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RESEARCH ARTICLE

Immunolocalization of Prostaglandin E2 Receptor Subtype 4 (EP_4) in the Cervix of Cyclic Bitches and Those with Pyometra

P Linharattanaruksa[1], K Chatdarong[1], S Ponglowhapan[1], M Khalid[3] and S Srisuwatanasagul[2]*

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

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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 E_2 (PGE_2) is considered to be involved in changes of extracellular matrix via coupling to prostaglandin E receptor subtype 4 (EP_4). This study investigated the expression of EP_4 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 EP_4 expression was observed at all the layers and the stages but the differences in EP_4 expression either among bitches in different stages of the estrous cycle and between open- and closed-cervix pyometra were limited to only surface epithelium (SE). In cyclic bitches during estrus and in open-cervix pyometra bitches, significantly higher ($P<0.05$) EP_4 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 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, EP_4 may be involved in stimulating dilation of the cervix in both estrus and open-cervix pyometra bitches.

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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 where 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 either during normal reproductive cycle or in pathological conditions such as pyometra is rare. As the bitch cervical canal is narrow and short, and is located between the uterus and the vagina; 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 was characterized by a simple columnar epithelium so it was called the uterine part, whereas the caudal part of the cervix was characterized by a stratified squamous epithelium and it was so-called the vaginal part (Khunkiti *et al*., 2011).

There are a 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 of collagen fibers, glycoproteins, proteoglycans, and glycosaminoglycans (Kjaer, 2004). Many studies support the concept that cervical patency in estrus is likely mediated by changes in the extracellular matrix (Kershaw-Young *et al.*, 2009; Cubas *et al.*, 2010) and prostaglandin E2 subtype 4 receptor (EP_4) 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 EP_4 mRNA expression which contributed to subsequent cervical relaxation (Kershaw-Young *et al.*, 2010). This leads to a suggestion that the circulating estrogens, cervical EP_4 content and ECM remodelling coordinately promote cervical dilation. Furthermore, the activation of EP_4 resulted in neutrophil infiltration and protease release that caused degradation of collagen, a component of cervical ECM, has been documented (Rath *et al.*, 1993). This study aimed at investigating the expression of EP_4 in the canine cervical tissues and determining if there were any variations in their expression among different reproductive cycles (anestrus, estrus, and diestrus) and between open- and closed-cervix pyometra.

MATERIALS AND METHODS

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 in ovaries and cornified cells of vaginal cytology were defined as estrus. Diestrus was characterized by the presence of corpora lutea in 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.

Hormonal analysis: 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.

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 immuno-histochemical evaluation.

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 EP_4 (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

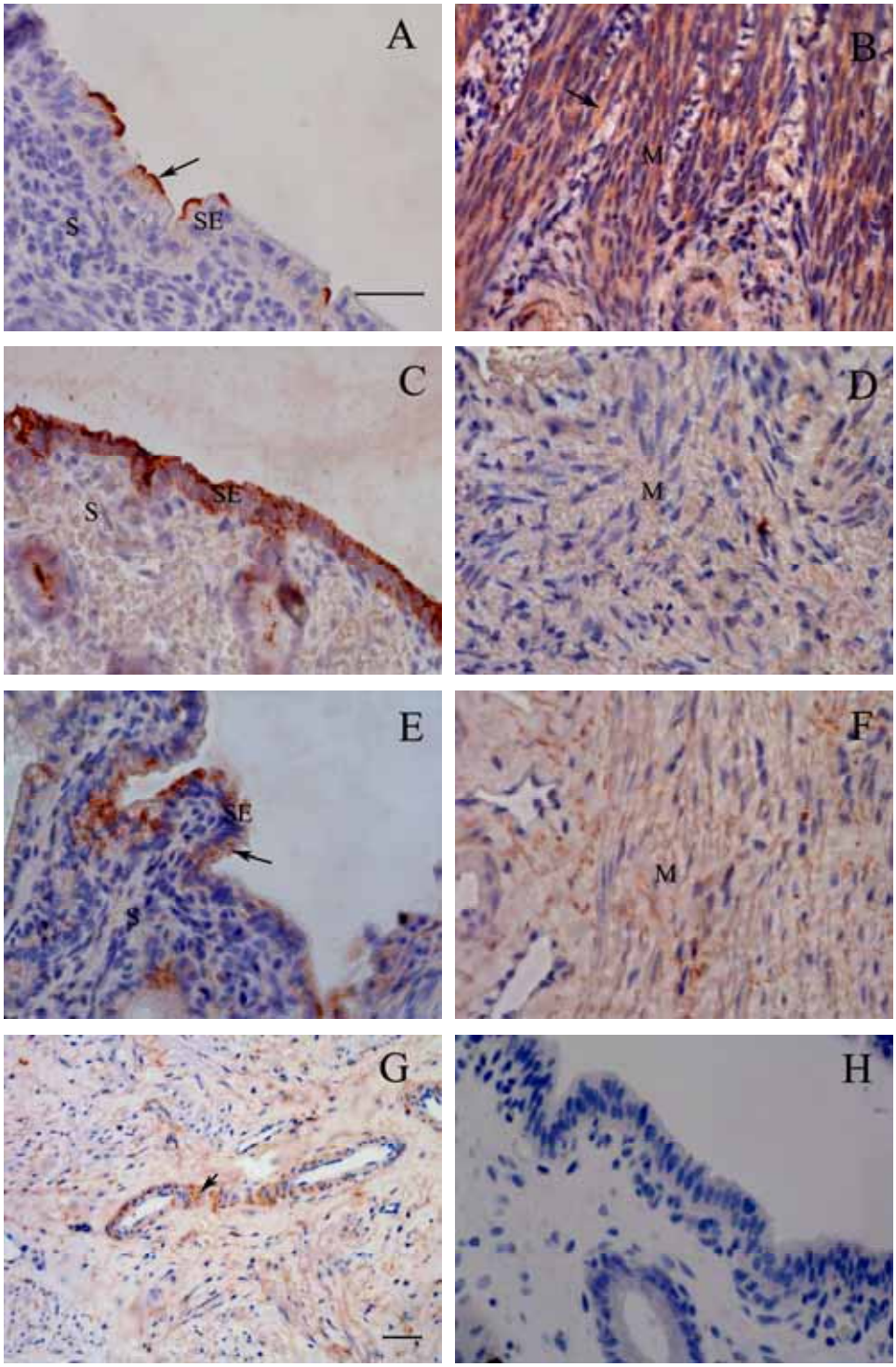

Fig. 1: EP_4 localization (red staining) in the uterine region of the cervix of healthy bitches. Positive staining (arrow) was observed in surface epithelium (SE) and stroma (S) in anestrus (A-B), estrus (C-D), and diestrus (E-F) stage of the estrous cycle. The expression of EP_4 is shown in cervical vessel (arrow; G) of muscular layer in cervix. H; Negative controls. Intense positive staining in the surface epithelium (arrow in A), moderate staining in the muscle (arrow in B) and weak staining in surface epithelium (arrow in E) can be noted (original magnification: 400x). Bars in figures A and G represent 20 μm.

Table 1: Expression index (mean ± S.E.M.) of EP_4 in different tissue layers of the uterine and vaginal regions of the canine cervix during different stages of the reproductive cycle

	Surface epithelium		Stroma		Muscular layer	
	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 in a row without a common subscripts differed ($P<0.05$).

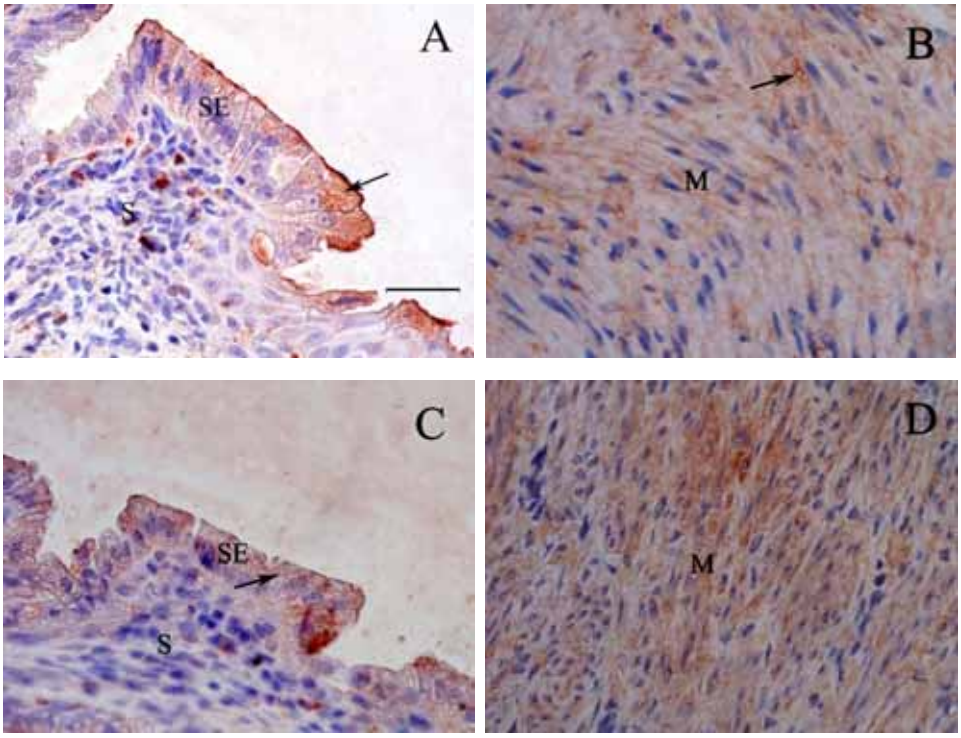

Fig. 2: EP_4 localization (red staining) in the uterine part of the cervix of bitches with pyometra (A-D). Positive staining was observed in surface epithelium (SE), stroma (S), and muscle layer (M) in open-cervix (A-B) and closed-cervix pyometra (C-D). Intense positive staining in the surface epithelium (arrow in A), moderate staining in the muscle (arrow in B) and weak staining in surface epithelium (arrow in C) can be noted (original magnification: 400x). Bars in figure A represents 20 μm.

Table 2: Expression index (mean ± S.E.M.) of EP_4 in different tissue layers of the uterine and vaginal regions of the cervix in open- and closed-cervix pyometra bitches

	Surface epithelium		Stroma		Muscular layer	
	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 in a row without a common subscripts differed (P<0.05).

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 EP_4 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 at each occasion of the immunohistochemical procedure. Evaluation of immunohistochemical staining was done in 3 different tissue layers, i.e. surface epithelium (SE), stroma (S), and muscular layer (M). Ten microscopic areas which corresponded to 0.0845 mm^2 of real tissue area with 400 × magnification were randomly chosen from each tissue layer. Expression of EP_4 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 EP_4 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).

Statistical analysis: Statistical analyses were performed using the Statistical Analysis System version 9.0 (SAS, Institute, 2002, Cary, NC). Data on EP_4 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 (surface 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 EP_4 between open- and closed-cervix pyometra groups were compared by student *t*-test. The level of significance was set at $P<0.05$.

RESULTS

Expression of EP_4 was observed in all the tissue layers (surface 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 EP_4

immunostaining and the control are shown in Fig. 1. Negative controls did not exhibit any EP_4 expression (Fig. 1 H). In addition, EP_4 was localized in the muscle layer of blood vessels (Fig. 1G).

EP_4 expression in the cervix of cyclic bitches: Significant differences ($P<0.05$) in the expression of EP_4 among reproductive cycles were found in only the surface epithelium. No differences were observed in the EP_4 expression in stroma and muscle layers either between the two cervical parts or among the different stages of the estrous cycle (Table1). In the surface epithelium of the uterine region, EP_4 expression differed significantly ($P<0.05$) among the stages of reproductive cycle; the lowest expression was observed in anestrus and highest expression in estrus ($P<0.05$; Table1). In the surface epithelium of vaginal region, EP_4 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 (Table1). Comparisons between the 2 regions of the cervix (uterine vs vaginal) showed that EP_4 expression was highly expressed ($P<0.05$) in the surface epithelium of the uterine region only in the estrous stage of reproductive cycle (Table1).

EP_4 expression in the cervix of pyometra bitches: Similar to cyclic bitches, significant differences in the expression of EP_4 between open and closed-cervix groups were found in only the surface epithelium. The expression in stroma and muscle layers did not differ significantly between uterine versus vaginal regions as well as between open- versus closed-cervix pyometra. In the uterine region, EP_4 expression in the surface epithelium of open-cervix group was significantly higher ($P<0.05$) than closed-cervix group (Table 2). Moreover, the EP_4 expression was significantly higher ($P<0.05$) in the surface epithelium of the uterine than the vaginal region of bitches with open-cervix pyometra (Table 2).

DISCUSSION

The results obtained have shown that expression of EP_4 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 EP_4 expression between the 2 regions of the cervix (uterine versus vaginal) and between different stages of reproductive cycle (anestrus, diestrus and estrus) were present in the surface epithelium and not in other tissue layers of the cervix examined. These findings suggested a possible role of EP_4 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 EP_4 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 PGE_2 in vasodilation in canine cervix via EP_4 with subsequent edema and leukocytic infiltration (Schmitz *et al.*, 2006).

In this study, higher expression of EP_4 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 EP_4 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 EP_4 expression in the surface 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 PGE_2 (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, PGE_2 secreted from the epithelial cells can regulate the muscle contraction or relaxation, depending on the type of receptor expressed. There are 4 subtypes of PGE_2 receptor which are EP_1, EP_2, EP_3 and EP_4; the EP_1 and EP_3 cause muscle contraction, while EP_2 and EP_4 are involved in muscle relaxation. In this study, we were interested in the expression of EP_4 in the canine cervix because the study in rat cervix demonstrated that EP_4 expressed near term parturition (Feltovich *et al.*, 2005). This suggests that PGE_2 plays a role in cervical dilation through EP_4 receptor. Significantly higher EP_4 expression in the surface epithelium at the periods of cervix opening, e.g. estrous stage of the reproductive cycle and open-cervix pyometra suggests the involvement of surface epithelium EP_4 in the underlying mechanism of cervical relaxation in the bitch. Moreover, the EP_4 expression in the surface epithelium and the synthesis of PGE_2 by cervical epithelium (Shemesh *et al.*, 1997) implies that epithelial cells not only secrete PGE_2 as triggered by some stimuli but also present EP_4 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.*, 1989). The higher expression of EP_4 during estrus observed in this study may be associated with high estrogen levels in the circulation. Such effects of estrogen on EP_4 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 EP_4 as demonstrated in the rat cervical tissue treated with progesterone (Hinton *et al.*, 2010). Similarly, in this study lower EP_4 expression in surface 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

findings that suppression of progesterone by the antiprogestin (RU-486) induces an increase in the cervical EP_4 expression and cervical relaxation in the rat (Hinton *et al.*, 2010).

In pyometra bitches, greater EP_4 expression in the surface epithelium of uterine regions observed in bitches developing open-cervix indicated that EP_4 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 EP_4 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 PGE_2 (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 EP_4 expression remains to be elucidated.

Conclusion: EP_4 is present in the canine cervix with higher expression being found in the surface epithelium of the uterine compared to vaginal region in estrous bitches and in bitches with open-cervix pyomtra. The results lead to a suggestion that EP_4 plays a role in regulating the canine cervical patency. Higher expression of EP_4 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.

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