การแสดงออกของตัวรับโพรสตาแกลนดินอี 2 (ซับไทป์ 2 และ 4) และ อัตราส่วนของเอกซ์ทราเซลลูลาร์ เมทริกซ์ ในคอมดลูกสุนัขที่ระยะต่างๆของวงรอบการเป็นสัด และในสุนัขที่มี ปัญหามดลูกอักเสบเป็นหนอง

นางสาวพิชนันท์ ลีฬหรัตนรักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาการสืบพันธุ์สัตว์ ภาควิชาสูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์
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ปีการศึกษา 2555

THE EXPRESSION OF PROSTAGLANDIN E2 RECEPTORS (EP2 AND EP4) AND THE PROPORTION OF EXTRACELLULAR MATRIX IN THE CERVIX OF CYCLIC BITCHES AND BITCHES WITH PYOMETRA

MISS PICHANUN LINHARATTANARUKSA

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy Program in Theriogenology

Department of Obstetrics, Gynaecology and Reproduction

Faculty of Veterinary Science

Chulalongkorn University

Academic Year 2012

Thesis Title	THE EXPRESSION OF PROSTAGLANDIN E2 RECEPTORS (EP2 AND EP4) AND THE PROPORTION OF EXTRACELLULAR MATRIX IN THE CERVIX OF CYCLIC BITCHES AND BITCHES WITH PYOMETRA
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พิชนันท์ ลีฬหรัตนรักษ์ : การแสดงออกของตัวรับโพรสตาแกลนดินอี 2 (ซับไทป์ 2 และ 4) และ อัตราส่วนของ เอกซ์ทราเซลลูลาร์ เมทริกซ์ ในคอมดลูกสุนัขที่ระยะต่างๆของวงรอบการเป็นสัด และในสุนัขที่มี ปัญหามดลูกอักเสบเป็นหนอง (THE EXPRESSION OF PROSTAGLANDIN E2 RECEPTORS (EP2 AND EP4) AND THE PROPORTION OF EXTRACELLULAR MATRIX IN THE CERVIX OF CYCLIC BITCHES AND BITCHES WITH PYOMETRA) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.สพ.ญ.ดร. เกวลี ฉัตรดรงค์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.น.สพ.ดร. สุดสรร ศิริไวทยพงศ์, ผศ.สพ.ญ.ดร. ศยามณ ศรีสุวัฒนาสกุล, 95 หน้า

การทดลองที่1 แบ่งกลุ่มสุนัขเป็น 2 กลุ่มการทดลอง กลุ่มที่ 1 เก็บตัวอย่างคอมดลูกจากสุนัขปกติในแต่ละระยะของวงรอบการเป็นสัด จำแนกเป็น 3 ระยะ ได้แต่ แอนเอสตรัส โปรเอสตรัส และไดเอสตรัส จำนวน 10, 7 และ 11 ตัวตามลำดับ กลุ่มที่ 2 เก็บตัวอย่างคอมดลูกจากสุนัขที่มีปัญหามดลูกอักเสบเป็นหนองจำนวน 26 ตัว จำแนกเป็น สุนัขที่มีปัญหามดลูกอักเสบเป็นหนองแบบคอมดลูกเปิดจำนวน 18 ตัว และสุนัขที่มีปัญหามดลูกอักเสบแบบคอมดลูกปิดจำนวน 8 ตัว ตรวจการเปลี่ยนแปลงของอัตราส่วนของเอกซ์ทราเซลลูลาร์ เมทริกซ์ด้วยวิธี Masson's trichrome staining เพื่อดูอัตราส่วนของคอลลาเจนกับกล้ามเนื้อเรียบในคอมดลูก และวิธี Alcian blue staining เพื่อตรวจปริมาณของไกลโคอะมิโนไกลแคนแต่ละชนิดในคอมดลูก จากผลการศึกษาพบว่า อัตราส่วนของคอลลาเจนต่อกล้ามเนื้อในคอมดลูกของสุนัขในระยะเอสตรัสมีอัตราส่วนสูงกว่าระยะแอนเอสตรัสอย่างมีนัยสำคัญทางสถิติ นอกจากนั้นในกลุ่มมดลูกอักเสบเป็นหนองแบบคอมดลูกเปิดมีค่าอัตราส่วนสูงมากกว่าในกลุ่มสุนัขที่มีปัญหามดลูกอักเสบแบบคอมดลูกปิด จากการทดลอง พบว่าปริมาณของไกลโคอมิโนไกลแคนแต่ละชนิดแตกต่างกันในคอมดลูกแต่ละระยะ และพบว่าไฮยาลูโรแนนเป็นองค์ประกอบที่พบมากสุดในคอมดลูกสุนัขปกติและพบมากในเยื่อบุผิวชั้นในของคอมดลูกในสุนัขระยะเอสตรัสเมื่อเทียบกับระยะแอนเอสตรัสแต่ไม่แตกต่างจากระยะไดเอสตรัส ในทางตรงกันข้ามกลับพบว่า ปริมาณของเคอราแทน ซัลเฟตและเฮปาแรน ซัลเฟตในคอมดลูกจากระยะแอนเอสตรัสสูงกว่าระยะเอสโรจสอย่างมีนัยสำคัญทางสถิติ แต่ในกลุ่มสุนัขที่มีปัญหามดลูกอักเสบพบว่า ปริมาณเคอราแทน ซัลเฟตและเฮปาแรน ซัลเฟตในกลุ่มคอมดลูกเปิดมีค่าน้อยกว่าในกลุ่มคอมดลูกปิดอย่างมีนัยสำคัญทางสถิติ ผลการศึกษาครั้งนี้พบว่าเมทาบอลิซึมของคอลลาเจนและไกลโคอมิโนไกลแคนในคอมดลูกสุนัขมีความเกี่ยวข้องกับระดับฮอร์โมนในแต่ละระยะของวงรอบการเป็นสัดและการเปิดปิดของคอมดลูกในสุนัขที่เป็นมดลูกอักเสบเป็นหนอง

การทดลองที่2 แบ่งกลุ่มสุนัขเป็นสองกลุ่มการทดลอง กลุ่มที่1 เก็บตัวอย่างคอมดลูกจากสุนัขปกติในแต่ละระยะของวงรอบการเป็นสัด จำแนกเป็น 3 ระยะ ได้แต่ แอนเอสตรัส โปรเอสตรัส และไดเอสตรัส จำนวน 6, 12 และ 6 ตัว ตามลำดับ กลุ่มที่ 2 เก็บตัวอย่างคอมดลูกจากสุนัขที่มีปัญหามดลูกอักเสบเป็นหนองจำนวน 26 ตัว จำแนกเป็น สุนัขที่มีปัญหามดลูกอักเสบเป็นหนองแบบคอมดลูกเปิดจำนวน 10 ตัว และสุนัขที่มีปัญหามดลูกอักเสบแบบคอมดลูกปิดจำนวน 10 ตัว ตรวจการแสดงออกของตัวรับพรอสตาแกลนดินอี2 ซับไทป์4 (EP4) ด้วยวิธี avidin-biotin-peroxidase complex (ABC) และคำนวณคะแนนของตัวรับพรอสตาแกลนดิน ผลการศึกษาพบว่า การแสดงออกของตัวรับ EP4 พบได้ในทุกชั้นเนื้อเยื่อของคอมดลูกสุนัขทั้งในสุนัขปกติและสุนัขที่มีปัญหามดลูกอักเสบเป็นหนอง แต่ความแตกต่างของการแสดงออกของตัวรับ EP4 พบแค่ในชั้นเยื่อบุผิวชั้นในสุด โดยพบว่า การแสดงออกของตัวรับ EP4 ในเยื่อบุผิวชั้นในส่วนที่ติดกับมดลูกในคอมดลูกจากสุนัขระยะเอสตรัสจะมีคะแนนมากกว่าในระยะแอนเอสตรัสและไดเอสตรัส รวมถึงในกลุ่มสุนัขที่มีปัญหามดลูกอักเสบเป็นหนองแบบคอมดลูกเปิดที่มีคะแนนของการแสดงออกตัวรับ EP4 มากกว่าสุนัขที่มีปัญหามดลูกอักเสบเป็นหนองแบบคอมดลูกปิด จากผลการศึกษาครั้งนี้พบว่า ปัจจัยที่มีผลต่อการเปิดปิดของคอมดลูกน่าจะอยู่ที่ส่วนที่ใกล้กับมดลูกและ ตัวรับ EP4 อาจมีส่วนเกี่ยวข้องกับกลไกการเปิดปิดของคอมดลูกในแต่ละระยะของวงรอบการเป็นสัดรวมถึงในสุนัขที่มีปัญหามดลูกอักเสบเป็นหนองเช่นกัน

การทดลองที่3 พรอสตาแกลนดินมีความสำคัญในการควบคุมการเปิดปิดของคอมดลูก การผลิตพรอสตาแกลนดินอี2 (PGE2) ควบคุมโดยเอนไซม์ไซโคลออกซีจิเนส (COX) และ พรอสตาแกลนดินอี ซินเทส (PGES) พรอสตาแกลนดินอี2 ทำงานผ่านการจับกับตัวรับฮอร์โมนพรอสตาแกลนดินอี2 (EP) ซับไทป์ EP2 และ EP4 ทำให้เกิดการคลายตัวของกล้ามเนื้อ ในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจหาระดับการแสดงออกของเมสเซนเจอร์อาร์เอ็นเอ คือ EP2 EP4 COX-2 และ PGES ในคอมดลูกของสุนัขระยะต่าง ๆ ของวงรอบการเป็นสัด และในสุนัขที่มีปัญหามดลูกเป็นหนอง เก็บคอมดลูกจากช่องเปิดด้านในถึงช่องเปิดด้านนอกหลังการทำหมันโดยตัดรังไข่และมดลูกออก สกัดอาร์เอ็นเอจากเนื้อเยื่อคอมดลูกแล้วนำมาตรวจหาระดับการแสดงออกของ EP2 EP4 COX-2 และ PGES ด้วยวิธี quantification real-time PCR (qPCR) นอกจากนั้นยังศึกษาการแสดงออกของโปรตีนด้วยวิธี western blot จากการศึกษาพบว่า การแสดงออกของเมสเซนเจอร์อาร์เอ็นเอที่สนใจไม่มีความแตกต่างกันในสุนัขในแต่ละระยะของวงรอบการเป็นสัด แต่การแสดงออกของเมสเซนเจอร์อาร์เอ็นเอของ PGES ในกลุ่มสุนัขที่มีปัญหามดลูกเป็นหนองแบบคอมดลูกเปิดมีค่ามากกว่าในกลุ่มคอมดลูกปิด ($p<0.05$) นอกจากนั้น การแสดงออกของโปรตีนของ EP2 EP4 และ COX-2 ไม่แตกต่างกันในแต่ละระยะของการเป็นสัด และในกลุ่มที่เป็นมดลูกอักเสบเป็นหนอง ดังนั้น ระดับฮอร์โมนที่เปลี่ยนแปลงในแต่ละระยะของวงรอบการเป็นสัด น่าจะไม่มีผลต่อการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างพรอสตาแกลนดินอี2 และตัวรับฮอร์โมนพรอสตาแกลนดินอี2 ซับไทป์ 2 และ 4 แต่ PGES น่าจะมีส่วนเกี่ยวข้องกับกลไกการเปิดของคอมดลูกในสุนัขที่มีปัญหามดลูกเป็นหนองแบบคอมดลูกเปิด

ภาควิชาสูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์
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ลายมือชื่อนิสิต.....
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ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....
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5175955231: MAJOR THERIOGENOLOGY

KEYWORDS: PROSTAGLANDINE2 RECEPTOR EXTRACELLULAR MATRIX CANINE CERVICAL TISSUE ESTROUS CYCLE PYOMETRA

PICHANUN LINHARATTANARUKSA: THE EXPRESSION OF PROSTAGLANDIN E2 RECEPTORS (EP2 AND EP4) AND THE PROPORTION OF EXTRACELLULAR MATRIX IN THE CERVIX OF CYCLIC BITCHES AND BITCHES WITH PYOMETRA ADVISOR: ASSOC. PROF. KAYWALEE CHATDARONG D.V.M., M.Sc., Ph.D., CO-ADVISOR: ASSOC. PROF. SUDSON SIRIVAIDYAPONG, D.V.M., Ph.D., ASST. PROF. SAYAMON SRISUWATANASAGUL, D.V.M., Ph.D., 94 pp.

Exp. I aimed to determine the proportion of collagen and smooth muscle using Masson's trichrome staining and Alcian blue staining was used to evaluate the relative distribution of cervical GAGs. The proportion of cervical collagen relative to cervical smooth muscle was higher at estrus compared to anestrus ($p \leq 0.05$). It was also higher ($p \leq 0.05$) in bitches with open- compared to those with closed-cervix pyometra. Overall, hyaluronan (HA) was the predominant GAG in the canine cervix and there were no differences in GAG composition in the stroma of healthy bitches and in the luminal epithelium and muscle of bitches with pyometra. In the luminal epithelium, HA was higher in estrus than in anestrus ($p \leq 0.05$) but not in diestrus ($p > 0.05$). On the contrary, the combined keratan sulfate (KS) and heparan sulfate (HS) content was higher in anestrus than estrus ($p \leq 0.05$). In bitches with pyometra, the combined KS and HS content was significantly lower in open- compared to closed-cervix pyometra ($p \leq 0.05$). Collectively, the different profiles of collagen and GAG observed at different stages of the estrous cycle as well as in pyometra suggest that the metabolism of both collagens and GAGs in the canine cervix is associated with hormonal statuses during the estrous cycle and cervical patency of bitches with pathological uterine conditions such as pyometra.

Exp. II aimed to investigate the expression of EP4 in the cervices of bitches during estrous cycle and those with pyometra. The expression of EP4 was observed at all the layers and all stages but the differences in EP4 expression either among bitches in different stages of the estrous cycle and between open- and closed-cervix pyometra were limited to only luminal epithelium (LE). In cyclic bitches during estrus and in open-cervix pyometra bitches, significantly higher EP4 expression was found in SE of uterine part than vaginal part. In SE of the uterine part, the expression was higher in the bitches during estrus than in anestrus and diestrus, and higher in the bitches affected by open-cervix than closed-cervix pyometra. The results suggest that regulation of cervical dilation appeared in the uterine part of the cervix. Moreover, EP4 may be involved in stimulating dilation of the cervix in both estrus and open-cervix pyometra bitches.

Exp. III aimed to investigate mRNA expressions of EP2, EP4, COX-2, and PGES in the bitch cervix. Two groups of bitches; normal cyclic bitches and bitches with pyometra were studied. RNA extracted from cervical tissue was determined for levels of EP2, EP4, COX-2, and PGES mRNA using a real-time qPCR. Western blot was performed to investigate the protein expression of EP2, EP4, and COX-2There were no differences of EP2, EP4, COX-2, and PGES mRNA expression in the bitch cervix among the stages of the estrous cycle. However, the expression of PGES mRNA was higher in the cervix of bitches with open-cervix than closed-cervix pyometra ($P<0.05$). However, the differences of protein expression were not observed in both normal cyclic bitches and bitches with pyometra. Our findings suggest that mRNA and protein expression of the enzymes involved in PGE2 synthesis and PGE2 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.

Department: Obstetrics Gynaecology and Reproduction
Field of Study: Theriogenology
Acedemic year: 2012

Student's signature……..……..
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Co-advisor's signature……..……
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ACKNOWLEDGEMENTS

This study was carried out at the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand and at The Royal Veterinary College, London, United Kingdom. The study was financially supported by Royal Golden Jubilee PhD program of Thailand Research Fund and Thesis Scholarship for Students Withdrawal Regulation.

First of all, I would like to express my gratitude to my main supervisor, Asso. Prof. Dr. Kaywalee Chatdarong for her supports, helps, and giving me the opportunity getting to know new knowledge about sciences. I am really appreciated for sustenance during my PhD, teaching me how to be a good scientist, and how to manage our thought to gain the achievement. I would like to deeply thank Assist. Prof. Dr. Sayamon Srisuwatanasagul, my beloved co-supervisor, for always giving me good advices not only about PhD things but also about life. I am really grateful for Dr. Muhammad Khalid for giving me an opportunity to experience new techniques and taking care of me when I was at RVC, UK. I would like to thank Dr. Anongnart Somchit-Assavacheep for giving me very useful suggestions on western blot. I would also like to thank Dr. Suppawiwat Ponglowhapan for his accompany and giving me valuable advices about my work. I also wish to express my appreciation to Dr. Sukanya Manee-in for aiding me the technique of immunohistochemistry and helping me collected samples. I am grateful for Dr. Theerawat Swangchan-uthai for teaching me everything about qPCR, the small little thing we do not even see! I also would like to thank Dr. Thanida Sananmuang for guidance in PCR methods.

I would like to thank Dr. Suparlark Nuntawan na Ayudhya for helping me setting up the technique of protein expression. A lot of thanks to Veterinarians and staffs at OGR clinic, Small animal hospital of Chulalongkorn Universtity, Dr. Attawit Samma, Dr. Phuwanai Parinamosot, and Dr. Chaitawach Manovit for kind assistances through all collecting samples time. Special thanks to all dogs providing me the valuable samples. Without you, this thesis would not even been started. I also would like to thank Dr. Paweena Thuwanut and Mimm, my beloved sister and friend, for their support, encouragement and be by my side during the tough time. To my lovely friends at OGR department for your warm friendship and laughing we had together (one of the best thing in PhD life). Last but not least, thanks to my best friends, Looknam, Nuj, Peng, Mook, and Mameow for supporting me during the unfavorable time.

Finally, I would like to thank the most important person of my life, mom and dad, for your unending love and support. Without your love, I would have not come this far. Thanks to my sister, you are sister, friend, parent, and whatever you can be to help me getting through all the impediments. Lastly, thanks to Eugene for your encouragement to make me stronger!! Without their support and love, this accomplishment would have not been happened.

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List of ABBREVIATIONS

˚c	degree Celsius
ABC	avidin biotin-complex
ANOVA	analysis of variance
APS	ammonium persulphate
a-SMA	alpha smooth muscle actin
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CEH	cystic endometrial hyperplasia
COX	cyclooxygenase
COX-1	cyclooxygenase 1
COX-2	cyclooxygenase 2
cq	quantification cycle
CS	chondroitin sulfate
DAB	diaminobenzidine
DNA	deoxyneucleic acid
dNTP	deoxynucleotide triphosphates
DPX	DPX mounting media
DS	dermatan sulfate
ECM	extracellular matrix
EP1	prostaglandin E2 receptor subtype 1
EP2	prostaglandin E2 receptor subtype 2
EP3	prostaglandin E2 receptor subtype 3
EP4	prostaglandin E2 receptor subtype 4

ERα	estrogen receptor alpha
Exp	experiment
fg	fentogram
FP	forward primer
g	gram
GAGs	glycosaminoglycans
GPCRs	G-protein-coupled receptors
h	hour
HA	hyaluronan
HRP	horseradish peroxidase
HS	heparan sulfate
IL-1	interleukin-1
IL-1β	interleukin-1β
IL-8	interleukin-8
KS	keratan sulfate
LE	luminal epithelium
LH	luteinizing hormone
M	molar
M	muscle
mA	milliampere
$MgCl_2$	magnesium chloride
MIE	mean integrated extinction
min	minute
mL	milliliter
MMP-2	matrix metalloproteinase-2

MMP-9	matrix metalloproteinase-9
MMPs	matrix metalloproteinases
mRNA	messenger Riboneucleic acid
NaCl	sodium chloride
ng	nanogram
NTC	non template control
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PGE2	prostaglandin E2
PGES	prostaglandin E2 synthase
PGG2	prostaglandin G2
PGH2	prostaglandin H2
PR	progesterone receptor
PVDF	polyvinylidine fluoride
qPCR	quantitative polymerase chain reaction
RN18S1	18S ribosomal RNA
RNA	riboneucleic acid
RP	reward primer
rpm	round per minute
RT	reverse transcriptase
S	stroma
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	second

SEM	standard error of mean
S-GAGs	sulfated glycosaminoglycans
SPARC	secreted protein acidic and rich in cysteine
TBS-T	tris buffer saline-tween
TEMED	tetramethylethylene-diamine
TGF-β	transforming growth factor beta
uPA	urokinase plasminogen activator
v	volt
w/v	weight by volume
β – ME	beta mercaptoethanol
μg	microgram
μl	microliter
μM	micromole
μm	micrometer

CHAPTER I

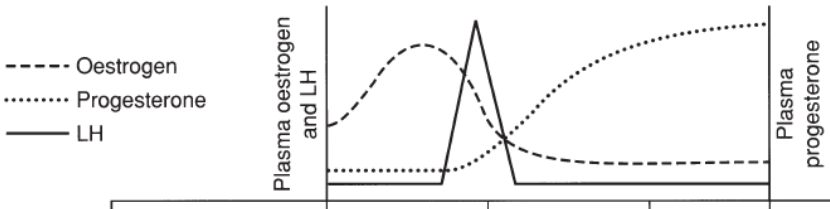
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In domestic dogs (*Canis familiaris*), the stages of reproductive cycle or so called "estrous cycle" refers to the completion of one estrus to the next estrus which comprises proestrus, estrus, diestrus, and anestrus. On average, dogs have two estrous cycles per year which ranges from one to four cycles. Dogs can come into estrus at any time of the year, so they are defined as non-seasonal breeders. Puberty in the female dog is the time when the capability to reproduce is achieved which begins between 6 and 10 months of age for dogs of smaller breeds, whereas it can be 2 years in larger breeds. The puberty is recognized as the onset of the first proestrus.

Proestrus accounts for the beginning of the cycle when the bitch is sexually attractive but rejects the copulation with male. The first day of proestrus is marked as the first presence of serosanguineous vulvar discharge and the swelling of vulvar lips. During proestrus, estradiol levels increase which account for clinical characteristics of this stage. Thereafter the levels of estradiol decrease before the bitch becomes receptive of mating (Concannon et al., 1975). The average range of proestrus is 9 days but can be ranged from 0 to 27 days (Bell and Christie, 1971). Estrus is recognized by the bitch's first willing to accept mating and the serum estradiol levels begin to fall with the concentrations of progesterone begin to rise (Beach et al., 1982). The vulvar size remains the same but it is normally softer than at proestrus. The average duration of this stage is about 9 days and can range from 4 to 24 days (Bell and Christie, 1971). The onset of diestrus begins when in vaginal smears show a decrease of superficial cells with an increase in the percentage of small intermediate and parabasal cells and a presence of neutrophils (Figure 1). Therefore, the onset of diestrus is recommended to be determined by the vaginal cytology rather than mating behavior. During diestrus, the serum progesterone concentrations increase rapidly and reach to the peak by 15 to 30 days. Anestrus is a quiescent period in term of the clinical signs or behavior with a variable duration of reproductive cycle. In this period, the bitch is not attractive to male

dogs. The mean interval from onset of one estrous cycle to the next is about 7 months, with a range of 4 to 12 months (Margaret, 2010).



Cell type		Pro-oestrus Early	Pro-oestrus Late	Oestrus Early	Oestrus Late	Early metoestrus	Anoestrus	Vaginitis pyometra
Para basal		+ + +	+	–	–	–	+ +	±
Small intermediate		+ + +	+ +	–	–	+	+	±
Large intermediate		±	+ +	+ + +	+ + +	–	–	±
Anuclear keratinised		–	+ +	+ + + +	+ + +	±	–	–
Red blood		+	+ + +	+ +	–	–	±	±
Neutrophilis		+	–	–	+	+ + +	+	+ + + +

Figure1. Changes of the types of vaginal cells in relative numbers during different stages of the estrous cycle in the bitch according to the peripheral plasma (Noakes et al., 2001b).

During the estrous cycle, there are changes in morphology, physiology and function of reproductive organs including the cervix. The cervix is an important barrier in both physiological and pathological processes. Closure of the cervix prevents ascending infection while opening allows passage of spermatozoa post mating and fetus at the time of parturition. The cervix maintains fetus in uteri throughout gestation and subsequently is involved in the parturition process. The cervical patency is believed to be regulated during estrous cycle by estrogen whereas closure of the cervix during diestrus is manipulated by progesterone levels (Silva et al., 1995). Associations between hormonal status and cervical relaxation are not clearly understood. However, a previous

study had demonstrated that the increase expression of progesterone receptor in the cervix during proestrus coincides with cervical relaxation, whereas the estrogen receptor alpha (ERα) expression does not seem to relate to cervical opening (Kunkitti et al., 2011). In the pathological condition likes pyometra, the cervix may remain closed or open. In cases with closed-cervix pyometra, more severe illness is speculated because of accumulation of pus in the uterus that may result in uterine rupture and peritonitis. Medical therapy may help to stabilize the patients prior to surgery. Enhancement of cervical relaxation in closed-cervix pyometra to allow the release of contents from the uterus has been accomplished using prostaglandin F2 alpha (Verstegen et al., 2008) and antiprogestin (Fieni, 2006) by increasing the uterine contractions and reducing the action of progesterone, respectively. These medical therapies may help to postpone the surgery until status of the patient is safe for anesthesia. Understanding the mechanism (s) by which patency of the cervix is regulated could be helpful for treatment of the uterine disease as well as for the control of parturition. Like in humans, prostaglandin E2 (PGE2) has been used to induce cervical ripening at term when fetal or maternal pathology occurs (Schmitz et al., 2001). PGE2 has also been applied for inducing cervical relaxation prior to artificial insemination in sheep (Candappa et al., 2009). Taken together, prostaglandins seem to be involved in the control of cervical patency to some extent. However, regulation of cervical relaxation has not been studied in dogs. Prostaglandin E2 exerts its role by coupling to prostaglandin E receptors, EP1, EP2, EP3 and EP4. EP1 and EP3 are related to the contraction of smooth muscle, whereas EP2 and EP4 receptors induce smooth muscle relaxation (Narumiya et al., 1999). Relaxation of cervix is likely mediated by EP4 via modification of cervical extracellular matrix (ECM) (Schmitz et al., 2001). PGE2 synthesis is regulated by cyclooxygenase (COX-2) and prostaglandin E2 synthase (PGES) also helps to convert PGH2 into PGE2 (Helliwell et al., 2004).

The cervix consists of smooth muscle and collagen which is supported by extracellular matrix (ECM). The ECM is a network made up with collagen bundles, glycoproteins, proteoglycans, glycosaminoglycans (GAGs) and water (Fosang et al.,

1990). Collagens are the most abundant protein in the ECM and account for structural support (Hasler et al., 1999; Kjaer, 2004). Proteoglycans are glycosylated glycoproteins while GAGs are specific types of polysaccharides and one part of proteoglycans. GAGs are made up with repeating disaccharides to form a variety of molecular species. They are classified into two groups; 1) the sulfated GAGs (S-GAGs) comprising of chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and heparan sulfate (HS), and 2) the non-sulfated GAG, hyaluronan or hyaluronic acid (HA) (Hileman et al., 1998). Cervical relaxation is mediated by reorganization of collagen bundles with a decrease in collagen concentration in the cervix (Feltovich et al., 2005). The fact that estradiol can induce collagen remodelling in the cervix of the pregnant ewe suggests that sex steroid hormones might affect cervical relaxation (Owiny et al., 1987).

In bitches with pyometra neutrophils are reported to be the crucial factor contributing to cervical relaxation in the open-cervix pyometra (Kunkitti at al., 2011). Positive correlation was also found between cervical patency and the interleukin-8 (IL-8) in women during parturition (Barclay et al., 1993). As IL-8 is a chemoattractant for neutrophils, level of extpression of IL-8 was also found positively associated with cervical patency and neutrophils in the bitches (Tamada et al., 2012).

This study aimed to investigate; 1) types of GAGs and proportion of collagen and smooth muscle, and 2) factors (PGE2 receptors (EP2 and EP4), COX-2 and PGES) that might potentially be associated with the cervical patency in normal healthy bitches and in pyometra bitches.

1.2 Literature review

1.2.1 Anatomy (macroscopic and microscopic) of the cervix

Cervix is the narrow portion of uterus that joins the cranial part of vagina and acts as a sphincter-like fibrous organ (Figure 2). The main function of this organ is to produce a mucus secretion (mucous plug) that is thought to aid in movement of the sperm within the female reproductive tract. This secretion is stimulated by estrogen and is produced by the simple columnar epithelial lining cells (Schatten and Constantinescu, 2007). Patency of the cervix is regulated by smooth muscle, fibrous tissue, and elastin

(Copeland, 1993). In bitch, the cervical canal is measured as 1.5-2 cm in length (Evans and Miller, 1993). The mucosa of the cervical canal in most mammals including rats and human is lined up with a single layer of columnar epithelial cells (Nalbandov, 1964; Cunningham and Leveno, 2001). At proestrus, the dorsal vaginal fold and cervix begin to hypertrophy and reach maximal size at estrus, then start to regress at early diestrus (Roszel, 1992). The cervical canal is in a nearly vertical position, with the external os in a close position to the ventral area of the vaginal cervix during proestrus (Evans and deLahunta, 1996). The radiographic study by placing the contrast medium at the vagina revealed the passage of the contrast medium through the cervix during proestrus and estrus but not during anestrus and diestrus (Linde, 1978). The opening of the cervix is reported to occur 2 days before the preovulatory LH peak and is likely to be induced by the maximal ratio of estradiol and progesterone levels, which decreases rapidly one day later (Concannon et al., 1977; Concannon et al., 1989). Accordingly, another study revealed that the cervical closing in the bitches occurred 6 days after the LH peaks or 3 days before the end of estrus (Silva et al., 1995). In cattle, the cervix opens during estrus and closes during diestrus and pregnancy (Arthur et al., 1989a). Furthermore, the thick cervical mucus stimulated by progesterone could be found during the closure of the cervix (Abusineina, 1962; Betteridge and Raeside, 1962; Grobbelaar and Kay, 1985). The cervical relaxation during follicular phase in the sheep is likely to involve periovulatory hormones, prostaglandins synthesis and remodeling of the extracellular matrix (Kershaw et al., 2005).

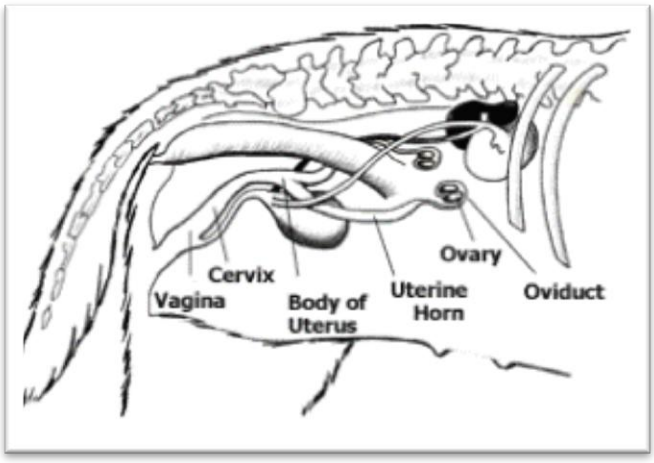


Figure 2. The female genital tract consists of the paired ovaries, uterus (uterine horn and uterine body), cervix, vagina, clitoris, and the female urethra. (http://www.petcaregt.com/dogcare/femaledogreproductivesystem.html)

1.2.2 Pyometra: the role of cervix

Pyometra is a common uterine disease that maybe linked to hormonal imbalance in which progesterone plays a major role (Noakes et al., 2001a) and mostly occurs during diestrus (Silva et al., 2010). Pyometra is a two-step process. The first pathologic change is cystic endometrial hyperplasia (CEH), a thickening of the uterine lining that occurs secondary to repeated estrous cycling. The unique estrous cycle of the bitch is characterized by high serum estrogen followed by prolonged elevation in progesterone (Margaret, 2010). The second pathologic change is infection. Infection invariable is due to an organism which is part of the normal vaginal flora and *Escherichia coli* is the most common isolate (Johnston et al., 2001). The importance of progesterone in the pathogenesis lies in its role in immunosuppression, stimulation of endometrial gland secretion which is suitable for bacterial growth, and functional closure of the cervix that inhibits drainage of uterine exudates (Chaffaux and Thibier, 1978; Austad et al., 1979). In some cases, although serum progesterone concentrations are found at basal level, uterine contraction and cervical relaxation are inhibited (Verstegen et al., 2008). The association between diestrus and pyometra has been well known. However, the real mechanism is poorly understood.

According to patency of the cervix, pyometra in the bitches is divided into closed- and open-cervix pyometra. In the open-cervix pyometra, the apparent clinical sign is yellow-green to pink or red-tinged, thick, odoriferous vulvar discharge. On the contrary, the bitches with closed-cervix pyometra show no clinical sign of vulvar discharge. Other clinical signs include depression, inappetence, and vomiting. While cervical relaxation is stimulated by intrauterine pressure of the fetus at the internal cervical os during the parturition process, it is questionable why the rise of intrauterine pressure by purulent fluid in the pyometra bitches does not provoke cervical relaxation. Regardless of the cervical patency, the treatment of choice for pyometra in the bitches is considered to be ovariohysterectomy, once the bitch has been adequately stabilized, since this technique can prevent recurrence of the disease. However, in valuable breeds of dogs or dogs in risk of anesthesia, medical therapy is an alternative choice. The low dose of prostaglandins such as prostaglandinF2α ($PGF_2\alpha$) given repeatedly alone or in combination with other drugs such as dopamine agonists (England et al., 2007) has been reported to reduce progesterone level which led to cervical relaxation, allowing drainage of uterine fluid and inducing uterine contraction (Verstegen et al., 2008). However, this therapy has important side effects in the bitches with closed-cervix pyometra as the spasmodic actions can cause higher risk of uterine rupture (Jackson, 1979). In many species, the supposition of prostaglandin E2 intravaginally can induce cervical relaxation by stimulating remodeling of extracellular matrix in cervix (Stys et al., 1981; Ledger et al., 1983; Rigby et al., 1998). In dogs, the use of misoprostol (prostaglandin E1 analog) that acts like prostaglandin E2 intravaginally has been reported to induce cervical relaxation. However, the scientific proof of misoprostol efficacy has not been scrutinized (Verstegen et al., 2008).

1.2.3 Cervical relaxation and changes in extracellular matrix

Cervical relaxation in women during the parturition process is characterized by edema, leukocyte infiltration, changes in extracellular matrix, and dispersion of collagen network that results from collagen degradation by matrix metalloprotienases (Schmitz et al., 2006). Extracellular matrix is composed of thick collagen bundles running in all directions, elastin, and glycosaminoglycans (GAGs) holding elastin together (Figure 3). GAGs are the main components that have been associated with cervical relaxation at parturition in the rat cervix (Golichowski et al., 1980). There are five types of GAGs which are chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS) and hyaluronic acid (HA). The major component of GAGs found in the ovine cervix is hyaluronan (Kershaw et al., 2005) which is mostly located in the stroma. An increased cervical content of hyaluronan plays a major role in edema. It has been shown that the increase of hyaluronan concentrations weaken the affinity of fibronectin to collagen (Stamatoglou and Keller, 1982) , therefore leading to the loosening of collagen at term. Furthermore, the increased of hyaluronan stimulates hydration of cervical tissues since hyarulonan has been implicated in increase water retention capacity in tissue (Mathews, 1975) which is associated with cervical relaxation (Cabrol et al., 1987). Many studies revealed that PGE2 treatment increases hyaluronan concentration in the cervix (Rath et al., 1993). Another important role of hyaluronan is inducing of interleukin-1 a strong inducer of polymorphonuclear leukocytes migration (Ito et al., 1987). Leukocytes infiltration is thought to play a fundamental role in cervical relaxation by increasing collagenase enzyme and collagen degradation (Ito et al., 1987).

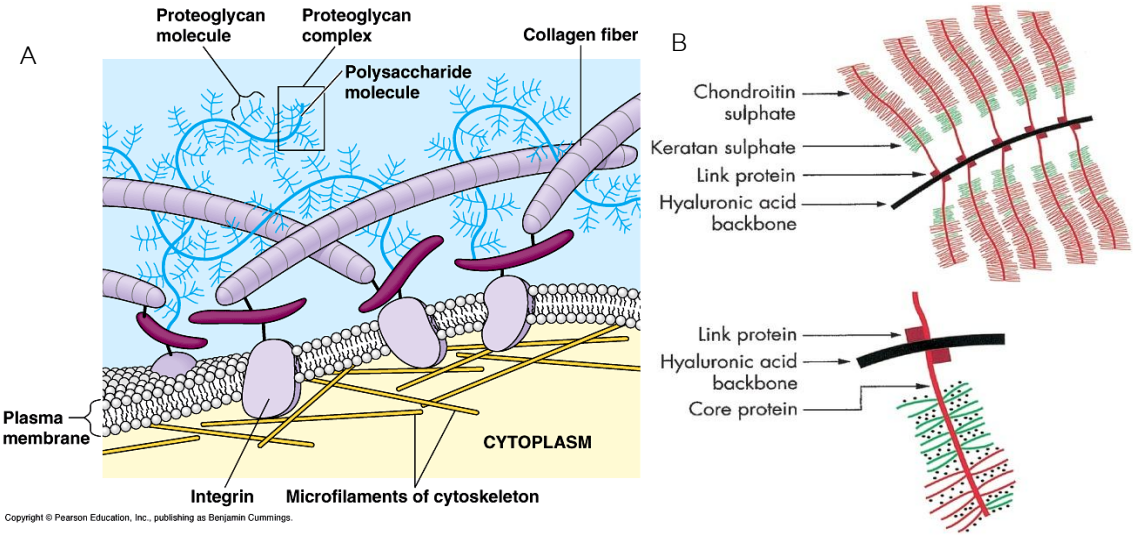


Figure 3. Schematic diagram of extracellular matrix (ECM) showing its components in animal tissue. ECM supports the cells and perform important functions (A). Proteoglycan molecules are composed of sulfated glycosaminoglycans attach to core protein and hyaluronan (B) (http://flipper.diff.org/app/pathways/info/3646, http://bjr.birjournals.org/content/76/911/777/F1.expansion.html).

In addition to hyaluronan, sulfated GAGs (chondroitin sulfate, dermatan sulfate, heparan sulfate and keratan sulfate) also influence the cervical relaxation in many species. For example, a decrease in dermatan sulfate in human (Osmers et al., 1993) implicated in the reorganization of collagen (Uldbjerg et al., 1983) as dermatan sulfate has the high affinity to the collagen (Engvall and Ruoslahti, 1977). Types of GAGs in the cervical tissue varies among species. In the sheep cervix, only dermatan sulfate was found (Kershaw-Young et al., 2009) while in the rat cervix, dermatan sulfate, heparan sulfate, and chondroitin sulfate were detected (Cubas et al., 2010; Akgul et al., 2012). During the estrous phase, the rat cervix had the highest amount of dermatan sulfate, suggesting that dermatan sulfate involved in cervical relaxation at estrus and is sensitive to hormonal chage (Cubas et al., 2010). In the human cervix, small amount of heparan sulfate was localized in the basement membrane of the blood vessels (Lindblom et al., 1989). Prostaglandin E2 (PGE2) has been postulated as the central mediator between the cervical relaxation and the sulfated GAGs. A decrease in sulfated GAGs component

was found after PGE2 treatment which led to the weakened interfibrillar interaction, the decline in cervical resistance and finally resulting in cervical relaxation (Ji et al., 2008).

1.2.4 Prostaglandin E2 and its role on cervical relaxation

Prostaglandins are metabolites of C-20 unsaturated fatty acid such as arachinodic acid which derived from cyclooxygenase (COX). COX has two consequential activities: catalyze activity which converts arachidonic acid to prostaglandinG2 (PGG2) and peroxidase activity which produces prostaglandinH2 (PGH2), a common precursor for synthesis of all protanoids (Figure 4) (Stocco and Deis, 1998). COX enzyme has 2 isoforms which are COX-1 and COX-2. COX-1 is responsible for physiological function and is expressed constitutively in many tissues. COX-2 is not commonly found but thought to be responsible for inflammatory processes which involve growth factors (Rocca and FitzGerald, 2002). Moreover, steroid hormones affect COX-2 expression (Wiltbank and Ottobre, 2003) by increasing COX-2 in intrauterine tissue, including cervix at term labor (Wu et al., 2004; Wu et al., 2005). In human enodometrium, COX-2 expression and PGE2 synthesis are associated with angiogenesis which was found maximum during the menstrual and proliferative phases. The expression of COX-2 and PGE2 synthesis were localized in epithelial and perivascular cell (Milne et al., 2001). The COX-2 mRNA expression increased in sheep cervix at estrus when estradiol concentration is greatest suggests that COX-2 stimulates PGE2 synthesis that acts through its receptors (Kershaw et al., 2007). In general, prostaglandins are produced in various cells in the body, released immediately and act in propinquity to sustain local homeostatis (Smith and Langenbach, 2001). Among prostaglandins, PGE2 is widely observed in domestic animals. PGE2 derives from PGH2 by prostaglandinE2 synthase (PGES) (Figure 4) and exerts its role via couple to G-protein-coupled receptors (GPCRs) in cell membrane which called prostaglandinE receptor (EP).

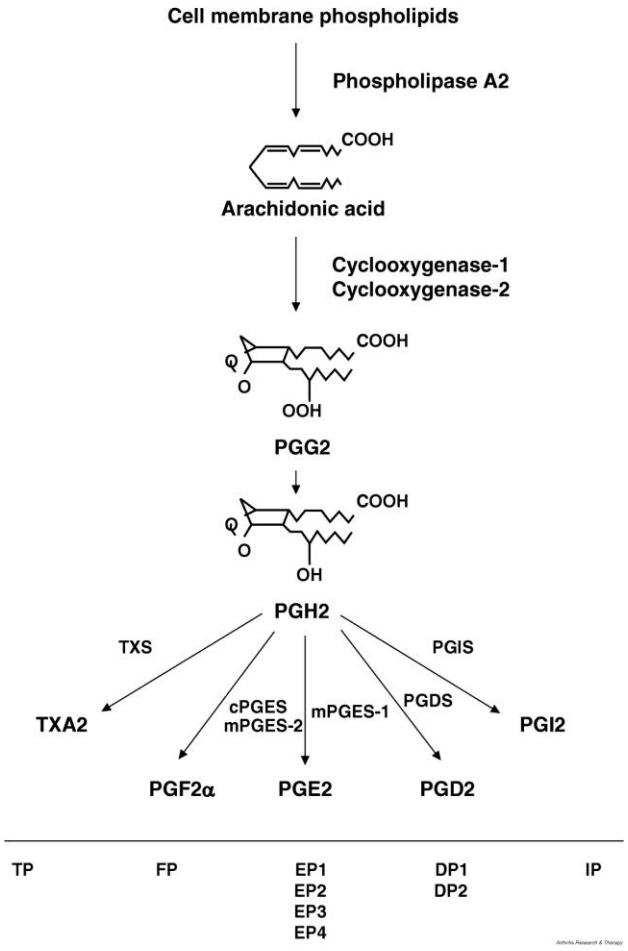


Figure 4. Diagram demonstrates prostaglandinE2 synthesis (Sampey *et al., 2005*)

There are four different subtypes of EP receptor which are EP1, EP2, EP3 and EP4 (Coleman et al., 1994; Narumiya et al., 1999) according to genes encoding and alternative mRNA splicing. EP1 mediates releasing of intracellular calcium (Ca2+) and diacyl glycerol, and activation of protein kinase C by coupling to phospholipase C (Arosh et al., 2004). EP2 and EP4 stimulate an increase in cAMP concentrations (Fujino et al., 2003; Regan, 2003). Although EP3 has wide range of actions, the major signaling pathway of the EP3 is an inhibition of adenylate cyclase. Furthermore, EP receptors can be categorized based on contractile activity into two groups. EP1 and EP3 are considered as contractile whereas EP2 and EP4 are believed to be relaxatory (Coleman et al., 1994). Therefore, PGE2 can induce contractility or relaxation of the muscle

depends on which receptor subtypes are expressed. Though, prostaglandins are thought to play an important role in regulation of cervical smooth muscle contractility (Lyons et al., 2003) , the mechanism is not clearly known. In ovariectomized ewes treated with estradiol, the decreases of the EP1 and EP3 expression which is contractile EP receptor promoted cervical ripening (Schmitz et al., 2006). Additionally EP4 can control matrix metalloproteinase expression and secretion in cultured cells (Weinreb et al., 1997) that may play a major role in collagen disruption in the cervix and consequently lead to cervical ripening. The study in human cervical fibroblasts cell culture demonstated that EP2 and EP4 receptors were presented and fuctional but only EP4 receptors can mediate PGE2 and stimulate GAGs production (Schmitz et al., 2001).

There are several studies showing the distribution of EP receptors related to cervical relaxation in different species. In rat, the study in rat cervical tissue demonstrated the localization of EP1, EP2, EP3, and EP4 in cervical epithelial cells and smooth muscle cells but EP1 localization in smooth muscle was not obviously seen (Hinton et al., 2010). In ewes, EP1, EP2, EP3 and EP4 receptors were widely distributed in all six different tissue layers which were endothelium and smooth muscle of cervical blood vessels, the epithelium of the cervical canal, the circular and longitudinal muscle layer, and the stroma (Schmitz et al., 2006). The expression of EP2 and EP4 mRNA in the sheep cervix throughout the estrous cycle suggests that PGE2 binds to these receptors on smooth muscle and fibroblast cells in the cervix in order to stimulate the relaxation of smooth muscle and hyaluronan-like glycoaminoglycan synthesis respectively (Kershaw-Young et al., 2009). Moreover, EP4 mRNA and protein expression was peak on day of parturition in pregnant rat cervical tissue suggesting that PGE2 induce cervical relaxation via activities through EP4 receptor (Chien and Macgregor, 2003). In human, it was shown that the increase of GAGs synthesis was mediated by EP4 receptors in cervical fibroblast (Schmitz et al., 2001). Furthermore, cervical ripening could be activated by PGE2 receptor agonist acting on EP4 which caused the decreased concentration of organized collagen and resulted in the decrease of cervical tensile strength in rat cervix (Feltovich et al., 2005).

1.2.5 Application of PGE2

The existance of a regid cervix at delivery time is a common problem for petient and therefore the induction of labor is needed. PGE2 has been used as intracervical gel (Prepidil) or intravaginal insertion (Cervidil) to induce cervical ripening and laboring in patients when cervix is unfavourable at the term (Norwitz et al., 2001). The study in mares showed that intracervical administration of PGE2 was beneficial to ripen the cervix prior to induction of parturition by increasing cervical relaxation (Rigby et al., 1998). Moreover, PGE2 has been used for cervical relaxation in trancervical artificial insemination in the sheep (Candappa et al., 2009) to increase fertility rates. PGE2 is likely to mediate cervical relaxation through the rearrangement of collagen bundles within cervical extracellular metrix (Feltovich et al., 2005) by disorganization of collagen bundles (Fosang et al., 1984; Owiny et al., 1987). Furthermore, in the rat cervix, it has been reported that PGE2-induced cervical tissue remodeling is mediated through the EP4 receptor that express maximally on the day of parturition and also first day after parturition (Chien and Macgregor, 2003).

1.3 Objectives of the study

1. To investigate the extracellular matrix (collagen and glycosaminoglycans) in the cervix of bitches during various stages of the estrous cycle and of bitches with pyometra.
2. To investigate mRNA expression of EP2, EP4, COX2 and PGES in the cervix of bitches during the stages of the estrous cycle and of bitches with pyometra

1.4 Hypothesis of the study

1. The mRNA and protein expression of EP2, EP4, COX2 and PGES, and collagen to smooth muscle ratio and glyclosaminoglycans profile in cervix of bitches vary during different stages of estrous cycle are different and also between group of bitches with open- or closed-cervix pyometra
2. Localization of EP4 in bitches during estrous cycle and in bitches with pyometra is related to cervical patency

1.5 **Keywords:** Canine, Cervical tissue, Estrous cycle, Extracellular matrix, ProstaglandinE2 receptor, Pyometra

CHAPTER II

COLLAGEN AND GLYCOSAMINOGLYCAN PROFILES IN THE CANINE CERVIX AT DIFFERENT STAGES OF THE ESTROUS CYCLE AND IN OPEN- AND CLOSED-CERVIX PYOMETRA

2.1 Abstract

The extracellular matrix of the cervix that comprises collagen, elastin, proteoglycans and glycosaminoglycans (GAGs), is thought to have an essential role in cervical relaxation. This study investigated the proportion of collagen and smooth muscle as well as the GAGs in healthy bitches at different stages of the estrous cycle and in bitches with open- and closed-cervix pyometra. Cervices were collected after ovariohysterectomy. The proportion of collagen and smooth muscle was determined using Masson's trichrome staining and Alcian blue staining was used to evaluate the relative distribution of cervical GAGs. The proportion of cervical collagen relative to cervical smooth muscle was higher at estrus compared to anestrus ($p \leq 0.05$). It was also higher ($p \leq 0.05$) in bitches with open- compared to those with closed-cervix pyometra. Overall, hyaluronan (HA) was the predominant GAG in the canine cervix and there were no differences in GAG composition in the stroma of healthy bitches and in the luminal epithelium and muscle of bitches with pyometra. In the luminal epithelium, HA was higher in estrus than in anestrus ($p \leq 0.05$) but not in diestrus ($p > 0.05$). On the contrary, the combined keratan sulfate (KS) and heparan sulfate (HS) content was higher in anestrus than estrus ($p \leq 0.05$). In bitches with pyometra, the combined KS and HS content was significantly lower in open- compared to closed-cervix pyometra ($p \leq 0.05$). Collectively, the different profiles of collagen and GAG observed at different stages of the estrous cycle as well as in pyometra suggest that the metabolism of both collagens and GAGs in the canine cervix is associated with hormonal statuses during the estrous cycle and cervical patency of bitches with pathological uterine conditions such as pyometra.

2.2 Introduction

The cervix has a crucial role during the reproductive cycle and the consistency of the cervix varies according to the stages of the estrous cycle. It is dilated during the estrous stage and closed during the anestrous and diestrous stages. In cows and mares (Hafez, 1973), the cervix is softer at estrus compared to its consistency in the luteal phase, whereas in sows (Meredith, 1977)and bitches (Silva et al., 1995) it is firmer at estrus compared to that at diestrus.

The cervix is mainly composed of connective tissue and smooth muscle, supported by extracellular matrix (ECM). The ECM is a network composed of collagen bundles, glycoproteins, proteoglycans, glycosaminoglycans (GAGs) and water (Fosang et al., 1990). Among those, collagen is the most abundant protein in the ECM and provides structural support (Hasler et al., 1999; Kjaer, 2004). The polymeric GAGs are made up of repeating disaccharides to form a family of related molecular species. They are classified into two groups; sulfated and non-sulfated. The sulfated GAGs (S-GAGs) are chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and heparan sulfate (HS), and 2) the non-sulfated GAG is hyaluronan (HA) or hyaluronic acid (Hileman et al., 1998). The major GAGs present in the human cervix are CS, DS and HA, although KS and HS are also found to a lesser extent (Osmers et al., 1993). However, until present, no data have been reported regarding the composition of the GAGs in the canine cervix.

Cervical relaxation is mediated by the reorganization of collagen bundles and decrease in the concentration of collagen (Feltovich et al., 2005). The fact that estradiol can induce collagen remodelling in the cervix of the pregnant ewe suggests that the ovarian steroid hormones have effects on cervical function (Owiny et al., 1987). During estrus, the bovine cervix relaxes, accompanied by an increase in its water content (Tsiligianni et al., 2001). In this respect, the content of HA, a hydrophilic GAG is thought to influence cervical hydration (Takemura et al., 2005). The prominent changes in human cervix observed during the peri-ovulatory phase are an increase in the total GAG and HA content, but a decrease in sulfated GAGs (DS and CS) (Winkler and Rath, 1999).

In the bitch, alterations in cervical relaxation are observed not only during the estrous cycle but also in some pathological conditions of the uterus such as pyometra. There are two types of pyometra defined by cervical patency; they are open-cervix pyometra and closed-cervix pyometra. To date, the mechanism of cervical relaxation in bitch remains poorly understood in both physiological (estrous cycle) and pathological (pyometra) conditions. Our previous study demonstrated a higher infiltration of neutrophils in the cervix of bitches with open- compared to closed-cervix pyometra (Kunkitti et al., 2011). In addition, increased infiltration of mast cells and macrophages was observed in the cervical stroma during estrus (Kemp et al., 1998) and in women, cervical polymorphonuclear cells were increased during parturition (Rath et al., 1994). Although cytokines released from neutrophils in the cervical stroma decreased collagen and increased GAG content (el Maradny et al., 1994) in the cervix, the effect of stromal neutrophils on cervical patency in bitches having open-cervix pyometra requires investigation.

In the ewe, the proportion of collagen to smooth muscle is an important determinant of the structural integrity of the cervix that relates closely to cervical function (Kershaw et al., 2007). In this study, our hypothesis was that collagen to smooth muscle ratio and GAGs content of the cervix vary during different stages of the estrous cycle as well as between open- and closed-cervix pyometra in dog.

2.3 Materials and methods

2.3.1 Experimental design

The study was performed in two parts. Cervical tissue was obtained from healthy cyclic bitches and bitches with pyometra. Healthy bitches were grouped according to their reproductive cycle and bitches with pyometra were classified as open- or closed-cervix. In Part I, sections of cervix were stained using Masson's trichrome to determine collagen to smooth muscle ratio. Part II types and proportions of cervical GAGs were determined using Alcian blue 8GX staining.

2.3.2 Animals

Twenty eight healthy adult nulliparous bitches with normal reproductive cycle and no history of previous hormonal use for reproductive control were used in the study. The average age was 2.1 ± 0.8 y (mean ± SEM, range 1-4 y). At the time of ovariohysterectomy 10 bitches were in anestrus, 7 in estrus and 11 in diestrus. Another group of 28 bitches with pyometra (open-cervix, n=18; closed-cervix, n=10) with a mean age of 7.0 ± 3.9 y (range, 2-15 y) were also included.

The stages of the estrous cycle were determined on the basis of the reproductive history, vaginal cytology, ovarian morphology (i.e., number and size of follicles and/or corpora lutea), and serum progesterone concentrations. A bitch was defined as in anestrus if her vaginal smear had >90% parabasal cells with no cornified cells, if serum progesterone concentrations were basal (<1 ng/mL) and if the ovaries were quiescent. A bitch was defined as estrus if the cornified cells in her vaginal smear were greater than 90% of all cells, if follicles were present on the ovaries and/or if serum progesterone concentrations were between 1 and10 ng/mL. Diestrus was identified by presence of corpora lutea on the ovaries and/or serum progesterone concentrations >10 ng/mL. A diagnosis of pyometra was based on clinical signs, hematology and an ultrasound scan of the uterus. Open- and closed-cervix pyometra were classified according to the presence or absence of purulent vulvar discharge, respectively.

2.3.3 Collection and processing of samples

Cervical tissue samples were collected surgically and prepared as previously described (Kunkitti et al., 2011). Briefly, the cervix was longitudinally cut from the internal to external os, fixed in 4% paraformaldehyde for 36-48 h and embedded in paraffin wax. Samples were sectioned at 4 μm and then placed on coated slides (3-aminopropyl-triethoxysilane, minimum 98%; Sigma-Aldrich, Taufkirchen, Germany) for Masson's trichrome or Alcian blue staining.

2.3.4 Hormone analyses

Blood samples were collected from the cephalic vein by venipuncture before the surgery; serum was separated and stored at -20 °C. Progesterone concentrations were determined using a chemiluminescent assay. The intra-assay coefficients of variation were 3.9% at 0.1 ng/mL and 6.5% at 36.1 ng/mL. The inter-assay coefficients of variation were 3.8% at 0.1 ng/mL and 16.3% at 36.1 ng/mL.

2.3.5 Staining with Masson's trichrome and the determination of collagen and smooth muscle

To determine the proportion of collagen and smooth muscle, tissue sections were deparaffinized and rehydrated with graded alcohols and immersed in warm Bouin's solution (Sigma-Aldrich, USA) (55-60 °C) for 2 h, washed in running tap water for 2 min followed by distilled water for 30-60 s. Thereafter, they were stained with Weigert Hematoxylin (Merck, Germany) for 10 min, and rinsed until only the cell nuclei were stained. Smooth muscle was stained red with Biebrich Scarlet-Acid-Fuschin (Sigma-Aldrich, USA) for 10 min, rinsed, and immersed in phosphomolybdic phosphotungstic acid (Sigma-Aldrich, USA) for 15 min. Subsequently, collagen was stained blue with Aniline Blue (Sigma-Aldrich, USA) for 10 min, and rinsed in distilled water, followed by immersion in 1% acetic acid for 5 min. Finally, the tissues were rehydrated with 95 and 100% ethanol, left to air dry, and mounted with mounting medium. All cervical tissue sections were stained in one time.

Color image analysis was performed under a light microscope at 40× magnification. A total of 5 to 10 reading fields were taken from each cervical section to cover the entire section. The blue (collagen) and red (muscle) stained area of the cervical tissue was measured using a software program (Image-Pro PLUS 6.0, Media Cybernetics, Inc, MD, USA). Proportion of collagen and muscle was calculated by the number of pixels of either blue (collagen) or red (muscle) color using an automated detection system, producing a percentage area for collagen and muscle in a tissue section. The mean proportion of collagen and muscle and standard errors of means was calculated from 5 to 10 readings per section.

2.3.6 The staining with Alcian blue 8GX and the determination of GAGs

Tissue GAGs were determined by Alcian blue staining as previously described (Kershaw-Young et al., 2009; Ponglowhapan et al., 2011). Briefly, tissue sections were de-waxed, rehydrated, and immersed in 0.025 M sodium acetate buffer, pH 5.8, containing 0.05% Alcian blue 8GX (Sigma, Poole, UK) with different concentrations of $MgCl_2$ (0.05 M, 0.4 M, and 0.8 M) for 18 h at room temperature. Sections were washed three times in the corresponding buffer containing $MgCl_2$, dehydrated and mounted in DPX.

Alcian blue stains different types of GAGs at different concentrations of $MgCl_2$ (Ponglowhapan et al. 2011). At low concentrations (0.05 M $MgCl_2$), all GAGs stain; at 0.4 M $MgCl_2$, all the sulfated GAGs (chondroitin sulfate, CS; dermatan sulfate, DS; heparan sulfate, HS; and keratan sulfate, KS) stain and at 0.8 M $MgCl_2$ only highly sulfated GAGs (KS and HS) stain.

Three cervical cell layers, i.e. luminal epithelium, stroma and muscle were separately analysed for the intensity of staining with Alcian blue using a Vickers M85A scanning and integrating microdensitometer (Kershaw-Young et al., 2009). Ten readings of each cell layer at each concentration of $MgCl_2$ were taken at 550 nm. Absolute Alcian blue staining intensity was expressed as the mean integrated extinction (MIE × 100) per unit area. The calculation of MIE of all GAGs in each layer was performed as previously described (Kershaw-Young et al., 2009). In brief, the percentage of sulfated GAGs (CS, DS, KS, and HS) was subtracted from the percentage of total GAGs (taken as 100%) to determine the percentage of non-sulfated GAGs (i.e. hyaluronan; HA). Similarly, percentage of highly sulfated GAGs (KS and HS) was subtracted from that for all the sulfated GAGs to determine the percentages of CD and DS and KS and HS. Results are expressed as (i) total GAGs, (ii) percentage of HA, (iii) percentage of total sulfated GAGs, (iv) percentage of CS plus DS and (v) percentage of KS plus HS (Kershaw-Young et al., 2009).

2.3.7 Statistical analysis

Data on the proportions of smooth muscle and collagen and the percentages of GAGs were analysed to compare the effect of the stage of the estrous cycle by analysis of variance using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). The Bonferroni test was performed for subsequent *post hoc* paired comparisons. The T-test was used for animals with open-cervix or closed-cervix pyometra comparing the proportions of smooth muscle and collagen and the percentages of GAGs between these two conditions. Level of significance was set at $p \leq 0.05$.

2.4 Results

2.4.1 Proportion of collagen and smooth muscle

Examples of the blue-stained collagen and red-stained smooth muscle in the cervix of a cyclic bitch and a bitch with pyometra are shown in Figure 5. As the technique used in this study determined the relative area of collagen to smooth muscle, the area of collagen was in an inverse relationship with the area of smooth muscle. Overall, the relative area occupied by collagen was lower ($p \leq 0.05$) during anestrus compared to estrus but did not differ between anestrus and diestrus ($p > 0.05$, Table 1). A higher proportion of collagen proportion was found in the cervical tissue of bitches with open- compared to closed-cervix pyometra ($p \leq 0.05$, Table 1).

Table 1. Proportion (%) of collagen in relative to smooth muscle (mean ± SEM) in the cervices of bitches at different stages of the estrous cycle, and with open- and closed-cervix pyometra.

Status of bitches		n	% Collagen
Healthy	Anestrus	10	47.03 ± 2.50^{a}
	Estrus	7	57.87 ± 2.39^{b}
	Diestrus	11	50.89 ± 2.14^{ab}
Pyometra	Open-cervix	18	39.57 ± 1.67^{x}
	Closed-cervix	8	26.49 ± 2.23^{y}

Values with different superscripts within healthy bitches (a and b) and bitches with pyometra (x and y) differed significantly ($p \leq 0.05$).

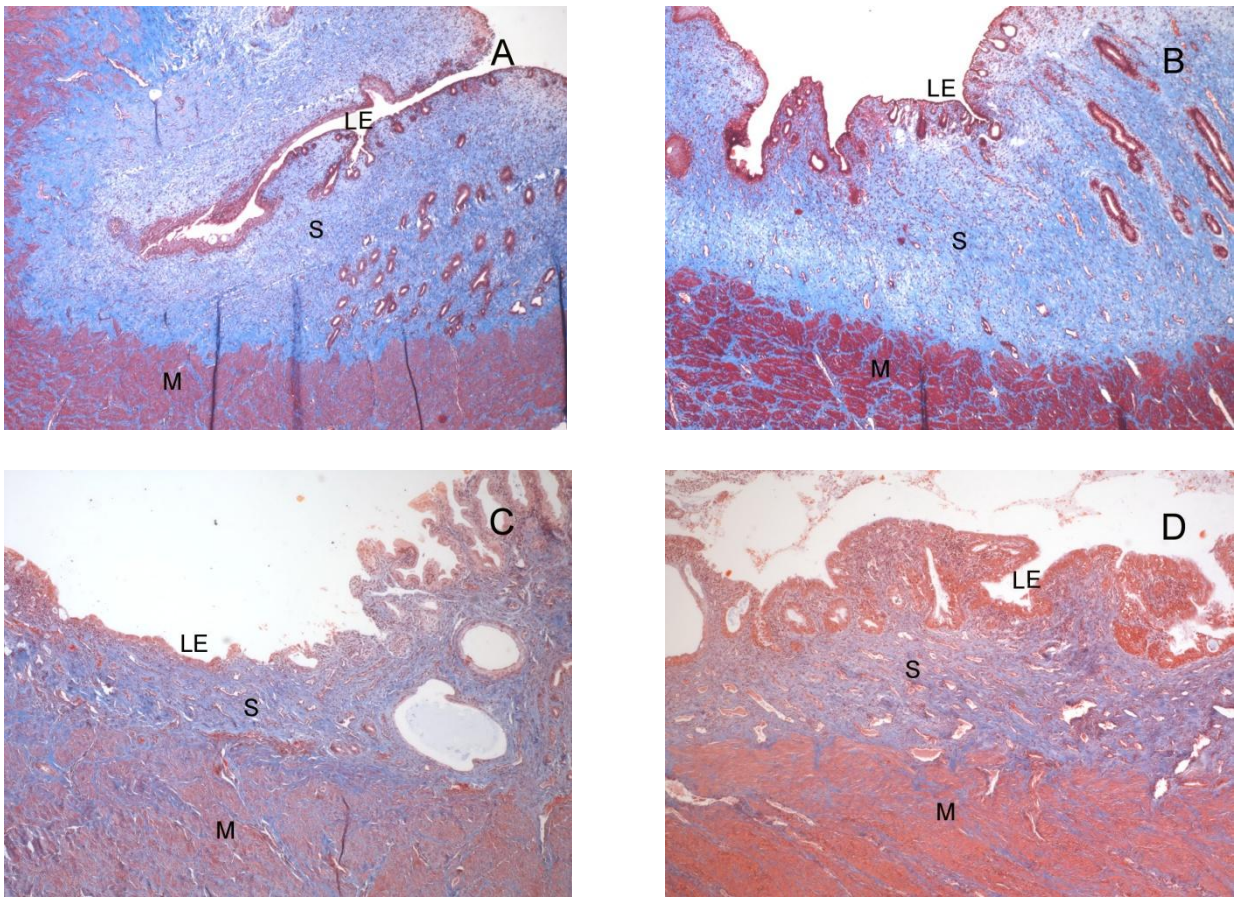


Figure 5. Masson's trichrome staining for blue-stained collagen and red-counterstained smooth muscle in the cervix of a healthy bitch during anestrus (A) and estrus (B), and in bitches with open- (C) or closed-cervix pyometra (D). Cervical cell layers: LE - Luminal epithelium, S – stroma, M - muscle. Bar = 100 μM.

2.4.2 Glycosaminoglycans

The pattern of staining with Alcian blue at three concentrations of $MgCl_2$ for three groups of GAGs; non-sulfated GAGs (mainly HA), sulfated GAGs (CS plus DS) and highly sulfated GAGs (KS plus HS) are shown in Figure 6. Regardless of type, the GAGs were primarily confined to the smooth muscle layers (Tables 2 and 3). The total GAGs in the cervix occupied about 46.8% of the muscle layer, 23.4% of the luminal epithelium and 28% of the stroma. Hyaluronan was the predominant GAG in the cervix of healthy bitches, irrespective of the stage of estrous cycle and also in bitches with pyometra (Tables 2 and 3). In the luminal epithelium and muscle layers, HA was the predominant GAG (69.0% of total GAGs). In the stroma, the percentage of HA was 47.5% of the total. In the luminal epithelium of healthy bitches, HA and KS plus HS were both significantly ($p \leq 0.05$) higher in estrus than anestrus (Table 2). In the muscle layer, CS plus DS were higher ($p \leq 0.05$) in estrus than diestrus. However, no significant differences were observed among the stages of the reproductive cycle in the stroma of healthy bitches (Table 2).

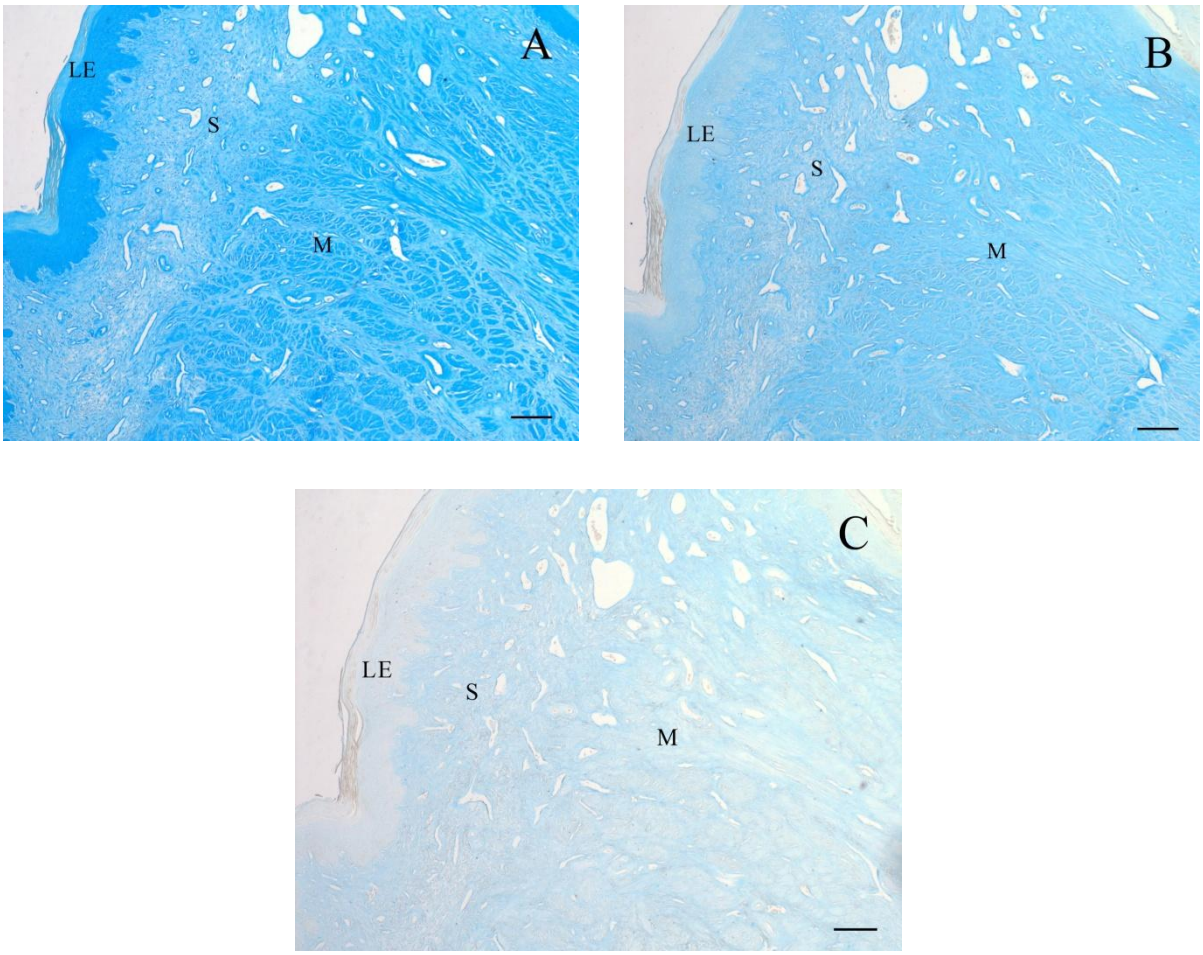


Figure 6. Alcian blue staining in body of the cervical tissue (A-C) of an anestrous bitch. Differences in staining intensity among the 3 varying concentrations of $MgCl_2$ can be noted; total GAGs at 0.05 $MgCl_2$ (A), total sulfated GAGs at 0.4 $MgCl_2$ (B) and highly sulfated GAGs at 0.8 $MgCl_2$ (C). Cervical cell layers are labeled: luminal epithelium (LE), stroma (S), and smooth muscle (M). Bar = 100 μM.

Table 2. Intensity of glycosaminoglycan (GAG) staining (MIE ± SEM) in different tissue layers of the bitch cervix during different stages of the estrous cycle.

Stage of estrous cycle	Luminal epithelium	Stroma	Muscle
Total GAGs			
Anestrus	10.58±1.09[a]	6.79±0.56[a]	16.50±2.23[a]
Estrus	15.84±2.09[b]	7.13±0.50[a]	19.31±1.83[a]
Diestrus	14.06±1.73[ab]	6.30±0.63[a]	17.53±1.67[a]
Hyaluronan (HA)			
Anestrus	7.31±1.30[a]	3.23±0.54[a]	10.27±2.29[a]
Estrus	12.80±2.30[b]	3.24±0.67[a]	12.15±2.19[a]
Diestrus	10.68±1.61[ab]	3.09±0.59[a]	11.89±1.55[a]
Total sulfated GAGs			
Anestrus	3.26±0.27[a]	3.56±0.28[a]	6.23±0.26[a]
Estrus	3.04±0.31[a]	3.88±0.33[a]	7.15±0.94[a]
Diestrus	3.38±0.45[a]	3.21±0.41[a]	5.64±0.56[a]
Chondroitin sulfate and dermatan sulfate (CS and DS)			
Anestrus	2.02±0.24[a]	2.06±0.14[a]	4.52±0.22[ab]
Estrus	2.30±0.31[a]	2.43±0.27[a]	5.56±0.83[a]
Diestrus	2.53±0.39[a]	1.85±0.33[a]	3.80±0.41[b]
Keratan sulfate and Heparan sulfate (KS and HS)			
Anestrus	1.24±0.14[a]	1.53±0.26[a]	1.71±0.22[a]
Estrus	0.74±0.0.8[b]	1.45±0.15[a]	1.59±0.18[a]
Diestrus	0.84±0.09[b]	1.35±0.21[a]	1.77±0.23[a]

Within columns values with different superscripts (a and b) differed significantly ($p \leq 0.05$).

In bitches with pyometra, all types of GAGs in the luminal epithelium and muscle layers were not different between open- and closed-cervix pyometra, However, in stroma the KS plus HS was higher ($p \leq 0.05$) in cervices from closed- compared to open-cervix pyometra (Table 3).

Table 3. Intensity of glycosaminoglycan (GAG) staining (MIE ± SEM) in different tissue layers of the cervices of bitches with open- and closed-cervix pyometra.

Status of pyometra	Luminal epithelium	Stroma	Muscle
Total GAGs			
Open	12.97±1.13[a]	8.31±0.57[a]	20.92±1.58[a]
Closed	12.07±1.65[a]	8.30±1.11[a]	19.54±2.50[a]
Hyaluronan (HA)			
Open	9.73±1.16[a]	4.21±0.53[a]	14.85±1.62[a]
Closed	8.48±1.72[a]	4.01±1.12[a]	13.37±2.4[a]
Total Sulfated GAGs			
Open	3.23±0.19[a]	4.09±0.18[a]	6.07±0.25[a]
Closed	3.58±0.27[a]	4.29±0.36[a]	6.17±0.50[a]
Chondroitin sulfate and dermatan sulfate (CS and DS)			
Open	2.34±0.18[a]	2.90±0.17[a]	4.40±0.21[a]
Closed	2.45±0.21[a]	2.38±0.27[a]	4.04±0.61[a]
Keratan sulfate and Heparan sulfate (KS and HS)			
Open	0.89±0.12[a]	1.18±0.10[a]	1.66±0.17[a]
Closed	1.13±0.17[a]	1.90±0.28[b]	2.12±0.19[a]

Within columns values with different superscripts (a and b) differed significantly ($P \leq 0.05$).

2.5 Discussion

The results of this study demonstrated that proportion of collagen to smooth muscle and GAG components in the cervix of healthy intact bitches differed according to the stage of the estrous cycle, i.e. estrus, diestrus and anestrus, suggesting an influence of sex steroids on the ECM of the canine cervix. In bitches with pyometra, the proportion of collagen was higher in those with open-cervix pyometra but the GAGs did not differ between open- and closed-cervix pyometra except for the highly sulfated GAGs, KS plus HS, in stroma where it was higher in cervical tissues from bitches with closed-cervix pyometra. These findings suggest that mechanisms regulating cervical patency in pyometra have a greater impact on collagen remodeling rather than on the patterns of GAGs.

In line with a recent study on the sheep cervix, the increase in cervical area occupied by collagen during estrus observed in the present study appeared to be associated with high estradiol concentrations (Kershaw et al., 2007). It has been suggested that the ovarian steroid hormones have a role in the synthesis and remodeling of collagen in the uterus of the rabbit (Goranova et al., 1993), the uterine cervix of women (Uldbjerg et al., 1983), the cervix of the ewe (Kershaw et al., 2007) and the canine lower urinary tract of the dog (Ponglowhapan et al., 2010). In this later case the proportion of collagen was greater in spayed compared to intact bitches (Ponglowhapan et al., 2010). Moreover, the estradiol-induced disruption of collagen bundles has been demonstrated in the cervix of pregnant (Owiny et al., 1987) and estrus (Zhang et al., 2007) ewes. The disruption of collagen bundles decreased the tensile strength of the cervix by increasing the interfibrillary distance in collagen fibrils (Feltovich et al. 2005). Although the proportions of collagen and smooth muscle in this study, differed among estrus, diestrus and anestrus, suggesting an effect of hormonal status on collagen remodeling, the involvement of collagen in cervical patency (open versus closed) in the dog remained an interesting subject for further investigation. In this respect it would be interesting to compare samples of cervix taken from estrous bitches when the cervix is open, approximately 2 days before the LH surge, or closed, 3 days

before end of the estrus (Silva et al., 1995), cervical tissue from pregnant and peripartum bitches would also be interesting.

In this study, the bitches affected with open-cervix pyometra had increased proportion of collagen in the cervix, in other words, a decreased proportion of smooth muscle, suggesting disruption of collagen leading to cervical relaxation (opening). This phenomenon associated with an inflammatory reaction in the uterus and also in the cervix as suggested by the higher degree of neutrophil infiltration in cervical tissues of bitches with open-cervix pyometra (Kunkitti et al., 2011). The disorganization of collagen in the cervix is characterized by a decrease in glycosaminoglycan-protein interaction probably induced by collagenolytic enzymes such as the matrix metalloproteinases (MMPs) from cervical neutrophils (Roughley and Lee, 1994). Increased densities of tissue-bound leukocytes, e.g. neutrophils, macrophages, during cervical relaxation/ripening in late pregnancy compared to during the first pregnant trimester have been reported for the human cervix, implying a role for these cells in cervical ripening (Bokstrom et al., 1997). Moreover, the levels of inflammatory cytokines such as IL-1 and IL-8 that recruit neutrophils into the human cervix, are increased at term labor (Winkler et al., 1999). In rabbits, local administration of IL-8 into the cervix induced an increase in neutrophil infiltration of the cervical stroma and decreased the collagen content (el Maradny et al., 1994). Although, the exact underlying mechanism(s) that alter cervical patency in bitches with pyometra is still unknown, neutrophils that induce collagenolytic enzymes and other cytokines are likely participants. Recently, positive correlations among the number of neutrophils in cervical stroma, the mRNA expression of IL-8 and cervical patency of bitches with pyometra have been reported (Tamada et al., 2012). However, it is worth noting that in normal bitches an association between increased neutrophil density and increased collagen in the cervix was not observed in estrous bitches (Kunkitti et al. 2011) where the proportion of collagen was increased but the number of tissue-bound neutrophils was not (Kunkitti et al., 2011).

In addition to IL-1 and IL-8 which are activated by neutrophils, matrix metalloproteinase (MMPs) produced by fibroblasts also have significant roles in

collagen remodeling (Sennstrom et al., 2000). Two subtypes of MMPs, MMP-2 and MMP-9, have been shown to degrade components in ECM, i.e. collagen and proteoglycans (Stygar et al., 2002). The activation of MMPs is dependent upon other regulatory mechanisms including the plasminogen-plasmin cascade, by which plasminogen is converted into active plasmin by urokinase plasminogen activator (uPA), resulting in the degradation of fibrin (Alexander and Werb, 1991). This study using cell culture, showed that the addition of plasminogen and uPA activated MMP-9 (Baramova et al., 1997). In addition, Secreted *Protein* Acidic and Rich in Cysteine (SPARC) which mediates cell-matrix interactions (Brekken and Sage, 2000) and induces MMP-9 production by fibroblasts (Tremble et al., 1993) are involved in the remodeling of ECM. Therefore, fibroblasts are likely to be important sources of modulators that induce remodeling the ECM and/or or collagen turnover. Interestingly, two phenotypes of fibroblasts, i.e. fibroblasts and myofibroblasts, have been detected in the cervix at the time of cervical ripening. The phenotype of fibroblasts was determined by the presence of α-smooth muscle actin (a-SMA) in myofibroblasts and a-SMA was higher in cervical tissue from non-pregnant women compared to women in labor. In addition, a cytokine, transforming growth factor β (TGF-β) regulated collagen turnover through the MMPs (Yan and Boyd, 2007) and induced the recruitment of neutrophils (Fava et al., 1991). To gain a better understanding of the mechanisms contributing to modulation of cervical patency in the dog, the roles of fibroblast phenotype, MMPs and other cytokines need to be studied further.

To our knowledge, the GAGs in the canine cervix have not been reported previously. This study revealed variation in distribution of the various GAGs among the tissue layers (luminal epithelium, stroma, and muscle) of the cervix. The findings that HA, the predominant GAG, and that it was more abundant in the muscle layers than in the luminal epithelium or the stroma was similar to findings reported for the sheep cervix (Fosang et al., 1984; Kershaw-Young et al., 2009). Our results in the cyclic bitches are also in line with previous reports for sheep (Kershaw-Young et al. 2009) and rats (Cubas et al., 2010) that showed that cervical HA was highest during estrus compared to other

stages of the estrous cycle. Cervical relaxation during estrus was related to changes in the ECM; an alteration of GAG contents (Toole, 2002) and increased water content (Fosang et al., 1984). Hyaluronan has hydrophilic properties which thus cause tissue edema, dissociation of collagen fibers, and decreased extensibility of cervical tissue, all of which assist cervical ripening/relaxation (Golichowski et al., 1980; Uldbjerg et al., 1983). In the cyclic bitches in this study, increased HA in the luminal epithelium during estrus were probably related to high estradiol concentrations at estrus. Human studies also support the suggestion that estradiol positively regulates HA synthesis in the cervix (Tanaka et al., 1997) while progesterone has the opposite (inhibitory) effect on HA synthesis (Uchiyama et al., 2005). Hyaluronan is involved in inflammatory processes and is ultimately linked to inflammatory cytokines. The local application of HA to the rabbit cervix resulted in a higher number of neutrophils that produce MMPs and thus regulates the remodeling collagen and facilitates cervical ripening (El Maradny et al., 1997). In cyclic bitches, during estrus, HA was increased whereas the number of neutrophils was not (Kunkitti et al., 2011); in bitches with pyometra bitches there was no difference in HA between open- and closed-cervix pyometra although there was an increased density of neutrophils in the bitches with open-cervix pyometra. Taken together, these findings suggest different regulatory roles of HA and/or neutrophils in regulating cervical ECM and cervical patency in normal cyclic bitches and bitches with pyometra.

Similar to our findings, the 4 sulfated GAGs, CS, DS, KS and HS, were present in human (Obrink, 1973; Cabrol et al., 1980; Kitamura et al., 1980; Uldbjerg et al., 1983) and rat cervical tissue (Osmers et al., 1993). However, no CS was detected in the sheep cervix (Kershaw-Young et al., 2009); thus the cervical distribution of GAGs may vary according to the species. Because sulfated GAGs increase the strength of electrostatic interactions between collagen fibers and the binding of DS to collagen (Obrink, 1973), a reduction in sulfated GAGs could weaken the cross-links between collagen fibers (Van Kuppevelt et al., 1987). These observations support our findings of significantly lower concentrations of the highly sulfated GAGs (KS and HS) in the cervical stroma of bitches with open-cervix pyometra. Furthermore, HS regulated leukocytic infiltration (Stringer

and Gallagher, 1997), and in genetically modified mice lacking HS, increased leukocyte recruitment was reported in cardiac muscle (Vanhoutte et al., 2007). The lower combined amount of HS and KS and increased neutrophil density observed in bitches with open-cervix pyometra may acted to rearrange collagen thus weakening it and causing cervical relaxation (Junqueira et al., 1980).

In conclusion, the results of this study have demonstrated that (i) the proportion of collagen to smooth muscle altered depending on stages of the reproductive cycle and suggest that these changes are related to the milieu of ovarian hormones, (ii) a higher proportion of collagen was found in bitches with open-cervix pyometra where the cervix was fully relax and a lower proportion of collagen was found in cases of closed-cervix pyometra where the cervix was tightly closed indicated different structural function of cervical collagen in dogs with a uterine pathology, and (iii) the distribution of cervical GAGs varied among tissue layers of the canine cervix, the stages of the reproductive cycle and between open- closed-cervix pyometra. Hyaluronan, the predominant cervical GAG, may have a role in remodeling collagen and in cervical relaxation via either an increase in the water content of the tissue or by acting in conjunction with tissue cytokines. It is interesting to note that our results suggest that HA is not involved in the patency of the cervix in bitches with pyometra but that the highly sulfated GAGs (KS and HS) along with cytokines produced by local neutrophils seem to be responsible.

CHAPTER III
IMMUNOLOCALIZATION OF PROSTAGLANDIN E2 RECEPTOR SUBTYPE 4 (EP4) IN THE CERVIX OF CYCLIC BITCHES AND BITCHES WITH PYOMETRA

3.1 Abstract

Cervix is an important part of the reproductive tract; in non-pregnant animals it remains closed during anestrus and diestrus and is open only during estrus. In pathological conditions like pyometra, the cervix may be open or closed but the control mechanism is not clearly known. Prostaglandin E2 (PGE2) is considered to be involved in changes of extracellular matrix via coupling to prostaglandin E receptor subtype 4 (EP4). This study investigated the expression of EP in the cervices of bitches during different stages of estrous cycle and those with pyometra. After ovariohysterectomy, cervices were collected from anestrus (n = 6), estrus (n = 12) and diestrus (n = 6), open- (n = 10) and closed-cervix pyometra (n = 10) bitches. Cervical EP4 expression was observed at all the layers and the stages but the differences in EP4 expression either among bitches in different stages of the estrous cycle and between open- and closed-cervix pyometra were limited to only luminal epithelium (LE). In cyclic bitches during estrus and in open-cervix pyometra bitches, significantly higher ($P<0.05$) EP4 expression was found in LE of uterine part than vaginal part. In LE of the uterine part, the expression was higher in the bitches during estrus than in anestrus and diestrus, and in the bitches affected by open-cervix than those with closed-cervix pyometra. The results suggest that regulation of cervical dilation appeared in the uterine part of the cervix. Moreover, EP4 may be involved in stimulating dilation of the cervix in both estrus and open-cervix pyometra bitches.

3.2 Introduction

Patency of the canine cervix is related to the stage of reproductive cycle and concentrations of circulating ovarian steroid hormones. The cervix is closed during diestrus when progesterone is dominant or during anestrus which is an inactive stage of ovarian activity. Cervical opening/ dilation is observed during estrus where estrogen is highly influential, approximately 2 days before LH peak up to 3 days before the end of estrus period (Silva et al., 1995). However, mechanisms by which ovarian hormones control cervical opening in the bitch are not clearly understood. Besides, dynamics of cervical patency (open or closed) are seen in bitches having pyometra, a uterine pathological condition commonly found in aged intact animals.

The information about the canine cervix is rare either during normal reproductive cycle or in pathological conditions such as pyometra. As the bitch cervical canal is narrow and short, and is located between the uterus and the vagina; therefore, the cervical canal can be divided longitudinally into two parts which are the cranial part or the uterine part and the caudal part or the vaginal part of the cervix. Though the gross appearance of these two parts of the bitch cervix is not clearly distinguishable unless connected to the uterus or the vagina, the histomorphology of the bitch cervix showed that the cranial part of the cervix characterized by a simple columnar epithelium so it was called the uterine part, whereas the caudal part of the cervix characterized by a stratified squamous epithelium and it is so-called the vaginal part (Khunkiti *et al*., 2011). Regarding different morphology of these two parts of the bitch cervical canal, a difference in the localization of prostaglandin E2 receptor is expected from the present study.

There are few studies on the regulation of cervical patency in the bitch. Previously we have demonstrated that cervical dilation was consistent with an increase in progesterone receptor (PR) levels in cervical tissues of cyclic bitches during proestrus and estrus stages of the reproductive cycle (Vermeirsch et al., 2000; Kunkitti et al., 2011). However, both the protein (Kunkitti et al., 2011) and mRNA levels (Tamada et al., 2012) of PR and estrogen receptor-α (ER-α) showed no relationship with cervical

patency in the bitches with pyometra. Interestingly, the number of neutrophils and level of interleukin-8 mRNA in the cervical tissues differ between open- and closed-cervix pyometra bitches (Kunkitti et al., 2011; Tamada et al., 2012). Taken together, the factors regulating the canine cervical patency is multifactorial and opening of the cervix in normal cyclic bitches and in bitches with pyometra seems to be controlled by different mechanisms (Kunkitti et al., 2011).

The cervix is structurally supported by extracellular matrix (ECM), a network made up with collagen fibers, glycoproteins, proteoglycans, and glycosaminoglycans (Kjaer, 2004). Many studies support the concept that cervical patency in estrus is likely to be mediated by changes in the extracellular matrix (Kershaw-Young et al., 2009; Cubas et al., 2010) and prostaglandin E2 subtype 4 receptor (EP4) is involved in the remodeling process of the ECM (Feltovich et al., 2005). In sheep, exogenous estradiol given to the ewe has been shown to stimulate cervical EP4 mRNA expression which contributed to subsequent cervical relaxation (Kershaw-Young et al., 2010). This leads to suggest that the circulating estrogens, cervical EP4 content and ECM remodelling coordinately promote cervical dilation. Furthermore, activation of EP4 resulted in neutrophil infiltration and protease release that caused degradation of collagen, a component of cervical ECM (Rath et al., 1993). This study aimed at investigating the expression of EP4 in the canine cervical tissues and determining if there were any variations in their expression among different stages of the reproductive cycle (anestrus, estrus, and diestrus) or between bitches with open- and closed-cervix pyometra.

3.3 Material and methods

3.3.1 Animals

Cervical tissues were obtained from bitches subjected to ovariohysterectomy at the Obstetrics and Gynaecology unit, Small Animal Teaching Hospital, Chulalongkorn University, Bangkok, Thailand. The ovariohysterectomy procedure in the bitch consists of laparotomy with ablation of the both ovaries, uterus, and some part of the cervix. The main objective of this procedure is generally for spaying, but it is also suggested in the

cases of pyometra, uterine tumors, and some other pathologies. These included 24 healthy adult nulliparous bitches with normal reproductive cycle and no history of previous hormonal use for reproductive control. Animals were of mixed breeds aged 1-4 years (1.9 ± 0.4 yrs, mean ± SEM). The stage of estrous cycle was determined based on the 4 criteria including reproductive history, vaginal cytology, ovarian structures and serum progesterone concentrations. Anestrus was characterized by quiescent ovaries and vaginal cytology showing basal cells. Bitches with the presence of follicles on ovaries and cornified cells of vaginal cytology were defined as estrus. Diestrus was characterized by the presence of corpora lutea on ovaries and intermediate cells of vaginal cytology. Serum progesterone concentrations used to define stage of estrous cycle were < 1 ng/mL: anestrus; 2-14 ng/mL: estrus; and > 15 ng/mL: diestrus as described previously (Kunkitti et al., 2011). Twenty four bitches were divided into 3 groups; anestrus (n = 6), estrus (n = 12), and diestrus (n = 6). In addition, 20 bitches, mixed breed, and aged 6.3 ± 3.0 yrs (range, 1-12 yrs) [open-cervix pyometra (n = 10), and closed-cervix pyometra (n = 10)] of different breeds diagnosed as having pyometra were included in the study. Cervical status of pyometra bitches was determined on the basis of the presence or absence of mucopurulent vulvar discharge. Open-cervix pyometra was defined as the presence of purulent vulvar discharge whereas bitches developing closed-cervix pyometra had absence of purulent vulvar discharge.

3.3.2 Hormone analyses

Blood samples were collected by cephalic vein before anesthesia, centrifuged and stored at -20 °C until analysed. Serum progesterone concentrations were determined by chemiluminescent assay. The intra-assay coefficients of variation were 3.9% at 0.1 ng/mL and 6.5% at 36.1 ng/mL. The inter-assay coefficients of variation were 3.8% at 0.1 ng/mL and 16.3% at 36.1 ng/mL.

3.3.3 Tissue collection

The cervical tissue samples were collected and prepared according to the protocol used in our previous study (Kunkitti et al., 2011). Briefly, the cervix from the internal to external os was longitudinally cut through the lumen and fixed in 4% paraformaldehyde for 36-48 h, embedded in paraffin wax and sectioned into slices of 4 μm thickness. Sections were placed on coated slides (3-aminopropyl-triethoxysilane, minimum 98%; Sigma-Aldrich, Taufkirchen, Germany) for immunohistochemical evaluation.

3.3.4 Immunohistochemical detection and quantification of EP4

The immunohistochemical procedure was performed by avidin-biotin method as described in ABC elite kit (Vectatain® ABC kit, Vector Laboratories, CA, USA). After tissue sections were deparaffinized and rehydrated with graded alcohol, antigen retrieval was performed in a microwave by immersing the slides in 0.01 M citric buffer (pH 6). Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide in methanol for 10 min at room temperature. Sections were then rinsed in phosphate buffer saline (PBS) and incubated in a humidified chamber. To prevent non-specific reactions, samples were incubated with normal horse serum for 30 min at room temperature. For immunohistochemical detection goat polyclonal anti-human primary antibody to EP4 (catalogue number: sc-16022, Santa Cruz biotechnology, CA, USA) was used at a dilution of 1:50 as described previously (Ponglowhapan et al., 2010). Sections were incubated with primary antibody in a humidified chamber at 4 °C for 20-22 h. After this, sections were rinsed with PBS and incubated with the biotinylated anti-goat antibody (Vector Laboratories, CA, USA) for 30 min. The sections were incubated with NovaRED peroxidase substrate (Vector Laboratories, CA, USA) and counterstained with Mayer's hematoxylin, followed by mounting in glycerin-gelatin. A canine cervical tissue in estrus known to react with EP4 antibody was used as a positive control. Negative controls were obtained by omitting primary antibody (Kunkitti et al., 2011).

Each longitudinal tissue section was evaluated separately in two regions: vaginal and uterine regions. The vaginal and uterine regions were characterized by a stratified squamous epithelium and simple columnar epithelium, respectively (Kunkitti et al., 2011). At least 3 tissue sections from each sample were used for immunohistochemical evaluation. Both negative and positive controls were included in each occasion of the immunohistochemical procedure. Evaluation of immunohistochemical staining was done in 3 different tissue layers, i.e. luminal epithelium (LE), stroma (S), and muscle (M). Ten microscopic areas which corresponded to 0.0845 mm2 of real tissue area with 400 × magnification were randomly chosen from each tissue layer. Expression of EP4 was evaluated using an expression index which was derived by a percentage expression and intensity score (Expression index = [% expression × intensity score]/100). The proportionate area showing cells expressing EP4 was rated to the nearest 5%. The intensity of staining was graded as 1=weak staining, 2=moderate staining and 3=strong staining (Ponglowhapan et al., 2010).

3.3.5 Statistical analysis

Statistical analyses were performed using the Statistical Analysis System version 9.0 (SAS, Institute, 2002, Cary, NC). Data on EP4 expression are presented as mean ± SEM. Analysis of variance (ANOVA) was performed using a linear mixed model (PROC MIXED) to compare the differences among the stages of the estrous cycle (anestrus, estrus, and diestrus), the cervical layers (luminal epithelium, stroma, and muscle layers) and between the cervical regions (uterine and vaginal). Stage of the estrous cycle, groups of pyometra, cervical layers, and cervical regions were regarded as fixed factors. Differences in the expression index of EP4 between open- and closed-cervix pyometra groups were compared by student *t*-test. The level of significance was set at $P<0.05$.

3.4 Results

Expression of EP4 was observed in all the tissue layers (luminal epithelium, stroma and muscle) in both the uterine and vaginal regions of cervices in the normal healthy bitches at different stages of reproductive cycle and in bitches with pyometra. Examples of positive EP4 immunostaining and the control were shown in Figure 7. Negative controls did not exhibit any EP4 expression (Fig. 7 H). In addition, EP4 was localized in the muscle layer of blood vessels (Fig. 7G).

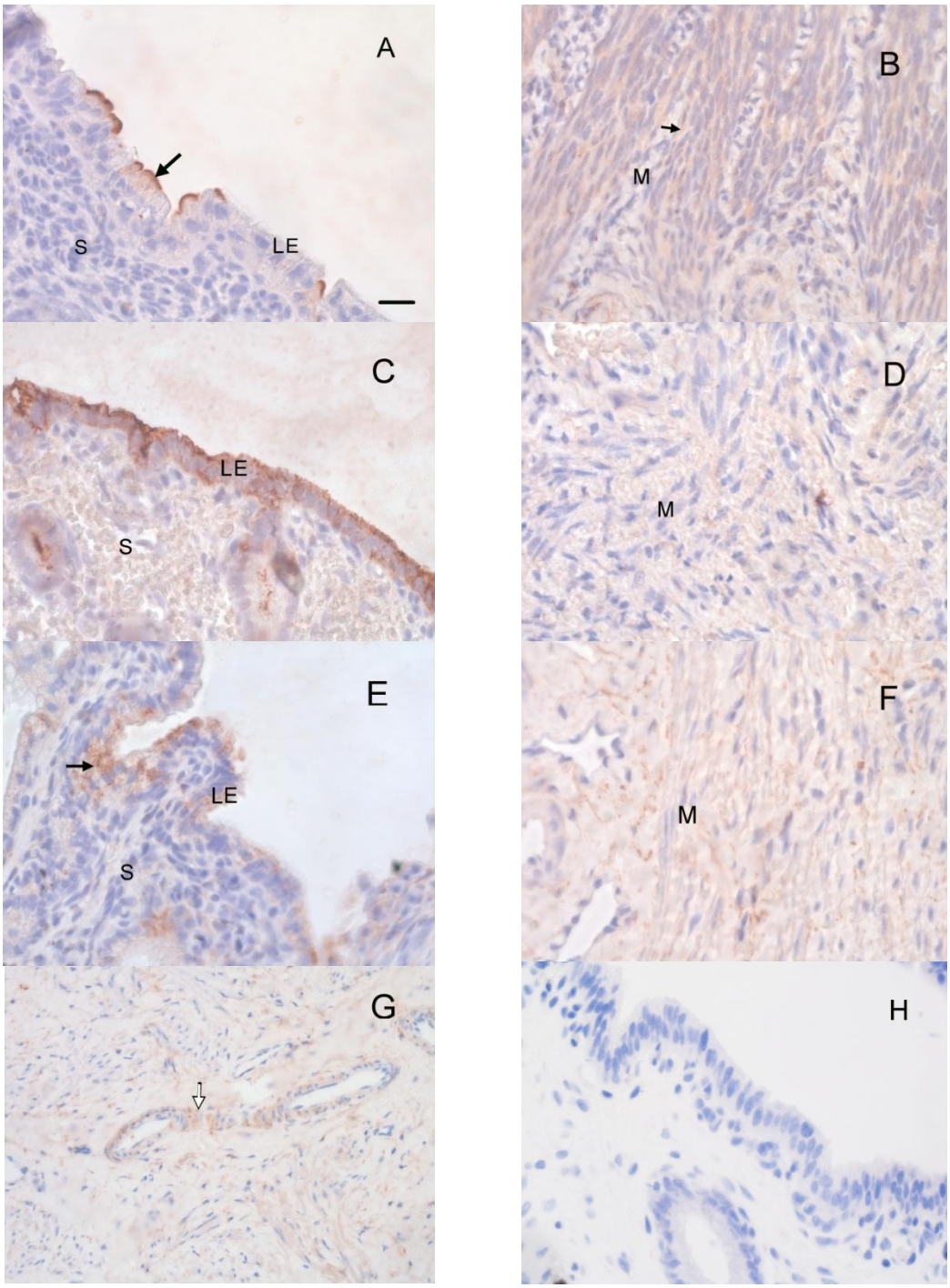


Figure 7: EP4 localization (red staining) in the uterine region of the cervix of healthy bitches. Positive staining (arrow) was observed in luminal epithelium (LE) and stroma (S) in anestrus (A-B), estrus (C-D), and diestrus (E-F) stage of the estrous cycle. The expression of EP4 is shown in cervical blood vessel (open arrow; G) of muscle layer in cervix. H; Negative controls. Intense positive staining in the luminal epithelium (arrow in A), moderate staining in the muscle (arrow in B) and weak staining in luminal epithelium (arrow in E) can be noted (original magnification: 400x).

3.4.1 EP4 expression in the cervix of cyclic bitches

Significant differences ($P<0.05$) in the expression of EP4 among different stages of the reproductive cycles were found in only the luminal epithelium. No differences were observed in the EP4 expression in stroma and muscles either between the two cervical parts or among the different stages of the estrous cycle (Table 4). In the luminal epithelium of the uterine region, EP4 expression differed significantly ($P<0.05$) among the stages of reproductive cycle; the lowest expression was observed in anestrus and highest expression in estrus ($P<0.05$; Table 4). In the luminal epithelium of vaginal region, EP4 expression was also significantly higher ($P<0.05$) in estrus and diestrus than anestrus, with no difference between estrus and diestrus stages of reproductive cycle (Table 4). Comparisons between the 2 regions of the cervix (uterine vs vaginal) showed that EP4 expression was highly expressed ($P<0.05$) in the luminal epithelium of the uterine region only in the estrous stage of reproductive cycle (Table 4).

Table 4. Expression index (mean ± S.E.M.) of EP4 in different tissue layers (LE; luminal epithelium, S; Stroma, M; muscle) of the uterine and vaginal regions of the canine cervix during different stages of the reproductive cycle

	Luminal epithelium		Stroma		Muscle	
	Uterine	Vaginal	Uterine	Vaginal	Uterine	Vaginal
Anestrus	$21.0\pm5.4^{a}_{x}$	$7.45\pm2.9^{a}_{x}$	$89.8\pm3.4^{a}_{x}$	$94.9\pm26.9^{a}_{x}$	$105.8\pm3.8^{a}_{x}$	$118.7\pm12.8^{a}_{x}$
Estrus	$227.9\pm32.2^{b}_{x}$	$59.5\pm14.1^{b}_{y}$	$72.5\pm12.9^{a}_{x}$	$81.5\pm14.2^{a}_{x}$	$80.2\pm11.0^{a}_{x}$	$66.7\pm21.1^{a}_{x}$
Diestrus	$101.9\pm38.8^{c}_{x}$	$66.2\pm26.9^{b}_{x}$	$62.5\pm26.7^{a}_{x}$	$52.7\pm20.65^{a}_{x}$	$105.1\pm26.0^{a}_{x}$	$102.8\pm4.6^{a}_{x}$

a,b Within a column, means without a common superscripts differed ($P < 0.05$).

x,y Within a layer, means without a common subscripts differed ($P < 0.05$).

3.4.2 EP4 expression in the cervix of pyometra bitches

Similar to cyclic bitches, significant differences in the expression of EP4 between open- and closed-cervix groups were found in only the luminal epithelium. The expression in stroma and muscle did not differ significantly between uterine versus vaginal regions as well as between open- versus closed-cervix pyometra. In the uterine region, EP4 expression in the luminal epithelium of open-cervix group was significantly higher ($P<0.05$) than closed-cervix group (Table 5). Moreover, the EP4 expression was significantly higher ($P<0.05$) in the luminal epithelium of the uterine than the vaginal region of bitches with open-cervix pyometra (Table 5).

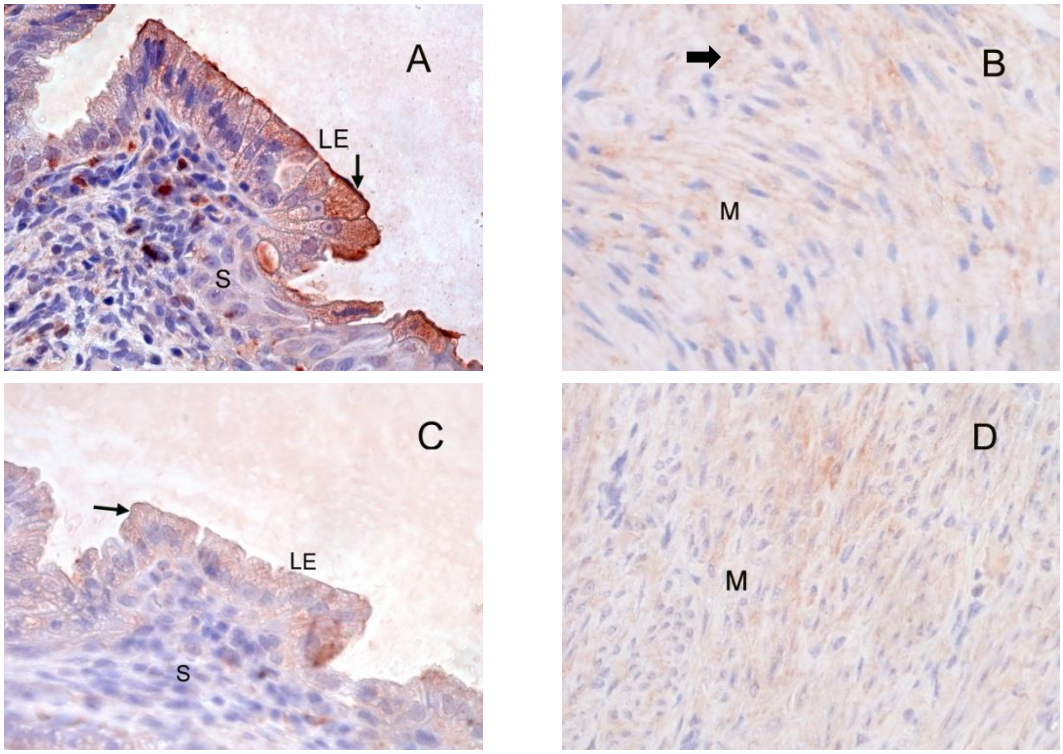


Figure 8. EP4 localization (red staining) in the uterine part of the cervix of bitches with pyometra (A-D). Positive staining was observed in luminal epithelium (LE), stroma (S), and muscle (M) in open-cervix (A-B) and closed-cervix pyometra (C-D). Intense positive staining in the luminal epithelium (arrow in A), moderate staining in the muscle (arrow in C) and weak staining in luminal epithelium (arrow in B) can be noted (original magnification: 400x).

Table 5 Expression index (mean ± S.E.M.) of EP4 in different tissue layers (LE; luminal epithelium, S; Stroma, M; muscle) of the uterine and vaginal regions of the cervix in open- and closed-cervix pyometra bitches

	Luminal epithelium		Stroma		Muscle	
	Uterine	Vaginal	Uterine	Vaginal	Uterine	Vaginal
Open-cervix	$83.8\pm16.7^{a}_{x}$	$37.3\pm12.2^{a}_{y}$	$93.6\pm12.7^{a}_{x}$	$68.8\pm13.0^{a}_{x}$	$110.4\pm10.0^{a}_{x}$	$94.1\pm8.2^{a}_{x}$
Closed-cervix	$28.6\pm15.5^{b}_{x}$	$27.9\pm14.0^{a}_{x}$	$59.9\pm12.8^{a}_{x}$	$75.6\pm19.7^{a}_{x}$	$78\pm7.3^{a}_{x}$	$95.3\pm14.5^{a}_{x}$

a,b Within a column, means without a common superscripts differed ($P < 0.05$).

x,y Within a layer, means without a common subscripts differed ($P < 0.05$).

3.5 Discussion

The results obtained have shown that expression of EP4 was consistently observed in the canine cervix regardless of the reproductive cycle and the cervical patency (open versus closed) of pyometra bitches. In addition, differences in EP4 expression between the 2 regions of the cervix (uterine versus vaginal) and between different stages of reproductive cycle (anestrus, diestrus and estrus) are present in the luminal epithelium and not in other tissue layers of the cervix examined. These findings suggested a possible role of EP4 in regulating cervical closure/opening during physiological changes of reproductive cycle in cyclic bithces and in bitches developing open- or closed-cervix pyometra. However, other factors like ovarian steroid hormones together with their receptors (Silva et al., 1995; Kunkitti et al., 2011) in cyclic bitches, as well as interleukin-8 mRNA and the number of neutrophils in cervical tissue of pyometra bitches (Tamada et al., 2012), have been reported to have regulatory role in cervical patency of the bitch. We also observed the EP4 expression in the smooth muscle of cervical blood vessels as reported previously in the ovine cervix (Wu et al., 2005) suggesting the possible role of PGE2 in vasodilation in canine cervix via EP4 with subsequent edema and leukocytic infiltration (Schmitz et al., 2006).

In this study, higher expression of EP4 was found in the uterine compared with the vaginal region of the cervix which is contrary to the study in the sheep (Kershaw-Young et al., 2009). The gradient expression of EP4 from uterine to vaginal part in the sheep cervix was postulated as being caused by the differences in cell density (Kershaw-Young et al., 2009). However, lower number of epithelial cells in the uterine compared with the vaginal region of the canine cervix has been reported (Goericke-Pesch et al., 2010). Hence, the higher EP4 expression in the luminal epithelium of the uterine part was not related to the number of epithelial cells but rather to the specific cell type. The uterine part of the cervix is lined by simple columnar cells, whereas, the vaginal part is covered with stratified squamous epithelium (Eurell and Frappier, 2006; Goericke-Pesch et al., 2010), proposing that the columnar cells in the uterine part might be more responsive than the squamous cells in the vaginal part.

Epithelial cells of human endometrium produce PGE2 (Smith and Kelly, 1988) which modulates smooth muscle contractility in response to signals from external stimuli such as hormones, chemicals, and bacteria (Ruan et al., 2011). Moreover, PGE2 secreted from the epithelial cells can regulate the muscle contraction or relaxation, depending on the type of receptor expressed. There are 4 subtypes of PGE2 receptor which are EP1, EP2, EP3 and EP4; the EP1 and EP3 cause muscle contraction, while EP2 and EP4 are involved in muscle relaxation. In this study, we were interested in the expression of EP4 in the canine cervix because the study in rat cervix demonstrated that EP4 expressed near term parturition (Feltovich et al., 2005). This suggests that PGE2 plays a role on cervical dilation through EP4 receptor. Significantly higher EP4 expression in the luminal epithelium at the periods of cervix opening, e.g. estrous stage of the reproductive cycle and open-cervix pyometra suggests the involvement of luminal epithelium EP4 in the underlying machanism of cervical relaxation in the bitch. Moreover, the EP4 expression in the luminal epithelium and the synthesis of PGE2 by cervical epithelium (Shemesh et al., 1997) implies that epithelial cells not only secrete PGE2 as triggered by some stimuli but also present EP4 receptors for it to bind and induce a signaling mechanism culminating at the cervical relaxation.

During estrus when the reproductive tract is under the major influence of circulating estrogen, the canine cervix relaxes (Silva et al., 1995) similar to other mammalian species such as mares and cows (Arthur et al., 1989b). The higher expression of EP4 during estrus observed in this study may be associated with high estrogen levels in the circulation. Such effects of estrogen on EP4 expression have been reported in the cervix and uterus of exogenous estrogen treated ovariectomized animals (Yang et al., 1997; Kershaw-Young et al., 2010). On the other hand, the progesterone seemed to suppress expression of EP4 as demonstrated in the rat cervical tissue treated with progesterone (Hinton et al., 2010). Similarly, in this study the lower EP4 expression in luminal epithelium was observed during diestrus when serum progesterone concentrations are comparatively higher than the other stages of reproductive cycle. These results are supported by the studies that report suppression of progesterone by the antiprogestin (RU-486) induces an increase in the cervical EP4 expression and cervical relaxation in the rat (Hinton et al., 2010).

In pyometra bitches, greater EP4 expression in the luminal epithelium of uterine regions observed in bitches developing open-cervix indicated that EP4 might also have a role in cervical patency in pathological conditions. Previous studies (Kunkitti et al., 2011; Tamada et al., 2012) have demonstrated a higher infiltration of neutrophils in the cervical tissues in open-cervix pyometra compared to closed-cervix pyometra suggesting that neutrophils are involved in cervical relaxation process. Neutrophils stimulate induction of inflammatory cytokines and prostaglandins production (Ito et al., 1988). Moreover, interleukin1-β (IL-1β) changes extracellular matrix via EP4 in human cervical fibroblasts leading to cervical relaxation (Schmitz et al., 2003). In addition to IL-1β, interleukin-8 (IL-8) which is stimulated by IL-1β and PGE2 (Ito et al., 1994; Denison et al., 1999) is a chemoattractant for neutrophils and stimulates degranulation of neutrophils, collagen degradation and connective tissue remodeling, thus, causing the cervical relaxation (Tamada et al., 2012). However, the relationship between IL-8 and EP4 expression remains to be elucidated.

In conclusion, EP4 is present in the canine cervix with higher expression being found in the luminal epithelium of the uterine compared to vaginal region in estrous bitches and in bitches with open-cervix pyomtra. The results lead to suggest that EP4 plays a role in regulating the canine cervical patency. Higher expression of EP4 during estrous period is potentially activated by estrogens in physiological conditions that prevail in normal cyclic bitches. It is possible that in pathological conditions like pyometra, inflammatory cytokines/chemokines may play a major role in this process.

CHAPTER IV

THE mRNA AND PROTEIN EXPRESSION OF PROSTAGLANDIN E2 RECEPTOR (EP2 AND EP4), CYCLOOXYGENASE-2 AND PROSTAGLANDIN E2 SYNTHASE IN CERVIX OF NORMAL CYCLIC BITCHES AND BITCHES WITH PYOMETRA

4.1 Abstract

Prostaglandins play a vital role in regulation of cervical patency. Prostaglandin E2 (PGE2) synthesis is regulated by cyclooxygenase (COX) and prostaglandin E synthase (PGES). PGE2 acts through prostaglandin E receptors (EP), EP2 and EP4, to stimulate muscle relaxation. The aim of this study was to investigate mRNA expressions of EP2, EP4, COX-2, and PGES 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 extracted from cervical tissue was determined for levels of EP2, EP4, COX-2, and PGES mRNA using a real-time qPCR. Western blot was performed to evaluate the protein expression of EP2, EP4 and COX-2. There were no differences of EP2, EP4, COX-2, and PGES mRNA expression in the bitch cervix among the stages of the estrous cycle. However, the expression of PGES mRNA was higher in the cervix of bitches with open-cervix than closed-cervix pyometra ($P<0.05$). However, the differences of protein expression were not observed in both normal cyclic bitches and bitches with pyometra. Our findings suggest that mRNA and protein expression of the enzymes involved in PGE2 synthesis and PGE2 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.

4.2 Introduction

Relaxation of the cervix is regulated by complex mechanism. Current knowledge on 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, an 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 are 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 E2 (PGE2) was shown to induce cervical ripening in human when administered intravaginally (Feltovich et al., 2005). Biosynthesis of PGE2 is regulated mainly by two enzymes, cyclooxygenase (COX) which converts arachidonic acid to prostaglandin H2 (PGH2), and prostaglandin E synthase (PGES) which converts PGH2 to PGE2. 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 have revealed that cervical glandular epithelial cells are 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 is highly expressed in the cervix at estrus when serum estradiol concentrations were high, indicating a relationship between PGE2 and estradiol (Kershaw et al., 2007). Prostaglandin E2 exerts its role by coupling to prostaglandin E receptors (EP); EP1, EP2, EP3 and EP4. The EP1 and EP3 are involved in the contraction of smooth muscle, whereas EP2 and EP4 function to induce smooth muscle relaxation (Narumiya et al., 1999). EP4 mRNA

expression in the rat cervix has been 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 from 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 not known. In our previous study, the number of neutrophils was found 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 has also been demonstrated in rabbits at the term time and is reported to be mediated by EP4 (Fukuda et al., 2007). Taken together, we hypothesized that there are associations between PGE2 [via its receptors (EP2 and EP4)], enzymes involved in PGE2 synthesis (COX-2 and PGES), and various stages of the estrous cycle which are 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 of EP2, EP4, COX-2 and PGES, and protein expression of EP2, EP4 and COX-2 in normal cyclic bitches during different stages of the estrous cycle and those with open- and closed-cervix pyometra.

4.3 Material and methods

4.3.1 Experimental design

The study contained two experiments.

Experiment I: The mRNA expression of EP2, EP4, COX-2 and PGES. The mRNA expression was determined using qPCR.

Experiment II: The protein expression of EP2, EP4, and COX2. This experiment was performed using the same cervical tissue from Exp I both the normal cyclic btiches and pyometra bitches. Western blot was used to define the level of protein expression.

4.3.2 Animals

Twenty eight bitches of various breeds with an average age of 2.1 ± 0.8 years (range 1-4 years) subjected to routine spaying were divided into 3 groups according to the 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 as outlined in table 6. Another group of 28 bitches diagnosed as having uterine content by ultrasonography were divided into 2 groups on the basis of whether cervix was open or closed open-cervix (n=18) and closed-cervix pyometra (n=10). The bitches affected with pyometra had an average age of 7.0 ± 3.9 years (range 2-15 years).

Table 6 Ovarian structures, vaginal cytology, serum progesterone level, and number of animals (N) used in each experiment in bitches during estrous cycle.

	Anestrus	Estrus	Diestrus
Ovarian structure	Absence of ovarian activity	Follicles	Corpora lutea
Vaginal cytology	Basal cells	Cornified cells	Intermediate cells
Serum progesterone level	< 1 ng/mL	2-15 ng/mL	> 10 ng/mL
(N)	10	7	11

4.3.3 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°C until used for RNA extraction.

4.3.4 Experiment I: Real-time PCR

4.3.4.1 RNA extraction

Cervical tissues were pulverized with a sterile mortar and pestle and total RNA was extracted from the cervical tissue using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) at room temperature following the manufacturer's instructions. The tissue powder were lysed in buffer RLT containing 1% β-Mercaptoethanol (β-ME) and was followed by homogenization using vortex. The lysate was centrifuged for 3 min at full speed and then carefully remove the supernatant by pipetting. The mixture was then transferred to a new microcentrifuge tube and added with 1 volume of 70% ethanol and mixed immediately by pipetting. Thereafter, the 700 µl of sample lysate was transferred to RNeasy spin column and centrifuge at ≥8,000 x g for 15 sec and then discarded the flow-through. Then, the RPE buffer was added to spin column and centrifuged at ≥8,000 x g for 15 sec. After centrifugation, the filtrate was discarded and buffer RPE was added to the re-seated spin cup and centrifuged at ≥8,000 x g for 2 minutes to wash the spin column membrane. The spin column was placed in new 1.5 ml collection tube and added with 30-40 µl of Rnase-free water and centrifuged at ≥8,000 x g for 1 min to elute RNA.

The RNA concentration and purity were accessed using spectrophotometer at 260 and 280 nm (Nanadrop ND-2000, Wilmington, Delaware, USA) where all samples had an acceptable 260/280 ratio of absorbance between 1.8-2.1. The RNA quality was assessed by visualization of 28s and 18s rRNA bands after electrophoresis using 1.5% agarose gel with ethidium bromide staining. After extraction RNA was stored at -80ºC. To ensure the 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˚C for 30 min.

4.3.4.2 Reverse Transcription (RT)

The single-stranded complementary DNA (cDNA) synthesis was performed using the 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 (100µM), 2 µl of 5 mM deoxynucleotide triphosphate (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 °C for 60 min. Selected negative control samples were prepared by including all reagents as above, minus the reverse transcriptase. A mastermix of RT reagent was prepared once to minimize potential variation.

4.3.4.3 Quantitative Real-time PCR

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 the 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 conventional PCR amplification using Platinum PCR supermix containing *Tag* polymerase (Invitrogen; Invitrogen Ltd. UK), 50 ng cDNA and 20 µM primers. 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 gene (EP2, EP4, 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 by conventional PCR amplification and then purified by QIA quick PCR Purification kits in accordance with

guidelines supplied by Qiagen (QIAGEN, Valencia, CA, USA). The concentration of purified PCR product was determined by the NanoDrop ND-1000 spectrophotometer. In the following stage, the optimal annealing temperature and melting curve analysis were assessed using a temperature gradient feature range from 50.0-65.0°C. The melting curve analysis was performed to ensure the absence of non-specific products.

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 Laboratories, 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). To minimize variation, all cDNA samples included in each analysis were derived from the same batch. Meanwhile, eight standards of a 10-fold serial dilution of the purified PCR product of each gene (range from 10 to 1 x 10-6 ng/ml) were diluted with nuclease-free water and run in parallel with unknown samples and the results used to generate a standard curve in each assay. The qPCR reactions for both standards and samples were performed in duplicate in PCR plate. Non Template Control (NTC) containing nuclease-free water was included in every assay. Data from samples which express below quantification cycle (cq) of the NTC were excluded. Thermal cycling conditions applied to each assay consisted of an initial *Taq* activation step at 95°C for 5 min followed by 38 cycles of denaturation at 95°C for 15 sec, annealing temperature as shown in Table 7, extension at 72°C for 20 sec followed by amplicon-specific fluorescence acquisition reading (range 74–84°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 Laboratories, Inc.) and are expressed as fg/µg reverse-transcribed RNA.

Table 7. Description of forward (FP) and reverse (RP) primers used to assess the expression of EP2, EP4, COX-2 and PGES genes in cervical tissues.

Primer	Accession number	Primer sequence	Annealing temperature (˚C)	Product-length (bp)
EP2	NM_001003170.1	F: 5'-TTC TCC TGG CTA TTA TGA CC-3' R: 5'-ATC TAC TGG CGT TTG ACT G-3'	62.5	273
EP4	NM_001003054.1	5'F: -GGT ACG GGT GTT CAT CAA C-3' 5'AGA R: AGA GGA GGG TCT GAG ATG TG-3'	62.5	323
COX-2	NM_001003354.1	5'F: -ACA GGA GAG AAG GAA ATG GC-3' 5'R: -GGA TTG AGG CAG TGT TGA TG-3'	64.1	250
PGES	NM_001122854.1	5'F: -ACC ATC TAC CCC TTC CTG T-3' 5'R: -CTG CTT CCC AGA CGA TCT-3'	62.5	214

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 Laboratories, Inc.) and express as fg/µg reverse-transcribed RNA. Real-time quantification of EP2 and EP4 mRNA transcripts were normalized to the endogenous normalizer (18s rRNA: 18s ribosomal RNA) content. Analysis of relative quantification from the ratios of the mRNA concentrations of the genes of interest to the reference genes were carried out in parallel with the absolute quantification.

4.3.5 Experimental II: Western Immunoblotting

4.3.5.1 Preparation of cervical tissue lysates

Tissue lysates

Frozen cervical tissues were taken from the – 80 °C freezer and ground to a powder with a mortar and pestle placed in liquid nitrogen to prevent tissue from thawing. Powdered cervical tissue was placed into a 1.5 ml microtube and added with lysis buffer (300-500 µl). The sample was then vortexed and centrifuged at 13,000 rpm at 4 °C for 10 min to remove pellet cellular debris. The supernatant was placed into 1.5 ml microtube, and boiled at 100 °C for 5 min in heating block. Two µl of cervical lysate solution was 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 (W/V; Sigma-Aldrich GmbH, Steinheim, Germany) and 0.02 % bromophenol blue (W/V; Sigma-Aldrich GmbH, Steinheim, Germany) for every 100 µl of lysate, and stored at -20 °C. The recipe of lysis buffer used was shown below.

Lysis buffer (1 ml):	(i) Tris-Glycine-sodium dodecyl sulphate buffer (Sigma-Aldrich GmnH, Sreinheim, Germany)	942 µl
	(ii) Sodium orthovanadate (200 mM) (Sigma-Aldrich GmnH, Sreinheim, Germany)	10 µl
	(iii) Protease inhibitor cocktail set 1 (Calbiochem, CN biosciences Inc., Darmstadt, Germany)	10 µl
	(iv) Distilled water	38 µl

4.3.5.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate the proteins in the cervical lysates. A mini gel electrophoresis system (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) was used with Tris/Glycine buffer system which was composed of 4% stacking gel and 12% resolving gel as shown below.

Gel electrophoresis solutions

4% stacking gel solution

(i)	Water	1.72 ml
(ii)	40% Acrylamide (A0008, Bio Basic Inc, Ontario, Canada)	275 µl
(iii)	4X Tris-Cl (pH 6.8)	312.5 µl
(iv)	10% Sodium dodecyl supphate (SDS) (Sigma-Aldrich GmnH, Sreinheim, Germany)	25 µl
(v)	10% Ammonium persulphate (APS) (Sigma-Aldrich GmnH, Sreinheim, Germany)	20 µl
(vi)	TEMED (N,N,N´,N´- Tetramethylethylene-diamine (Sigma®, Poole, Dorset, UK)	6 µl

12% stacking gel solution

(i)	Water	1.9 ml
(ii)	40% Acrylamide (A0008, Bio Basic Inc, Ontario, Canada)	1.55 ml
(iii)	4X Tris-Cl (pH 8.8)	1.25 ml
(iv)	10% Sodium dodecyl supphate (SDS) (Sigma-Aldrich GmnH, Sreinheim, Germany)	50 µl
(v)	10% Ammonium persulphate (APS) (Sigma-Aldrich GmnH, Sreinheim, Germany)	30 µl
(vi)	TEMED (N,N,N´,N´- Tetramethylethylene-diamine (Sigma®, Poole, Dorset, UK)	10 µl

Note that APS was always made fresh on the day the gel was run, and APS and TEMED were added to the solution last (just before pouring off the solution).

The resolving gel was poured into an electrophoresis mini gel set (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) (8 x 10 cm per gel). Isopropanol was added on top of the resolving gel layer until it set. After the gel has set (approximately 30 min) the isopropanol was poured off and then rinsed the gel with

distilled water. The access water was soaked up with thin filter paper. A comb was placed to the top of gel after adding with stacking gel and leave until stacking gel was set (approximately 30 min). The gel plates with clamp holders were placed into a gel cassette. Tank buffer (Tris Glycine buffer) was then poured into the gel cassette and the comb was gently taken off the stacking gel.

Protein extracts (30 µg) were loaded into lane 2-8 (one sample per lane). Lane 1 was loaded with 10 µl of specified molecular weight marker (PageRulerTM Prestained Protein Ladder, Fermantas, Maryland, USA). Lane 9 was loaded with 5 µl of sample buffer as negative controls (sample buffer: 7.25ml distilled water; 1.25ml 0.5 M Tris pH 6.8; 1ml glycerol; 0.01g bromophenol blue; 0.2g SDS; 0.5 ml β-mercaptoethanol). Lane 10 was loaded with a quality control sample as positive control (EP2; JAR cell lysate, EP4; HeLa whole cell lysate, Santa Cruz Biotechnologies, Inc., Santa Cruz, California, USA). Electrophoresis was carried out at 150 V and 400 mA for 60 min.

4.3.5.3 Immunoprobing

Immunoproblotting

Following gel electrophoresis, the stacking gel was cut off. The remaining gel was approximately 5 x 9 cm in size. The gel was soaked in blotting buffer for 15 min (6 g Tris, 28.8g Glycine, 2g SDS; then made up to 1.6L) in 400 mL methanol. A polyvinylidine fluoride (PVDF) membrane (BioTraceTM PVDF transfer membrane, P/N 66543, Pall Corporation, MI, USA) was cut to the same size as the gel, soaked with methanol and then soaked with blotting buffer for 5 min. Four filter papers (Whatman®, Lot number; 1001-125, New Jersey, USA) were cut to the same size as the gel and soaked in blotting buffer. The blotting layers were arranged on the blotter (Trands-Blot, SD semi-dry Electrophoretic transfer Cell, Bio-Rad, Laboratories, Hemel Hempstead, Hertfordshire, UK) as followed: two filter papers, PVDF membrane, gel, and two filter papers on the top of the stack. Air bubbles were removed using a small bread roller pressed onto the blotting sandwich. The blotting layer was the blotted at 100 V and 500 mA for 120 min.

Blocking

Following the blotting, the PVDF membrane was placed in a blocker solution (5% BSA in TBS-T) and incubated at room temperature for 1 h with gentle agitation (200 rpm). (Blocker solution; 5g BSA in 100ml of Tris buffer saline-Tween (TBS-T); (10x stock solution; 24.28g Tris, 87.66g NaCl, 5ml Tween; made up to 1L adjusted to pH 7.6 and diluted from 10x to 1x by adding distilled water). Following blocking, the PVDF membrane was probed with a primary antibody against either EP2 or EP4 or COX-2. The β-actin used as an internal control.

Probing with primary antibody

Goat Polyclonal Antihuman EP2 (sc-22196), Goat Polyclonal Antihuman EP4 (sc-16022), (Santa Cruz Laboratories Inc., 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) were used as the primary antibodies at working dilution of 1:250, 1:250, 1:250 and 1:5,000 respectively, in blocker solution. The PVDF membrane was placed in a plastic bag and 5 ml of primary antibody poured into the bag, any air bubbles were removed before the plastic bag was sealed. This bag was incubated overnight at 4 °C on the shaker set at 200 rpm. After the incubation overnight with primary antibody, the PVDF membrane was then taken off from the plastic bag and washed with TBS-T for 3 x 10 min on a shaker set at 200 rpm.

Probing with secondary antibody

Horseradish Persoxidase (HRP)-Conjugated Horse Anti-Goat Immunoglobulin Polyclonal Antibody (PI-9500, Vector Laboratories, California, USA) and Polyclonal Rabbit Anti-Mouse Immunoglobulin (DAKO, Glostrup, Denmark) were used as the secondary antibodies at a working solution of 1:500 and 1:1,000 in TBS-T respectively. The PVDF membrane was incubated at room temperature in the plastic bag with the secondary antibody for 5 h on a shaker set at 200 rpm. The membrane was washed in TBS-T for 3 x 10 min on a shaker.

Developing the color

Immunoreactive proteins were detected using diaminobenzidine (DAB) (ImmPACTTM DAB Peroxidase Substrate, Vector Laboratories, California, USA). The membrane was soaked with DAB for 5 min until the brown color of protein band developed and then washed with distilled water for 5 min. After leaving the membrane till dry, the membrane was scanned with the HP scanner (HP Scanjet 2400c, HP, USA) to get the image.

Optical density measurement

The optical density of EP2, EP4 and COX-2 bands was measured by using Quantity One Software Program version 4.4.0 (Bio-Rad Laboratories). The specific density of each sample for EP2, EP4 and COX-2 was calculated by subtraction of the background density and divided by the specific density of quality control band.

4.3.6 Statistical analysis

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 expression and protein expression (EP2, EP4, COX-2 and PGES) in different stages of the estrous cycle (anestrus, estrus and diestrus) in Ext I and II. Data were tested for homogeneity of variance using a Levene's test. Post hoc comparisons were performed using Bonferroni correction to test for the significances. All data were expressed as mean ± SEM. A *t*-test was used to analyze the differences of mRNA expression between open-cervix and closed-cervix pyometra groups using SPSS. Values were considered to be statistically significant at $P \leq 0.05$.

4.4 Results

4.4.1 Experimental I: the mRNA expression of EP2, EP4, COX-2, and PGES

The cervical expression of EP2, EP4, COX-2, and PGES mRNA in cyclic bitches: The mRNA for EP2, EP4, 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 Figure. 9. The melting curve analysis showed no primer-dimers or nonspecific products in all assays (Fig. 10 to 13)

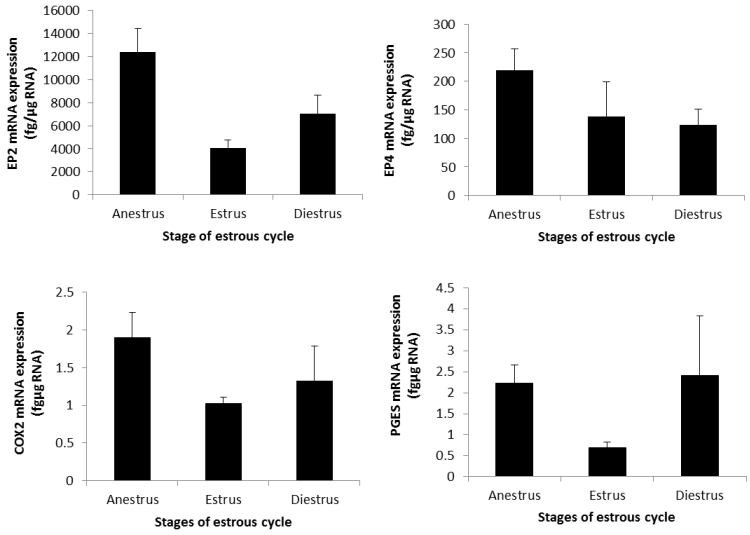


Figure 9. Mean (± S.E.M.) mRNA expression for EP2, EP4, COX2 and PGES in cervix of healthy bitches during various stages of the estrous cycle. Mean values with a different superscript differed significantly ($P<0.05$).

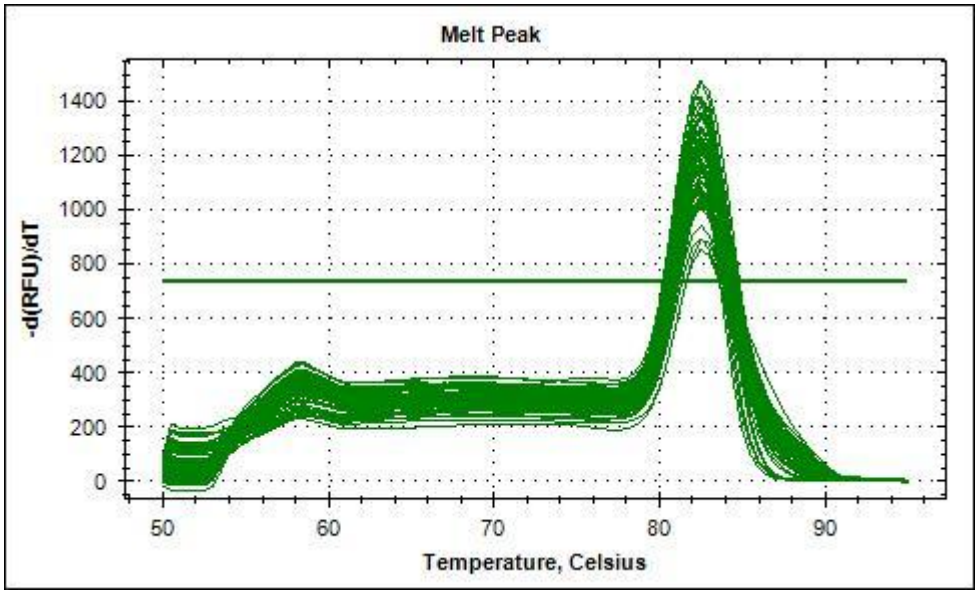


Figure 10. Melting curve analysis of RN18S1 performed at the end of the real-time PCR. There were no significant primer-dimers or nonspecific products present on the curve.

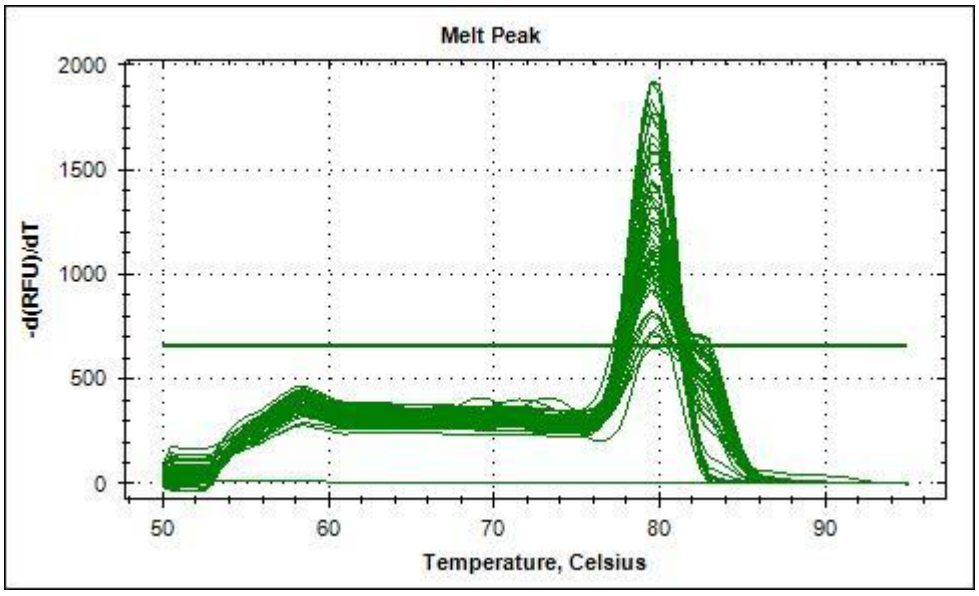


Figure 11. Melting curve analysis of EP2 performed at the end of the real-time PCR. There were no significant primer-dimers or nonspecific products present on the curve.

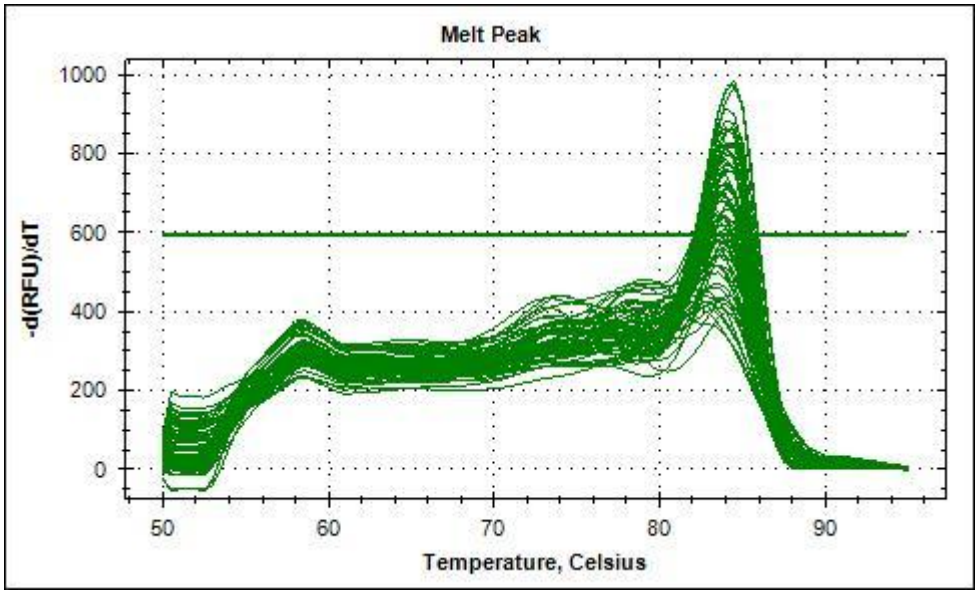


Figure 12. Melting curve analysis of EP4 performed at the end of the real-time PCR. There were no significant primer-dimers or nonspecific products present on the curve.

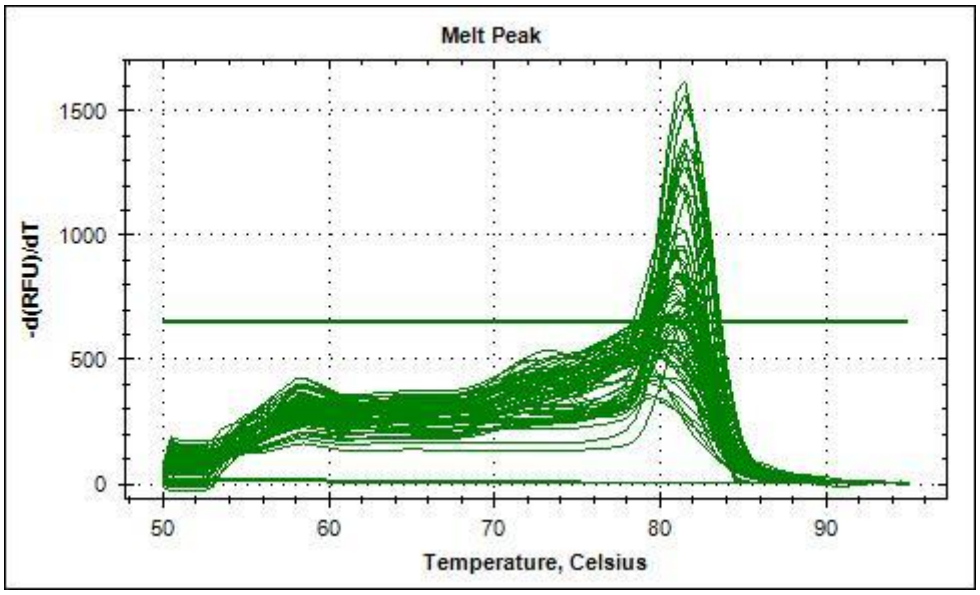


Figure 13. Melting curve analysis of COX-2 performed at the end of the real-time PCR. There were no significant primer-dimers or nonspecific products present on the curve.

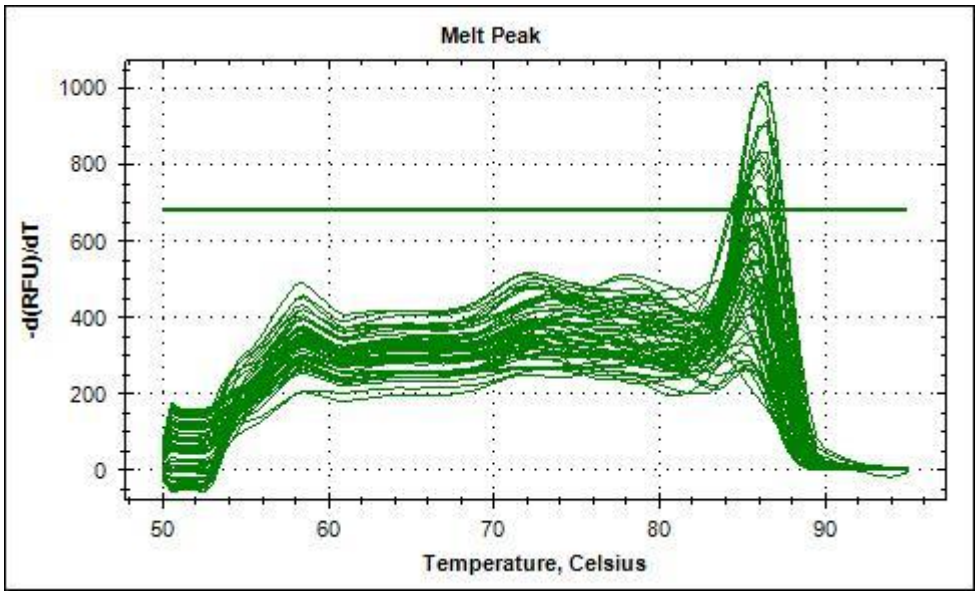


Figure 14. Melting curve analysis of PGES performed at the end of the real-time PCR. There were no significant primer-dimers or nonspecific products present on the curve.

The cervical expression of EP2, EP4, COX-2, and PGES mRNA in bitches with pyometra: 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 EP2, EP4, and COX-2 did not differ between the two groups (Fig. 15).

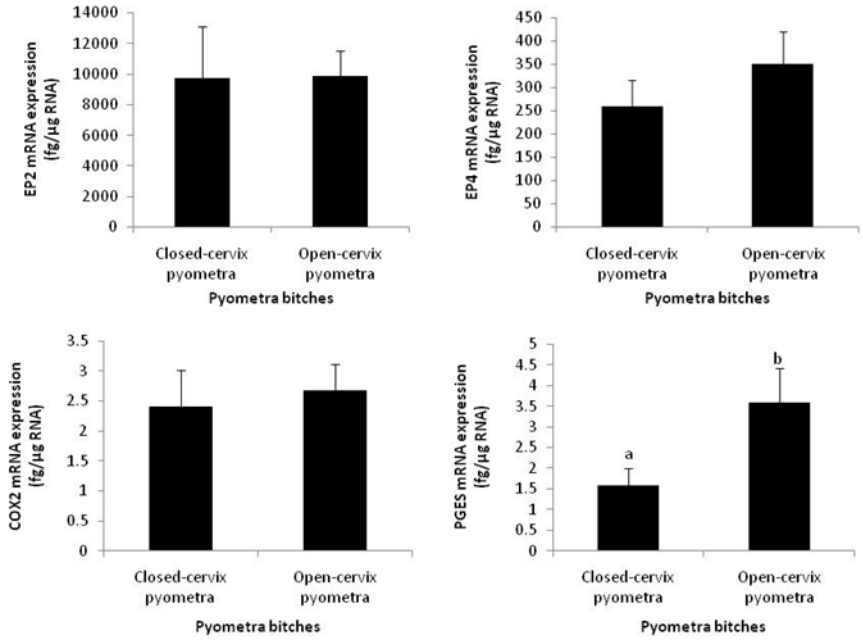


Figure 15. Mean (± S.E.M.) mRNA expression for EP2, EP4, COX2 and PGES in cervix of bitches with pyometra. Mean values with a different common superscript differed significantly (P<0.05).

4.4.2 Experimental II: Protein expression of EP2, EP4, and COX-2

The results from western blot confirmed the protein expression of EP2, EP4, and COX-2 in canine cervical tissue collected from during estrous cycle and from bitches with pyometra. EP2, EP4 and COX-2 protein was expressed in anestrus, estrus and diestrus although the no differences were observed among them (p>0.05) (Figure 16). Similarly, there were no differences in EP2, EP4 and COX-2 protein expression between open-cervix and closed-cervix pyometra (p>0.05) (Figure 17). The protein bands of EP2, EP4, and COX-2 and molecular weight (kDa) were shown in Figure 18.

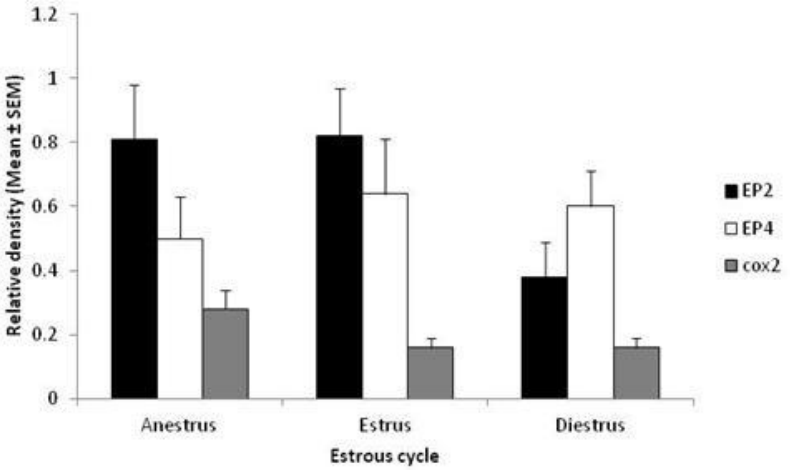


Figure 16. The relative density (Mean ± SEM) of EP2 receptor protein expression of canine cervix at estrous cycle.

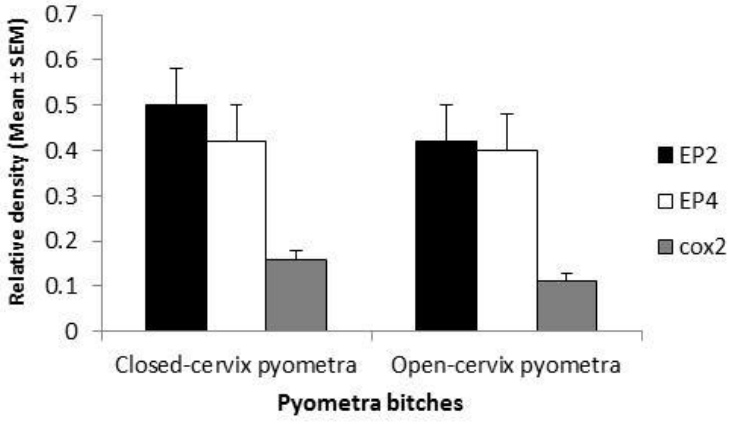


Figure 17. The relative density (Mean ± SEM) of EP4 receptor protein expression of canine cervix in bitches with open-cervix pyometra and closed-cervix pyometra.

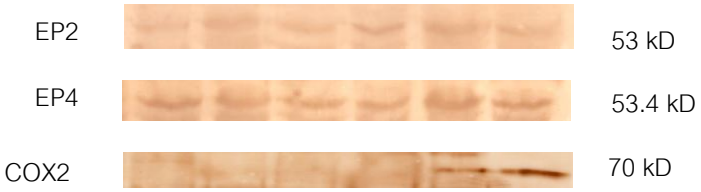


Figure 18. The protein band of EP2, EP4, and COX-2 showed at molecular weight of 54, 53.4 and 70 kD respectively.

4.5 Discussion

In the present study, the cervical mRNA and protein expression of EP2, EP4, COX-2, and PGES (mRNA only) was 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 (Silva et al., 1995; 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 has been 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 did not increase in cervix threated 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 in pre-LH surge was found (Falchi and Scaramuzzi, 2013). One possible explanation is the modulation of COX-2 expression may be multifactorial regulation. Although the differences in EP2 mRNA expression were not statistically significant between the estrous than estrous periods, the considerably lower EP2 mRNA expression during estrus might have resulted from a down regulation by the estradiol during the estrous stage. Similar findings were demonstrated in the

baboon cervix in which EP2 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 EP2 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 EP2 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 EP2 mRNA expression in the myometrium after treatment with progesterone (Dong and Yallampalli, 2000). However, the protein expression of EP2 did not differ among stages of estrous cycle suggesting that EP2 protein expression may not regulated by ovarian steroids. Our results of a lack of differences in EP4 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 EP4 in the bitch cervix in the present study, the immunohistochemical study revealed a higher EP4 expression in the luminal epithelium of the bitch cervix during estrus than anestrus in our previous study (chapter III). Moreover, the protein expression of EP4 in rat cervix increased with advancing gestation and it was highest on the day of parturition suggesting the role of EP4 in cervical relaxation (Chien and Macgregor, 2003). This leads 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 EP4 expression become masked.

It has been known that PGE2 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 (Uldbjerg and Ulmsten, 1990). 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 (Naftolin and Stubblefield, 1980). Although the protein expression of

EP4 did not differ between open- and closed-cervix pyometra in this study, our previous study (chapter III) based on immunolocalization EP4 expression was higher in the open-cervix than closed-cervix. These finding support the notion that EP4 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 did not differ between open- and closed-cervix pyometra while the PGES did, suggesting 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 in the cervix of bitches with pyometra compared to the normal cyclic bitches, indicating an up-regulated gene transcription. This has also been 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 (Schatten and Constantinescu, 2007). MMPs are a family of enzymes that are classified according to substrates such as collagenase, gelatinase, and stromelysin (Birkedal-Hansen et al., 1993) that can degrade components of the extracellular matrix. The expression of IL-8 in the cervix is stimulated by PGE2 (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 lead to cervical relaxation. From our results, we propose that the increase of PGES mRNA expression is stimulated by proinflammatory cytokines, resulting in the enhanced production of PGE2 that

subsequently stimulates neutrophil infiltration through the IL-8 and released 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 EP2 and EP4 mRNA. Moreover, the biosynthesis of PGE2 by COX-2 and PGES was not influenced by the stages of the estrous cycle. In addition, PGES seems to control the production of PGE2 to regulate cervical relaxation in the bitches with open-cervix pyometra.

CHAPTER V
GENERAL DISCUSSION

Cervical relaxation during estrus and parturition in many species is thought to be involved in changes in extracellular matrix, PGE2 and inflammatory process (Liggins et al., 1977; Stys et al., 1981; Chien and Macgregor, 2003; Nanda et al., 2007; Cubas et al., 2010). Nonetheless, the mechanism of cervical relaxation in cyclic and pyometra bitch is poorly understood. The present study revealed the changes in collagen to smooth muscle ratio and GAGs at estrus in the normal cyclic bitch and also in bitches with open-cervix pyometra. To gain more understanding about the mechanism of cervical relaxation, the expression of PGE2 receptors (EP2 and EP4) and enzymes involved in PGE2 synthesis (COX-2 and PGES) were investigated.

The extracellular matrix is a molecular nest containing collagen, glycoproteins, proteoglycans and GAGs (Cubas et al., 2010). It maintains structure of the organ. Moreover, the collagen degradation implies a change in the strength of extracellular matrix and is characterized by a decrease in cross-links between collagen fibers (Bank et al., 1997) and increase the interfibrillary distance in collagen fibrils (Feltovich et al. 2005). In chapter II, our results showed that the proportion of the area occupied by collagen was higher at estrus compared to the other stages of the estrous cycle which may be caused by an increase of interfibrillary distance of collagen. This is supported by the study in sheep cervix which showed the greater area of collagen prior to preovulatory LH surge (Kershaw et al., 2007). The current study also revealed that cervical tissue at estrus had the highest hyaluronan content compared to the other stages of the estrous cycle (chapter II). Hyaluronan has hydrophilic properties which cause tissue edema that result in dissociation of collagen fibers and decreased extensibility of cervical tissue, all of which assist cervical ripening/relaxation (Golichowski et al., 1980; Uldbjerg et al., 1983). Furthermore, hyaluronan synthesis is regulated by estradiol through PGE2 (Umscheid et al., 1998). On the other hand, sulfated GAGs did not seem to play a role in cervical relaxation in the bitch during the

estrous cycle in this study since there were no differences in sulfated GAGs among different stages of estrous cycle.

PGE2 has been shown to induce cervical relaxation by the activation through its receptors (EP2 and EP4) (Feltovich et al., 2005; Kershaw-Young et al., 2009). The study in sheep cervix revealed that mRNA expression of EP4 was higher in sheep cervix treated with estradiol (Kershaw-Young et al., 2010) supporting the role of estradiol in EP4 expression. Although the mRNA and protein expression of EP4 using western blot analysis in the current study did not differ among the stages of the estrous cycle (chapter IV), the immunolocalization revealed a significantly higher expression of EP4 in the luminal epithelium during estrus (chapter III). This suggests that EP4 in the luminal epithelium may play an important role in canine cervical relaxation during estrus which is modulated by estradiol. The observed lack of difference in EP4 expression on the basis of results obtained through western blot may reflect the fact that luminal epithelium is only a small part of the cervical wall where it was mainly localized when studied by immunohistochemistry. The contribution of PGE2 to collagen degradation and cervical relaxation is less well understood, although it does seem to play a key role in cervical relaxation, as demonstrated by the activation of PGE2 (Feltovich et al., 2005). Biosynthesis of PGE2 is regulated mainly by two enzymes, cyclooxygenase (COX) which converts arachidonic acid to prostaglandin H2 (PGH2), and prostaglandin E synthase (PGES) which converts PGH2 to PGE2. There are two forms of COX; COX-1 and COX-2. COX-1 is a constitutive, whereas COX-2 is specific to inflammatory processes (Tamada et al., 2012) and can be regulated by hormonal changes (Kowalewski et al., 2006). The mRNA and protein expression by western blot of COX-2 and PGES did not differ among stages of the estrous cycle implied that COX-2 and PGES expression might not be regulated by ovarian hormones or alternatively like EP4, COX-2 and PGES are also expressed mainly in epithelium which is only a small part of the cervical wall. From our results, the higher area of collagen to smooth muscle ratio, hyaluronan content and greater expression of EP4 in luminal epithelium at estrus compared to anestrus and diestrus, may suggest that the high estradiol concentrations during estrus provoked

collagen degradation by increasing PGE2 which coupling to EP4 that increased hyaluronan to initiate cervical relaxation in bitch at estrus.

In the pyometra bitches, previous studies in our laboratory demonstrated the higher number of neutrophils in open-cervix than closed-cervix pyometra (Kunkitti et al., 2011) suggesting the association between inflammatory reaction and cervical patency in uterine disease like pyometra. Moreover, the positive correlation among number of neutrophils in cervical stroma, the mRNA expression of proinflammatory cytokine such as IL-8 and cervical patency of bitches with pyometra have been reported (Tamada et al., 2012). Matrix metalloproteinase (MMPs) such as MMP-1 and MMP-8 have been shown to degrade collagen which is produced by fibroblasts, endothelial cells, and neutrophils (Tamada et al., 2012). Together with our results, the observed increase in the area occupied by collagen in the open-cervix (chapter II) suggested that cervical relaxation in the pyometra bitches was associated with collagen degradation induced by MMPs which might have been released from neutrophils. Furthermore, the sulfated GAGs form cross-links between collagen fibers and strengthens collagen bundles (Obrink, 1973; Kershaw-Young et al., 2009), and a reduction in sulfated GAGs as observed in our study could weaken the cross-links between collagen fibers (Van Kuppevelt et al., 1987). These observations support our findings that there were significantly lower concentrations of the highly sulfated GAGs (KS and HS) in the cervical stroma of bitches with open-cervix pyometra (chapter II). To our knowledge, the association between PGE2 and inflammatory reactions in the cervix of bitches with pyometra has not been investigated. Our results showed that differences in EP2, EP4, and COX-2 mRNA expression between open- and closed-cervix pyometra were not observed except the mRNA expression of PGES which had higher expression in the open-cervix than in closed-cervix pyometra (chapter IV). Although the differences of protein expression in EP2, EP4, and COX-2 were not observed between two groups of pyometra by western blot (chapter IV) the protein expression by immunohistochemistry showed the higher expression of EP4 in the luminal epithelium in open-cervix pyometra than closed-cervix pyometra (chapter III). Therefore, PGE2 might modulate the cervical

relaxation in the pyometra bitches via EP4 in the luminal epithelium. In addition, PGE2 and IL-1β increase IL-8 production in cervical tissue (Ito et al., 1994; Denison et al., 1999) and IL-8 also activates the degranulation of neutrophils (Tamada et al., 2012). Taken together, we propose that the activation of PGE2 via EP4 stimulate collagen degradation (increase in area occupied by collage)n and decrease in sulfate-GAGs, and therefore, causing cervical relaxation.

Future directions

Although, the mRNA and protein expression results from this study demonstrated that COX-2, and PGES did not differ among stages of estrous cycle and between pyometra groups, it may not necessary mean that COX-2, and PGES are not involved in cervical relaxation. In Moreover, higher expression of COX-2 and PGES have been reported in the endometrium of pyometra bitches (Silva et al., 2009). Immunolocalization of COX-2 and PGES may provide better understanding of whether there are any differences in the proteins expression among layers of cervix during stages of estrous cycle and in pyometra bitches.

From our results, we can suggest that PGE2 plays a role in cervical relaxation in normal cyclic and pyometra bitches. The concentrations of PGE2 in serum and diameter of cervical canal can be measured in order to evaluate the relationship between cervical patency and PGE2 concentrations. In pyometra bitches, the medical treatment that normally used is PGF that can induce the release of pus from the uterus. However, side effects such as vomiting, diarrhea, and panting are observed. Thus PGE2 might be an alternative choice for medical treatment in pyometra bitches because its intravaginal application may reduce the side effects observed for PGF. In fact, misoprostal (prostaglandin E1 analogue) is used to dilate cervix and induce labor in women near the term (Justus Hofmeyr, 2003). Nevertheless, there are reports of misoprostal used in pyometra bitches to induce cervical relaxation (Verstegen et al., 2008) but without scientific proof of its efficacy.

Conclusions

The study of extracellular matrix, prostaglandin E receptor expression (EP2 and EP4), and enzymes involved in PGE2 synthesis (COX-2 and PGES) in cervix of normal cyclic bitches and bitches with pyometra is the beginning of the understanding of factors associated with cervical patency in normal cyclic bitches and in pathological conditions like pyometra. This is the first report that revealed the possible mechanism of cervical relaxation in cervix of normal cyclic and pyometra bitches. The similar findings observed at estrus and in open-cervix pyometra in this study were the increase in area occupied by collagen and the higher expression of EP4 in luminal epithelium indicating that PGE2 might acts through EP4 to degrade collagen leading to cervical relaxation. We propose that the activation of collagen degradation in physiological (estrus) and pathological (pyometra) conditions may be achieved through different mechanisms. The collagen degradation at estrus seems to be mediated by ovarian hormones that change throughout the estrous cycle. On the other hand, inflammatory process seems to play a major role in collagen degradation in pyometra bitches.

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Appendix

List of publication and conferences

1. Linharattanaruksa, P., Ponglowhapan, S., Muhammad, K., Srisuwatanasagul. S., and Chatdarong, K., 2013. Collagen and glycosaminoglycan profiles in the canine cervix at different stages of the estrous cycle and in open- and closed-cervix pyometra, Manuscript submitted.
2. Linharattanaruksa, P., Swangchan-uthai, T., Muhammad, K., Srisuwatanasagul. S., and Chatdarong, K., 2013. The mRNA expression 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, Manuscript submitted.
3. Linharattanaruksa, P., Chatdarong, K., Ponglowhapan, S., Muhammad, K. and Srisuwatanasagul. 2013. Immunolocalization of prostaglandin E2 receptor subtype 4 (EP_4) in cervix of cyclic bitches and those with pyometra. Pak Vet J: In Press.
4. Linharattanaruksa, P., Pitsillides, A., Muhammad, K., Srisuwatanasagul, S. and Chatdarong, K. 2011. Comparison of glycosaminoglycans (GAGs) in cervices of bitches with open- and closed-cervix pyometra. **World Congress of Reproduction Biology 2011 (WCRB)**. Cairns, Australia, 9^{th}-12^{th} October 2011, p112.
5. Linharattanaruksa, P., Pitsillides, A., Muhammad, K., Srisuwatanasagul, S. and Chatdarong, K. 2011. Comparison of glycosaminoglycans (GAGs) in cervices of bitches during estrous cycle. **The 1^{st} Symphosium of The Thai Society of Animal Reproduction**, Bangkok, Thailand, 29^{th}-30^{th} September 2011, p20.
6. Linharattanaruksa, P., Chatdarong, K., Sirivaidyapong, S. and Srisuwatanasagul, S. 2011. The protein expression of prostaglandin E2 receptor subtype 4 (EP4) in canine cervix in anestrus and estrous stage of cycle. **The 10^{th} Chulalongkorn**

University Veterinary Annual Conference, Bangkok, Thailand, 21st - 22nd April 2011, p36.

7. Linharattanaruksa, P., Chatdarong, K., and Srisuwatanasagul, S. 2011. The protein expression of prostaglandin E2 receptor subtype 4 (EP4) in canine cervix during anestrus and estrus. **13th Royal Golden Jubilee congress**, Bangkok, Thailand, 20th April 2011, p20.
8. Linharattanaruksa, P., Srisuwatanasagul, S., Ponglowhapan, S., Sirivaidyapong, S. and Chatdarong, K. 2010. Prostaglandin E2 receptor subtype 4 (EP4) in canine cervical tissue of bitches with pyometra. **7th EVSSAR (The European Veterinary Society for Small Animal Reproduction)**, Louvain-La-Neuve, Belgium, 14th -15th May 2010, p75.
9. Linharattanaruksa, P., Chatdarong, K., Sirivaidyapong, S. and Srisuwatanasagul, S. 2010. The proportion of smooth muscle and collagen in the cervical tissue in bitches during various stages of estrus and bitches developing pyometra. **The 9th Chulalongkorn University Veterinary annual conference**, Bangkok, Thailand, 1st April 2010, p129.
10. Linharattanaruksa, P., Chatdarong, K., Sirivaidyapong, S. and Srisuwatanasagul, S. 2010. Localization of prostaglandin E2 receptor subtype 4 (EP4) in the canine cervical tissue during estrous cycle. **11th Royal Golden Jubilee congress**, Bangkok, Thailand, 24th February 2010, p39.
11. Linharattanaruksa, P., Chatdarong, K., Sirivaidyapong, S. and Srisuwatanasagul, S. 2009. Localization of prostaglandin E2 receptor subtype 4 in the bitches developing pyometra. **4th Asian society of veterinary pathologists**, Bangkok, Thailand, 20th Novermber, 2009.

VITAE

Ms. Pichanun Linharattanaruksa was born on October 22nd 1983 in Bangkok province, Thailand. She graduated with Degree of Doctor of Veterinary Medicine (DVM) with the 2nd honour from Faculty of Veterinary Medicine, Kasetsart University, in 2007. In 2008, she received the scholarship from the Royal Golden Jubilee PhD program of Thailand Research Fund to perform a PhD program of Theriogenology at Department of Obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Her focus research is about the mechanism of cervical relaxation related to prostaglandin E2 in bitches during stages of estrous cycle and also bitches with pyometra, which aims to find out the association of extracellular matrix, PGE2, and cervical relaxation.