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Factors associated with patency of the uterine cervix in bitches with pyometra

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ABSTRACT

This study examined factors involved in the patency of uterine cervices in the bitch with pyometra. The uterine cervices were obtained from the bitches with pyometra at the time of ovariohysterectomy. Cervical patency was measured by inserting the stainless steel rods with different diameter into cervical canals. Collagen concentration and collagenase activity (for type I collagen) in the tissue were determined and the number of neutrophils, which contain the enzymes related to collagen metabolism, and morphological changes in collagenous fibers were studied by histological examination. Levels of mRNA expressions for hormonal factors, estrogen receptor-α (ER-α), progesterone receptor (PR), relaxin (Rlx) and an attractant of neutrophils, interleukin-8 (IL-8), were determined by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). In the statistical analysis, the cervical patency positively correlated with the collagenase activity, and negative correlation was found between the cervical patency and collagen concentration. Histological examination indicated distinct positive correlation between the cervical patency and the number of neutrophils in the cervical stroma and that the collagenous fiber in the uterine cervix became thinner and degraded with increase of the cervical patency. Although there was no relationship between the cervical patency and the level of mRNA for ER-α, PR or Rlx, IL-8 mRNA level has significant positive correlation with the cervical patency and the number of neutrophils in the cervical stroma.

These results suggest that the increased number of neutrophils in the uterine cervix, which could be related to the local expression of IL-8, may be involved in collagen degradation and connective tissue remodeling to increase cervical patency in the bitch with pyometra.

1. Introduction

In the bitch, pyometra occurs when bacteria colonize a uterus with progressive hormone-mediated cystic hyperplasia of the endometrium and is frequently seen in older animals during luteal phase (Miller-Liebl et al., 1994; Dow, 1958). Clinical signs in the bitch with pyometra vary depending on patency of the cervix. Cervical patency is associated with the presence of vulvar discharge, and the impatency is more commonly associated with abnormal distension of the uterus by accumulation of the pus. Although onset of clinical signs may be acute or gradual, more severe clinical signs are associated with cervical impatency (Jones and Joshua, 1982; Feldman and Nelson, 1987; Pretzer, 2008). Although ovariohysterectomy (OHE) is the first choice for the treatment of pyometra, medical therapy, treatment with prostaglandin (PG) $F_{2\alpha}$ or antiprogestins is sometimes performed for the dog that has problems in receiving operation or is anticipated for reproduction. Since it has been suggested that the treatment with drugs that stimulate uterine contraction may cause uterine rupture or leakage of purulent material through the friable uterine wall, treatment with $PGF_{2\alpha}$ or its analogue for animals with closed cervix pyometra is not recommended (Jones and Joshua, 1982; Feldman and Nelson, 1987; Kustritz, 2005; Smith, 2006). Taken together, cervical patency is an important factor for the diagnosis, severity of clinical signs and choice of medical therapy for pyometra in bitches. The mechanism how patency of uterine cervix is regulated in bitches with pyometra is not known, whereas regulation of the patency could be helpful for diagnosis and therapy for this disease.

Some associations between hormonal status and cervical dilatation are known. Generally estrogen opens cervical canals, and progesterone closes them. Consistently, the canal of the canine uterine cervix dilates at estrus and closes when the blood progesterone level increases (Silva et al., 1995). In the bitches with open-cervix pyometra, however, blood progesterone level is not critical for the patency of the uterine cervix (Renton et al., 1993). Although it has been suggested that progesterone receptor but not estrogen

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receptor in the uterine cervix is related to the patency (Vermeirsch et al., 1999, 2000), expression of the receptors for progesterone and estrogen has not been studied in the bitches with pyometra. Another hormonal factor that is closely related to the patency of uterine cervices is relaxin. Our previous study showed the local expression of relaxin in the canine uterine cervix (Tamada et al., 2010). Since the dog with pyometra does not have the placenta, the major organ that affects the blood level of relaxin in this species (Tsutsui and Stewart, 1991), the local expression may be critical for the patency.

Many reports indicated that increase in collagenase activity (Rath et al., 1987; Rajabi et al., 1988, 1991), decrease in tissue collagen concentration (Rath et al., 1994) and infiltration of neutrophils (Junqueira et al., 1980), which are likely to be inflammatory response, occur in the uterine cervix associated with ripening at the time of parturition. Collagenases belong to a family of matrix metalloproteinases (MMPs), and MMP-1, MMP-8 and MMP-13 are well known as the enzymes which degrade collagen. Among these, MMP-1 and MMP-8 specifically degrade the collagen type I and III (Hulboy et al., 1997), which predominantly exist in the uterine cervix (Minamoto et al., 1987). The site of production for MMP-1 is the fibroblast or endothelial cell, and that for MMP-8 the neutrophil. Neutrophils also contain elastase that is another enzyme degrading collagen type I and III (Starkey, 1977). Results of recent studies indicate that local expression of interleukin-8 (IL-8), a chemoattractant for neutrophils, is associated with cervical ripening and associated neutrophil infiltration (Barclay et al., 1993; Uchiyama et al., 1992; El Maradny et al., 1994, 1996; Chwalisz et al., 1994). However, the relationship between patency of the cervix and collagen structure or neutrophil infiltration in bitches with pyometra has not been reported.

This study examined some factors to advance understanding how to control cervical patency in bitches with pyometra. The factors studied include collagen concentration, collagenase activity, neutrophil infiltration, expression of mRNA for ER-α, PR, relaxin and IL-8, all of which could be involved in the dilatation of uterine cervix at parturition.

2. Materials and methods

2.1. Animals

The tissue samples excised by ovariohysterectomy from 21 bitches with pyometra (4 mongrels, 2 Shiba inu dogs, 2 Shetland sheep dogs, 2 Maltese dogs, 2 Yorkshire terriers, 2 Shih Tzus, a golden retriever, a Siberian husky, a toy poodle, a Pomeranian, a pug, a Westy and a miniature dachshund, 2.8–13 years old) were kindly provided by the dog owners at our Veterinary Teaching Hospital and the other animal hospitals close to our laboratory, and no animal was operated upon or killed specifically for this study. After confirming that whole uterine cervices were removed by ovariohysterectomy, 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 of the uterine cervix. The patency was defined as ratio of the diameter of the thickest stainless steel rod that passed through the canal against the diameter of the uterine cervix, which was determined on the round cross section including the cervical canal. Whole or a part of the uterine cervix was frozen in liquid nitrogen and stored at −80 °C until the following determinations. In some samples, the tissue was fixed in Bouin's fluid for histological examination. These tissues were cut from perimetrium to the center of the canal by straight line to include mucosa, myometrium and perimetrium at the constant ratio. Since the amount of tissue obtained was not enough for all determinations, all samples were not used for the determination of each factor described below. The numbers of corpora lutea and follicles greater than 3 mm in diameter were counted in each ovary. Cervix type of pyometra (closed or open), the interval between sample collection and the previous estrus (months after estrus), age and body weight were recorded in each dog.

2.2. Determination of collagen and collagenase activity

The uterine cervix (approximately 100 mg) was freeze-dried for 24 h and weighed. Hydroxyproline was determined by the procedure of Berg (1982), and the value was multiplied by 8.27 to estimate the quantity of collagen. Collagen concentration (content/dry weight) was calculated. Correlation between the values determined and patency in the uterine cervix was examined.

Collagenase was extracted by the procedure of previous reports (Rajabi et al., 1991; Sellers and Woessner, 1980; Lenhart et al., 2001). Briefly, the tissue (approximately 100 mg) was cut into small pieces, homogenized in 10 volumes of 0.25% Triton X-100 solution containing 10 mM $CaCl_2$ at 2 °C, centrifuged (6000*g*) at 2 °C for 20 min, and the supernatant was taken as 'Triton extract'. To the precipitate, 10 volume of 50 mM Tris–HCl (pH 7.5) containing 100 mM $CaCl_2$ and 200 mM NaCl was added, and the mixture was heated at 60 °C for 2 min and centrifuged (10,000*g*) at 2 °C for 20 min. The supernatant was taken as 'Heat extract'. 'Triton extract' and 'Heat extract' were demineralized with Micro Bio-Spin chromatography columns (P-6, Bio-Rad Laboratories, CA). Collagenase in the solutions was activated by trypsin prior to the determination of activity. To the demineralized solution (62.5 μL), 50 mM Tris–HCl (pH 7.5) containing 10 mM $CaCl_2$ and 200 mM NaCl (387.5 μL) as well as trypsin (12.5 μg; 14,100 BAEE units/mg) were added, and the mixture was incubated at 37 °C for 3 min. The reaction was terminated by adding trypsin inhibitor (50 μg; 9000–18,000 BAEE units/mg). The activity of collagenase was determined by type I collagenase activity determination kit (Yagai, Yamagata, Japan) according to the manufacturer's protocol. Briefly, the samples were mixed with the solution containing fluorescence-labeled type I collagen, and the mixture was incubated at 37 °C for 22 h. The reaction was stopped by adding 'stop solution', and the mixture was centrifuged (2000*g*) for 15 min to separate unchanged collagen from decomposed collagen. The fluorescence (excitation wavelength 495 nm, fluorescence 520 nm) for the supernatant containing decomposed collagen was determined using fluorescence photospectrometer. The value where collagenase was inactivated by keeping the sample tube in boiling water for 5 min was defined as 'Blank', and the value where all collagen was decomposed by keeping the fluorescence-labeled collagen tube in boiling water for 1 min as 'Total'. Collagenase activity was calculated by the following formula.

Collagenase activity (% degradation of collagen) = (Sample–Blank)/(Total–Blank) × 100. Combined activity for 'Heat extract' and 'Triton extract' were used as the value for each sample. Correlation between the determined value and patency in the uterine cervix was calculated.

2.3. Histological examination

The tissue fixed in Bouin's fluid was paraffin-embedded and sectioned at 7 μm. The sections were stained with hematoxylin and Eosin. The number of neutrophils in the stroma of mucosae was examined microscopically (400×). The number of all neutrophils were counted in each field (0.197 mm^2) that occupies only stroma. For each uterine cervix, 3 sections were examined, and 3 fields were investigated for each section. The average count for each section was used to calculate the average count for each uterine cervix. Correlation between the counts and patency in the uterine cervix was calculated.