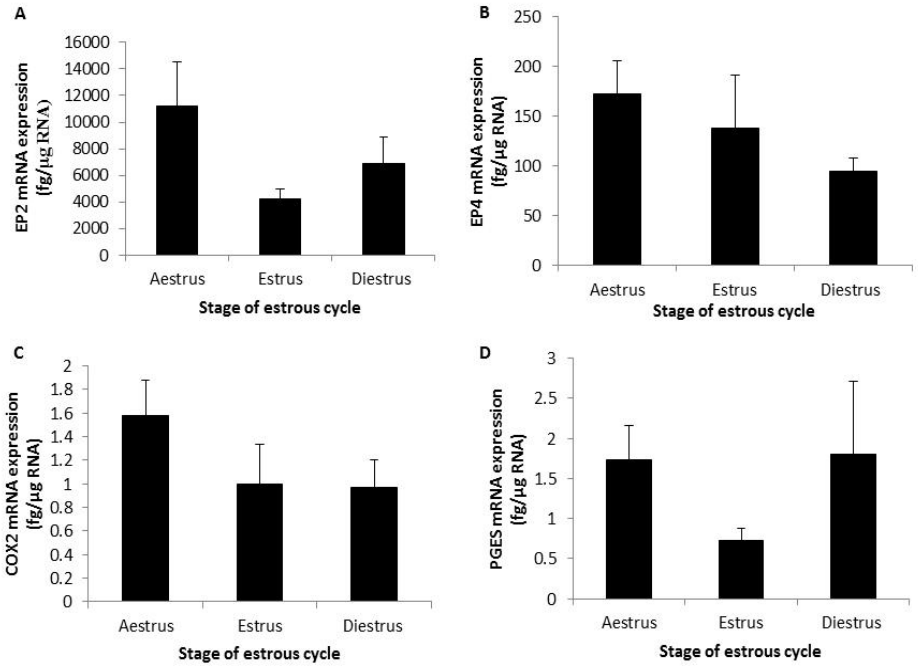

Figure 1 Mean (±SEM) mRNA expressions of EP_2, EP_4, COX-2 and PGES in cervices of healthy bitches during different stages of the estrous cycle

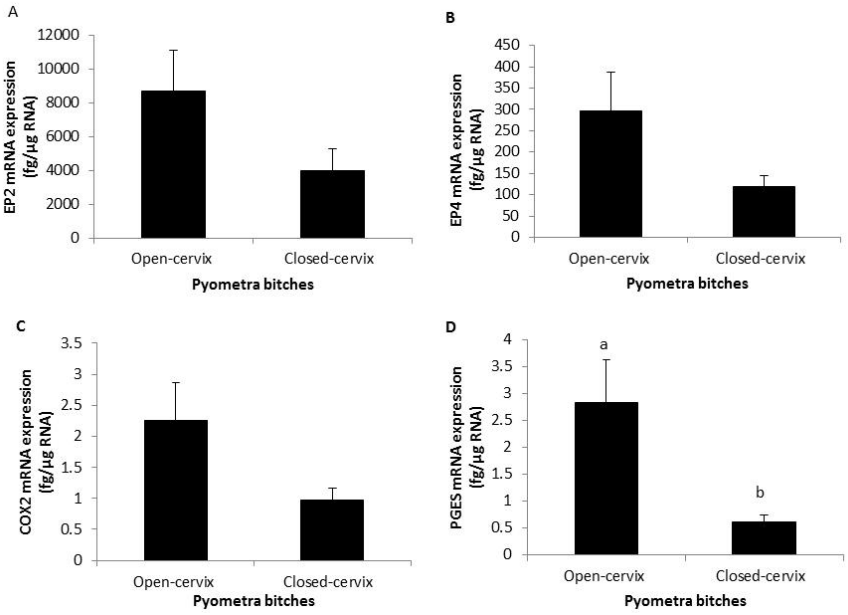

Figure 2 Mean (±SEM) mRNA expression of EP_2, EP_4, COX-2 and PGES in cervices of bitches with open- or closed-cervix pyometra. Bars with different superscripts show significant ($p < 0.05$) differences.

Results

Cervical expression of EP_2, EP_4, COX-2, and PGES mRNA in cyclic and pyometra bitches: The mRNA for EP_2, EP_4, COX-2 and PGES was expressed in cervical tissue of the normal cyclic bitches during all stages of the estrous cycle. However, no differences were observed in the expression of any gene studied among the stages of the estrous cycle when analyzed either as relative number (after normalized with RN18S1) or absolute concentrations (fg cDNA/µg RT RNA). The data are presented as absolute numbers in Fig 1. In the bitches with pyometra, PGES mRNA expression in the cervical tissue of open-cervix pyometra was higher than closed-cervix pyometra ($p < 0.05$) whereas the expression of EP_2, EP_4, and COX-2 did not differ between the two groups (Fig 2).

Cervical expression of EP_2, EP_4, and PGES proteins in cyclic and pyometra bitches: The results from western blot confirmed the protein expressions of EP_2, EP_4, and COX-2 in canine cervical tissue during estrous cycle and in bitches with pyometra. EP_2, EP_4, and COX-2 proteins were expressed in anestrus, estrus and diestrus although the differences among them were not observed ($p > 0.05$) (Fig 3). Similarly, there were no differences of EP_2, EP_4, and COX-2 protein expressions between open-cervix and closed-cervix pyometra ($p > 0.05$) (Fig 4).

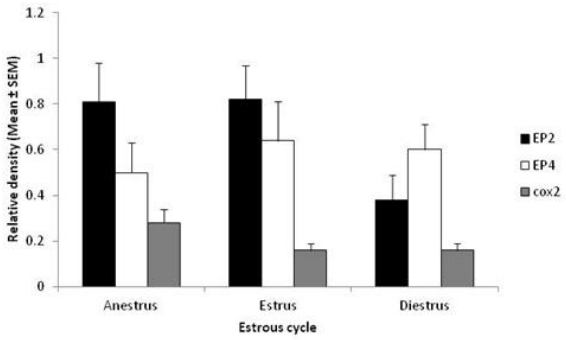

Figure 3 Relative density (Mean±SEM) of EP_2 receptor protein expression of canine cervix at estrous cycle

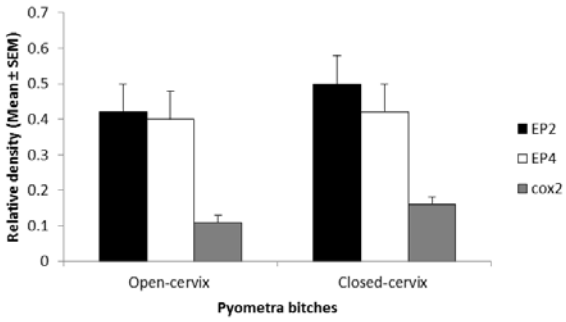

Figure 4 Relative density (Mean±SEM) of EP_4 receptor protein expression of canine cervix in bitches with open-cervix pyometra and closed-cervix pyometra

Discussion

In the present study, the cervical mRNA and protein expressions of EP_2, EP_4, COX-2, and PGES were studied in bitches during the various stages of the estrous cycle and those affected with pyometra with the aim to help the understanding of the prostaglandin associated cervical relaxation.

Estradiol and progesterone are two major steroids functioning during the estrous cycle to regulate cervical patency possibly through prostaglandins production (Kershaw et al., 2005; Kershaw et al., 2007; Kershaw-Young et al., 2009). In the present study mRNA expression of COX-2 in the cervix was not influenced by dominated hormones during the estrous cycle. Our results are concordant with the study of COX-2 mRNA expression in the bitch uterus, demonstrating no differences between anestrus and diestrus (Silva et al., 2009), whereas they differ from the studies in sheep, in which estradiol was shown to significantly elevate the COX-2 mRNA expression in the cervix (Zhang et al., 2007; Kershaw-Young et al., 2010). The protein expression of COX-2 in this study did not differ among the stages of estrous cycle. The study in sheep cervix demonstrated that COX-2 protein expression did not increase in cervix treated with estradiol compared to control group (Zhang et al., 2007), which is concordant with our result. On the contrary, the high level of COX-2 protein expression in pre-LH surge was found compared to luteal phase (Falchi and Scaramuzzi, 2013). One possible explanation is that the modulation of COX-2 expression may involve multifactorial regulation. Although the differences in EP_2 mRNA expression were not statistically significant between the estrous and diestrous periods, the considerably lower EP_2 mRNA expression during estrus might resulted from a down regulation by the estradiol during the estrous stage. Similar findings were demonstrated in the baboon cervix in which EP_2 mRNA was lower during labor when the cervix was relaxed than the animals that were not in labor (Smith et al., 2001). Such differences were also observed in the rat uterus (Brodt-Eppley and Myatt, 1998). Interestingly, the EP_2 mRNA expression was regulated by changes in estrogen and progesterone in the mouse uterus in the fashion that the estrogen down-regulated the level of EP_2 mRNA whereas progesterone up-regulated the expression of this receptor (Lim and Dey, 1997). This was also supported by the report in the rat, showing the increase of EP_2 mRNA expression in the myometrium after treatment with progesterone (Dong and Yallampalli, 2000). However, the protein expression of EP_2 did not differ between estrus and diestrus, suggesting that the regulation of EP_2 expression might be modulated at the translation stage. Our results of a lack of differences in EP_4 mRNA expression throughout the estrous cycle are similar to the previous study in the sheep (Kershaw-Young et al., 2009). Although the circulating estradiol concentrations did not seem to influence mRNA and

protein expression of EP_4 in the bitch cervix in the present study, the immunohistochemical study revealed a higher EP_4 expression in the luminal epithelium of the bitch cervix during estrus than anestrus in our previous study (Linharattanaruksa et al., 2013). Moreover, the protein expression of EP_4 in rat cervical tissue increased with advancing gestation and was found highest on the day of parturition when cervix relaxed (Chien and Macgregor, 2003). These lead us to propose that the differential expression of EP4 during the different stages of the estrous cycle are localized in certain regions and with the whole cervix together tissue, such differences in EP_4 expression become masked.

It has been known that PGE_2 plays an important role in various inflammatory responses (Rocca and FitzGerald, 2002) including cervical relaxation during labor by modification of extracellular matrix, i.e. a decrease in collagen content and an increase in collagen remodeling in the cervix (Rodriguez et al., 2003). In mammalian cervix, proinflammatory cytokines responsible for the changes in extracellular matrix are secreted by various cells including cervical fibroblasts, endothelial cells, and leukocytes such as neutrophils. Although the protein expressions of EP_2 and EP_4 protein did not differ between open- and closed-cervix pyometra in this study, our previous study revealed that immunolocalization of EP_4 expression was higher in the open-cervix than closed-cervix pyometra (Linharattanaruksa et al., 2013). These finding support the notion that EP_4 protein expression in specific tissue layer (in this case luminal epithelium) may be involved in cervical relaxation in the bitches.

In the present study, expression of COX-2 mRNA and protein did not differ between open- and closed-cervix pyometra while the PGES mRNA did, suggesting that a post-COX enzyme mechanism is likely to be involved in the determination of final prostaglandin product (Hafez and Smith, 1982).

In our previous study, the number of neutrophils in the cervix was higher in bitches with open- than closed-cervix pyometra (Kunkitti et al., 2011). In this study, higher COX-2 and PGES mRNA levels were observed in the cervix of bitches with pyometra compared to the normal cyclic bitches, indicating an up-regulated gene transcription. This was also reported in the endometrium of the bitches with pyometra (Silva et al., 2009). COX-2 and PGES are said to be stimulated by proinflammatory cytokines and endotoxins such as lipopolysaccharides (LPS) (Murakami et al., 2000; Helliwell et al., 2004). Moreover, the main proinflammatory cytokine in cervical relaxation is interleukin-8 (IL-8) (Uchiyama et al., 1992; el Maradny et al., 1994; El Maradny et al., 1996), which has chemotactic effect on neutrophils which are the major source of matrix metalloproteinases (MMPs) (Stygar et al., 2002). Furthermore, IL-8 stimulates the activation and degranulation of neutrophils, thus provoking the release of MMPs (van Engelen et al., 2009). MMPs are a family of enzymes that are classified according to substrates such as collagenase, gelatinase, and stromelysin (Sorsa et al., 2004) that can degrade components of the extracellular matrix. The expression of IL-8 in the cervix is stimulated by PGE_2 (Ito et al., 1994) and the increase in IL-8 concentrations stimulates degranulation of MMPs from neutrophils (Kelly, 2002; Tamada et al., 2012). This, in turn, results in the changes in collagen turn over in extracellular matrix and leads to cervical relaxation. From our results, we propose that the increase in PGES mRNA expression is stimulated by proinflammatory cytokines, resulting in the enhanced production of PGE_2 that subsequently stimulates neutrophil infiltration through the IL-8 and releases MMPs to degrade extracellular matrix causing cervical relaxation.

In conclusion, the cervix of normal bitches during various stages of the estrous cycle expressed the same levels of EP_2 and EP_4 mRNA. Moreover, the biosynthesis of PGE_2 by COX-2 and PGES was not influenced by the stages of the estrous cycle. In addition, PGES seems to control the production of PGE_2 to regulate cervical relaxation in the bitches with open-cervix pyometra.

Acknowledgements

The study was financially supported by the Royal Thai Golden Jubilee (RGJ) Ph.D. program, Thailand Research Fund and the Research Fund Project, Faculty of Veterinary Science, Chulalongkorn University. We thanks the Research Unit of Obstetrics and Reproduction in animals for providing parts of laboratory equipment. We would like to thank Dr. Thanida Sanammuang for her laboratory technical assistance.

References

Brodt-Eppley J and Myatt L 1998. Changes in expression of contractile FP and relaxatory EP_2 receptors in pregnant rat myometrium during late gestation, at labor, and postpartum. Biol Reprod. 59(4): 878-883.

Chien EK and Macgregor C 2003. Expression and regulation of the rat prostaglandin E_2 receptor type 4 (EP_4) in pregnant cervical tissue. Am J Obstet Gynecol. 189(5): 1501-1510.

Dong YL and Yallampalli C 2000. Pregnancy and exogenous steroid treatments modulate the expression of relaxant EP(2) and contractile FP receptors in the rat uterus. Biol Reprod. 62(3): 533-539.

el Maradny E, Kanayama N, Halim A, Maehara K, Sumimoto K and Terao T 1994. Interleukin-8 induces cervical ripening in rabbits. Am J Obstet Gynecol. 171(1): 77-83.

El Maradny E, Kanayama N, Halim A, Maehara K, Sumimoto K and Terao T 1996. Biochemical changes in the cervical tissue of rabbit induced by interleukin-8, interleukin-1beta, dehydroepiandrosterone sulphate and prostaglandin E2: a comparative study. Hum Reprod. 11(5): 1099-1104.

Falchi L and Scaramuzzi RJ 2013. The expression of

ERalpha, OTR, cPLA(2), COX-2, and PPARgamma in the cervix of the ewe during the estrous cycle. Theriogenology. 79(1): 40-47.

Feltovich H, Ji H, Janowski JW, Delance NC, Moran CC and Chien EK 2005. Effects of selective and nonselective PGE2 receptor agonists on cervical tensile strength and collagen organization and microstructure in the pregnant rat at term. Am J Obstet Gynecol. 192(3): 753-760.

Fieni F, Marnet PG, Martal J, Siliart B, Touzeau N, Bruyas JF and Tainturier D 2001. Comparison of two protocols with a progesterone antagonist aglepristone (RU534) to induce parturition in bitches. J Reprod Fertil Suppl. 57: 237-242.

Fukuda Y, Sugimura M, Suzuki K and Kanayama N 2007. Prostaglandin E2 receptor EP4-selective antagonist inhibits lipopolysaccharide-induced cervical ripening in rabbits. Acta Obstet Gynecol Scand. 86(11): 1297-1302.

Hafez ESE and Smith JP 1982. Carcinoma of the Cervix : Biology and Diagnosis. Springer, New York. 272.

Helliwell RJ, Adams LF and Mitchell MD 2004. Prostaglandin synthases: recent developments and a novel hypothesis. Prostaglandins Leukot Essent Fatty Acids. 70(2): 101-113.

Ito A, Imada K, Sato T, Kubo T, Matsushima K and Mori Y 1994. Suppression of interleukin 8 production by progesterone in rabbit uterine cervix. Biochem J. 301(Pt 1): 183-186.

Kelly RW 2002. Inflammatory mediators and cervical ripening. J Reprod Immunol. 57(1-2): 217-224.

Kershaw-Young CM, Khalid M, McGowan MR, Pitsillides AA and Scaramuzzi RJ 2009. The mRNA expression of prostaglandin E receptors EP2 and EP4 and the changes in glycosaminoglycans in the sheep cervix during the estrous cycle. Theriogenology. 72(2): 251-261.

Kershaw-Young CM, Scaramuzzi RJ, McGowan MR, Pitsillides AA, Wheeler-Jones CP and Khalid M 2010. The effect of estradiol on COX-2, EP2, and EP4 mRNA expression and the extracellular matrix in the cervix of the hypogonadotrophic, ovariectomized ewe. Theriogenology. 73(5): 620-628.

Kershaw CM, Khalid M, McGowan MR, Ingram K, Leethongdee S, Wax G and Scaramuzzi RJ 2005. The anatomy of the sheep cervix and its influence on the transcervical passage of an inseminating pipette into the uterine lumen. Theriogenology. 64(5): 1225-1235.

Kershaw CM, Scaramuzzi RJ, McGowan MR, Wheeler-Jones CP and Khalid M 2007. The expression of prostaglandin endoperoxide synthase 2 messenger RNA and the proportion of smooth muscle and collagen in the sheep cervix during the estrous cycle. Biol Reprod. 76(1): 124-129.

Kowalewski MP, Schuler G, Taubert A, Engel E and Hoffmann B 2006. Expression of cyclooxygenase 1 and 2 in the canine corpus luteum during diestrus. Theriogenology. 66(6-7): 1423-1430.

Kunkitti P, Srisuwatanasagul S and Chatdarong K 2011. Distribution of estrogen receptor alpha and progesterone receptor, and leukocyte infiltration in the cervix of cyclic bitches and those with pyometra. Theriogenology. 75(6): 979-987.

Lim H and Dey SK 1997. Prostaglandin E2 receptor subtype EP2 gene expression in the mouse uterus coincides with differentiation of the luminal epithelium for implantation. Endocrinology. 138(11): 4599-4606.

Linharattanaruksa P, Chatdarong K, Ponglowhapan S, Khalid M and Srisuwatanasagul S 2013. Immunolocalization of prostaglandin E2 receptor subtype 4 (EP4) in the cervix of cyclic bitches and those with pyometra. Pak Vet J. in press.

Murakami M, Naraba H, Tanioka T, Semmyo N, Nakatani Y, Kojima F, Ikeda T, Fueki M, Ueno A, Oh S and Kudo I 2000. Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. J Biol Chem. 275(42): 32783-32792.

Narumiya S, Sugimoto Y and Ushikubi F 1999. Prostanoid receptors: structures, properties, and functions. Physiol Rev. 79(4): 1193-1226.

Pretzer SD. 2008. Clinical presentation of canine pyometra and mucometra: A review. Theriogenology. 70(3): 359-363.

Rocca B and FitzGerald GA 2002. Cyclooxygenases and prostaglandins: shaping up the immune response. Int Immunopharmacol. 2(5): 603-630.

Rodriguez HA, Kass L, Varayoud J, Ramos JG, Ortega HH, Durando M, Munoz-De-Toro M and Luque EH 2003. Collagen remodelling in the guinea-pig uterine cervix at term is associated with a decrease in progesterone receptor expression. Mol Hum Reprod. 9(12): 807-813.

Silva E, Leitao S, Ferreira-Dias G, Lopes da Costa L and Mateus L 2009. Prostaglandin synthesis genes are differentially transcripted in normal and pyometra endometria of bitches. Reprod Domest Anim. 44(Suppl 2): 200-203.

Silva LD, Onclin K and Verstegen JP 1995. Cervical opening in relation to progesterone and oestradiol during heat in beagle bitches. J Reprod Fertil. 104(1): 85-90.

Smith GC, Wu WX and Nathanielsz PW 2001. Effects of gestational age and labor on expression of prostanoid receptor genes in baboon uterus. Biol Reprod. 64(4): 1131-1137.

Sorsa T, Tjaderhane L and Salo T 2004. Matrix metalloproteinases (MMPs) in oral diseases. Oral Diseases. 10(6): 311-318.

Swangchan-Uthai T, Walsh SW, Alexander SL, Cheng Z, Crowe MA, Evans AC and Wathes DC 2011. Comparison of mRNA for IGFs and their binding proteins in the oviduct during the peri-oestrous period between dairy heifers and lactating cows. Reproduction. 142(3): 457-465.

Tamada H, Kawata N, Kawate N, Inaba T, Kida K, Hatoya S, Akune A, Nakama K, Kohsaka T, Takahashi M and Sawada T 2012. Factors associated with patency of the uterine cervix in bitches with pyometra. Res Vet Sci. 93(3): 1203-1210.

Timmons B, Akins M and Mahendroo M 2010. Cervical remodeling during pregnancy and parturition. Trends Endocrinol Metab. 21(6): 353-361.

Uchiyama T, Ito A, Ikesue A, Nakagawa H and Mori Y 1992. Chemotactic factor in the pregnant rabbit uterine cervix. Am J Obstet Gynecol. 167(5): 1417-1422.

van Engelen E, de Groot MW, Breeveld-Dwarkasing VN, Everts ME, van der Weyden GC, Taverne MA and Rutten VP 2009. Cervical ripening and parturition in cows are driven by a cascade of pro-inflammatory cytokines. Reprod Domest Anim. 44(5): 834-841.

Verstegen J, Dhaliwal G and Verstegen-Onclin K 2008. Mucometra, cystic endometrial hyperplasia, and pyometra in the bitch: advances in treatment and assessment of future reproductive success. Theriogenology. 70(3): 364-374.

Wu WX, Ma XH, Coksaygan T, Chakrabarty K, Collins V, Rose J and Nathanielsz PW 2004. Prostaglandin mediates premature delivery in pregnant sheep induced by estradiol at 121 days of gestational age. Endocrinology. 145(3): 1444-1452.

Wu WX, Wolf R, Chakrabarty K, Collins V, Unno N, Nathanielsz PW and Rose JC 2005. Induction of uterine prostaglandin H synthase 2 by estradiol following fetal adrenalectomy. Endocrine. 26(2): 153-159.

Zhang Q, Collins V, Chakrabarty K, Rose JC and Wu WX 2007. Regulation of the prostaglandin enzymatic system by estradiol and progesterone in nonpregnant sheep cervix. Reproduction. 133(5): 1027-1034.