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NOTE

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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Running head: PATENCY IN CANINE UTERINE CERVIX
ABSTRACT.

Factors involved in patency of uterine cervices in the bitch with pyometra remain to be clarified. This study examined the 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 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
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) E₂, 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 PGH₂, the immediate substrate for a series of cell specific PG, and PGE synthase (PGES) converts PGH₂ 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 cervixes 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 (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 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-1 was 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 cervixes.

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

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

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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>sense</td>
<td>5'- AGAAACAACAGGAACCTACCA</td>
<td>661</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5'- CTCCAGGATGTTGTAGCGC</td>
<td></td>
</tr>
<tr>
<td>COX-1</td>
<td>sense</td>
<td>5'- CACCCGCTCATGCCAGACTCC</td>
<td>356</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5'- CCCGGGTAGAATTCCAAGGCATCA</td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>sense</td>
<td>5'- TGAGCGGTTATTCAGAGACGAGCAG</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5'- CCAACCCCGCAGCCATTTCTTCT</td>
<td></td>
</tr>
<tr>
<td>PGES</td>
<td>sense</td>
<td>5'- CACCGGAACGACATGGAGACCATC</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5'- CAGAGCCATGGAGGCGCAGGGGAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BC008280 (human)</td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>sense</td>
<td>5'- TGGTTGATCCTGCCAGTAGCA</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5'- ATGAGCCATTCGCAGTTTCACT</td>
<td></td>
</tr>
</tbody>
</table>
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).