NOTE *Theriogenology*

Positive correlation between patency and mRNA levels for cyclooxygenase-2 and prostaglandin E synthase in the uterine cervix of bitches with pyometra

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ABSTRACT. Factors involved in patency of uterine cervices in the bitch with pyometra remain to be clarified. This study examined relationship between patency and mRNA levels for inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-1, COX-2 and prostaglandin E synthase (PGES) in the uterine cervix of bitches with pyometra. Cervical patency was measured by inserting the stainless steel rods with different diameter into cervical canals. Levels of mRNA expression were determined by semi-quantitative reverse transcription-polymerase chain reaction. The cervical patency was positively correlated with mRNA levels for COX-2 and PGES, but not those for iNOS and COX-1. The results suggest that gene expression of COX-2 and PGES may be involved in the regulation of patency in the uterine cervix of bitches with pyometra.

KEY WORDS: canine, iNOS, PGE, pyometra, uterine cervix

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In canine pyometra, patent uterine cervices are generally associated with vulvar discharge, whereas impatent ones more commonly cause uterine distension by accumulation of the pus, resulting in severe clinical signs [5, 11]. Although cervical patency affects not only clinical signs but also choice of medical therapy [13], the mechanism how patency of the uterine cervix is regulated remains to be elucidated. Recently, we have reported that increased number of neutrophils, which could be attracted by the local expression of interleukin (IL)-8, may cause collagen degradation and connective tissue remodeling to increase cervical patency in the uterine cervix of bitches with pyometra in a similar way to ripening cascade of uterine cervix during parturition [15]. On the other hand, a number of studies have shown that not only IL-8 but also nitric oxide (NO) and prostaglandin (PG) E_2, are involved in ripening of the uterine cervix [1–4, 10, 14, 16, 17].

Evidence for involvement of the enzymes producing NO and PGE in regulation of cervical patency has also been reported [9, 10, 14, 17]. Cervical expression of inducible NO synthase (iNOS), which is stimulated by inflammatory events, increases in association with the ripening in women [17] and rats [10]. Among the enzymes relating to PG production, cyclooxygenase (COX) catalyzes a key step in the conversion of arachidonate to PGH_2, the immediate substrate for a series of cell specific PG, and PGE synthase (PGES) converts PGH_2 to PGE. Expression of COX-1 is constitutive, and that of COX-2 is inducible and involved in pro-inflammatory stimuli [8]. Expression of mRNA for COX-2 and intensity of stromal immunostaining for COX-1 as well as COX-2 in the uterine cervix increase around parturition in humans [14]. Recently, Linharattanaruksa *et al*. [9] showed that mRNA level of PGES but not of COX-2 in the uterine cervix of the pyometra-infected dog with vulvar discharge is higher than in that without the discharge, and suggested involvement of PGES in patency of the uterine cervix. However, presence or absence of vulvar discharge could be determined by not only cervical patency but also intrauterine pressure which is dependent upon amounts of the pus and uterine capacity and contractility. Therefore, accurate analysis of the cervical patency requires its direct measurement. This study examined relationship between degree of the patency and mRNA levels of inducible NO synthase (iNOS) as well as PG-related enzymes, COX-1, COX-2 and PGES in the uterine cervix of bitches with pyometra.

Table 1 shows information of the dogs with pyometra whose tissue samples were excised by ovariohysterectomy. The samples were kindly provided by the dog owners at our Veterinary Teaching Hospital and the other animal hospitals. Some samples, which are shown by the dog number with an asterisk in Table 1, were the same as used in the previous report [15]. Uterine cervices were cut, and immediately, the stainless steel rods with a diameter of 1.5, 3.0, 4.0 or 5.5 mm were inserted into the canal. The diameter of the thickest stainless steel rod that passed through the canal was divided by the diameter of respective uterine cervix, and obtained value was defined as patency. Mean (± SE) value of patency in the bitch with open-cervix pyometra (n=16) was 0.33 (± 0.02), and that in the bitch with closed-cervix pyometra

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Table 1. Breed, type and patency of the uterine cervix, age and body weight in 24 bitches with pyometra

Bitch no.	Breed	Cervix type[a)]	Patency	Age (years)	Body weight (kg)
1*	Mongrel	Closed	0.11	8.9	12.7
2*	Mongrel	Closed	0.13	10.0	6.3
3	Golden retriever	Closed	0.17	6.0	33.1
4	Papillion	Closed	0.19	10.0	3.3
5	Miniature Schnauzer	Open	0.19	6.3	8.7
6	Shetland sheep dog	Open	0.20	10.0	14.9
7*	Maltese dog	Closed	0.21	7.5	3.2
8*	Yorkshire terrier	Open	0.21	13.9	2.6
9*	Yorkshire terrier	Closed	0.25	7.0	3.5
10	Shih Tzu	Closed	0.25	7.0	5.7
11	Collie	Open	0.26	10.0	27.9
12*	Shetland sheep dog	Open	0.27	11.0	19.0
13	Labrador retriever	Open	0.29	7.3	32.9
14	Shetland sheep dog	Open	0.30	9.8	10.3
15*	Shiba inu dog	Closed	0.32	2.8	15.0
16	Golden retriever	Open	0.32	ND	ND
17*	Toy poodle	Open	0.33	12.0	ND
18	Shih Tzu	Open	0.34	8.6	8.7
19	Shih Tzu	Open	0.39	8.7	6.5
20	Chihuahua	Open	0.41	8.2	4.1
21	Miniature dachshund	Open	0.42	7.5	4.4
22*	Mongrel	Open	0.42	13.0	15.6
23*	Pomeranian	Open	0.43	13.0	3.7
24*	Siberian husky	Open	0.53	9.0	14.9

ND: Not determined. a) The type was classified by the presence or absence of vulvar discharge. *The same samples were used in our previous report (Tamada *et al*., 2012).

Table 2. Oligonucleotide sequences of the primer pairs used for the RT-PCR, the size of the product and the reference based on the construction of the primers

Primer	Sequence	Size (bp)	Reference
iNOS		661	AF077821 (dog)
sense	5′- AGAAACAACAGGAACCTACCA		
antisense	5′- CTCCAGGATGTTGTAGCGC		
COX-1		356	AF535138 (dog)
sense	5′- CACCCGCTCATGCCAGACTCC		
antisense	5′- CCCGGGTAGAATTCCAAGGCATCA		
COX-2		500	AY044905 (dog)
sense	5′- TGAGCGGTTATTCCAGACGAGCAG		
antisense	5′- CCAACCCCGCAGCCATTTCCTTCT		
PGES		213	AY057096 (horse)
sense	5′- CACCGGAACGACATGGAGACCATC		AY032727 (cattle)
antisense	5′- CAGAGCCATGGAGGCGCAGGGGAG		BC008280 (human)
18S rRNA		96	Hatoya *et al*. [6]
sense	5′- TGGTTGATCCTGCCAGTAGCA		
antisense	5′- ATGAGCCATTCGCAGTTTCACT		

(n=8) was 0.20 (± 0.02). There was significant difference between the two groups (P<0.003, Student's t test). These results suggest that closed-cervix pyometra and open-cervix one associate with lower and higher patency, respectively, although some bitches with closed-cervix pyometra showed higher values of patency than in those with open-cervix pyometra.

The uterine cervix was frozen in liquid nitrogen and stored at −80°C. Based on the previous report [15], extraction of mRNA and reverse-transcription (RT) polymerase-chain reaction (PCR) for iNOS, COX-1, COX-2 and PGES were performed using 18S ribosomal RNA (rRNA) as an internal standard. Table 2 shows sequences of the primer pairs used and related information for PCR products. PCR was performed with 200 nM (iNOS, COX-1, COX-2 and PGES) or 20 nM (18S rRNA) primer pairs. The PCR conditions for

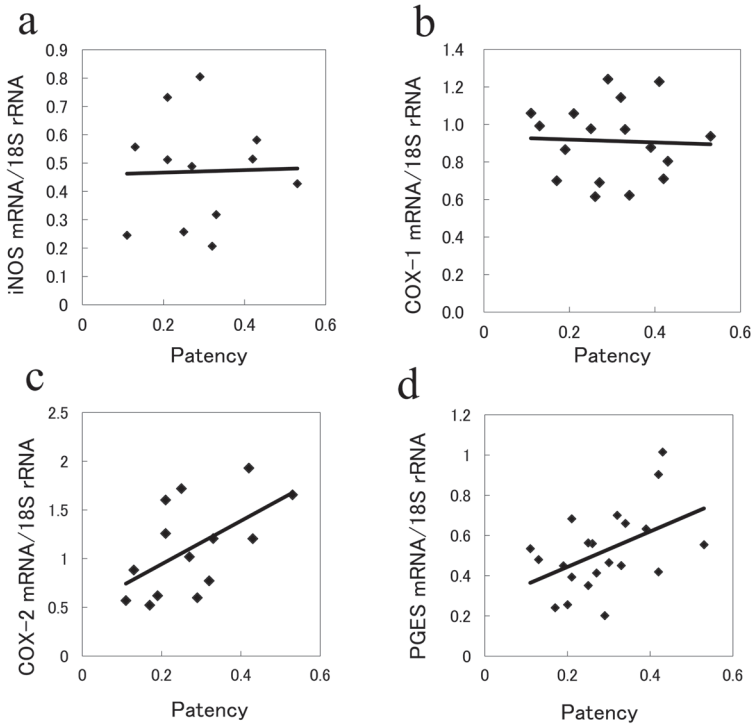

Fig. 1. Relationship between patency and mRNA level of inducible nitric oxide (iNOS) (a), cyclooxygenase (COX)-1 (b), COX-2 (c) or prostaglandin E synthase (PGES) (d) in the uterine cervix of bitches with pyometra. Coefficients of correlation (number of determinations) between patency and mRNA level for iNOS, COX-1, COX-2 and PGES were −0.03 (n=12), 0.04 (n=17), 0.57 (n=14) and 0.47 (n=21), respectively. Significant correlation was found between the patency and mRNA levels of COX-2 and PGES (P<0.05).

COX-1, COX-2 and PGES were 2 min at 94°C for denaturation, followed by specified number of cycles of 30 sec at 94°C, 30 sec at 67°C (for COX-1 and PGES) or 60°C (for COX-2) and 60 sec at 72°C. The PCR condition for iNOS was 3 min at 95°C for denaturation, followed by specified number of cycles of 40 sec at 94°C, 60 sec at 62°C and 120 sec at 72°C. After the PCR reaction, the products were electrophoresed through agarose gel containing ethidium bromide, and bands were examined by a UV transilluminator. Bands of the expected sizes were found in respective RT-PCR, and a negative control, in which the reverse transcriptase was omitted, yielded no PCR bands for any of the target mRNAs. The PCR products for iNOS were cut by the restriction enzyme Kpn I (Gibco Laboratories, Grand Island, NY, U.S.A.) to two fragments of the expected size, and those for COX-1 and COX-2 were by Pst I (Takara, Otsu, Japan). The PCR product for PGES was extracted using a QIAEX II GEL Extraction Kit (QIAGEN, Hilden, Germany) and was sequenced by Takara. After completing the sequence analysis, the sequence of complete cDNAs for canine PGES mRNA has been reported (Accession: EF063141.1). The sequence of the present PCR product between primers displayed 100% homology with that of reported cDNAs for canine PGES mRNA. Proper but not saturated expression of mRNAs for 18S rRNA in the concurrent PCR amplification of iNOS, COX1, COX2 or PGES was obtained by delaying the addition of the primer pairs for 18S rRNA by 22, 17, 19 and 21 cycles, respectively. The relative densities of the bands were determined by densitometric scanning using NIH Image™ software (NIH, Bethesda, MD, U.S.A.), and the intensities of objective products were normalized by that of 18S rRNA. Preliminary experiments settled the PCR condition in which linear relation between densitometric intensity of the RT-PCR products and amounts of RNA was seen. The cycle numbers and intermediate amounts of RNA within the linear relation, which were used for the semi-quantitative RT-PCR, were 36, 31, 29 and 34 cycles and 0.125, 15, 100 and 45 ng RNA, for iNOS, COX-1, COX-2 and PGES, respectively. Using Statcel (the add-in forms on Excel, 1st ed.; OS Ltd., Tokorozawa, Japan), the relationship between 2 factors was analyzed by regression and correlation coefficients.

Figure 1 shows relationship between patency and mRNA expression for iNOS, COX-1, COX-2 and PGES in the uterine cervix. Although there was no significant correlation between patency and mRNA level of iNOS or COX-1, the correlation between patency and mRNA level of COX-2 or PGES was significant (P<0.05). Expression of iNOS mRNA in the rat uterine cervix is correlated with parturition [10], whereas that in the dog with pyometra in this study had no relation with cervical patency. The reason for this difference in relationship between patency and iNOS mRNA expression is not clear. However, infection could markedly stimulate

iNOS expression above the normal value in the cervix as has been reported in the uterus [7], while the expression level was decreased during pregnancy and was below the value of nonpregnant rats even around parturition [10]. It may be possible that relationship between patency and iNOS expression in the cervix during ripening is fundamentally different from the relationship in pyometra-affected bitches. On the other hand, results of the present study suggest that gene expression of PGE-related enzymes, COX-2 and PGES, is involved in patency of the uterine cervix of bitches with pyometra. Partly consistent with this, Linharattanaruksa *et al*. [9] reported that mRNA level of PGES but not of COX-2 in the uterine cervix of dogs with open-cervix pyometra was higher than in that with closed-cervix pyometra. In this study, the rate of cervical patency was directly measured, and mRNA levels of both PGES and COX-2 were correlated with the cervical patency. Endometrial gene transcription for COX-2 and PGES but not for COX-1was significantly higher in the dog with pyometra than in normal bitches, suggesting that inflammation in the uterus enhances the expression of these genes [12]. Taken together, inflammation may stimulate expression of COX-2 and PGES in the endometrium and uterine cervix, which in turn affects the patency of uterine cervices.

Although production of PGE_2 by human cervical explants increases in response to NO [1], there was no correlation between mRNA level of iNOS and that of COX-2 or PGES in this study. Gene expression of iNOS could not be related to mRNA levels for PGE-related enzymes, which may be involved in patency of the uterine cervix. Since PGE_2 stimulates IL-8 synthesis in the human uterine cervix during parturition [1], the relationship between PGE and IL-8 in the uterine cervix of bitches with pyometra remains to be studied.

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