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Expression of oxytocin, progesterone, and estrogen receptors in the reproductive tract of bitches with pyometra

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ABSTRACT

Canine pyometra is considered a serious and life-threatening condition. Due to the relationship among sex steroid hormones, oxytocin receptor (OTR) expression, and canine pyometra pathogenesis, this study aimed to investigate the expression of oxytocin, progesterone, and estrogen receptors in the reproductive tissues of canines with pyometra by real-time PCR and immunohistochemistry. A total of 27 pyometra bitches were classified into open- and closed-cervix pyometra groups based on the presence of vaginal discharge. Moreover, 15 normal bitches in the luteal phase served as a control group. The results showed that *OTR* gene expression in the ovary of pyometra bitches was higher than that of normal bitches, whereas the level of *OTR* gene expression in the cervix of pyometra bitches was less than that of normal bitches ($P < 0.05$). Conversely, a lower OTR H-score in ovarian follicles was observed in pyometra bitches compared with normal bitches, whereas a higher percentage of OTR-positive immunostaining in uteri and cervices were found in pyometra bitches compared with normal bitches ($P < 0.05$). Moreover, the H-scores of estrogen receptor alpha in uteri and cervices of pyometra bitches were less than that of normal bitches ($P < 0.05$). However, the localization of the OTR and sex steroid receptors between groups of pyometra bitches was not different. Our findings suggest that pyometra pathogenesis is associated with a change in expression of OTR and sex steroid receptors in the canine reproductive tract. However, cervical dilation in bitches with pyometra was not influenced by the expression of OTR and sex steroid receptors.

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1. Introduction

In older or middle-aged intact bitches, pyometra is the most common disease of the uterus found after one or more nonpregnant estrous cycles [1]. The pathogenesis of canine pyometra is not fully understood [2]. Many studies in bitches suggested that an excessive quantity of progesterone or an oversensitivity of the uterus to progesterone can lead to pyometra [3,4]. Moreover, pyometra usually occurs during the period of diestrus, as high levels of progesterone during that stage promote endometrial proliferation and glandular activity, whereas suppressing myometrial contractions and leukocyte inhibition in the uterus [5]. Estrogen may also be a regulator in canine pyometra because high levels of estrogen during proestrus and estrus can increase uterine sensitivity to progesterone in the following stages of the estrous cycle [6]. Therefore, exogenous estrogen therapy for mismating and progesterone therapy for contraception have been associated with increased risk for pyometra [7]. Canine pyometra can be classified into two groups: closed- and open-cervix pyometra. The clinical signs of closed-cervix pyometra cases seem to be more severe than in open-cervix pyometra cases because bitches are suffering from an enlarged uterus

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and endotoxemia; moreover, they are at risk of uterine rupture and the systemic inflammatory response syndrome [7]. Regarding the pathogenesis of pyometra, many investigators have studied the expression of sex steroid receptors including estrogen receptor alpha (ERα) and progesterone receptor (PR) in the canine reproductive tract of pyometra cases [8–12]. When the sex steroid receptor expressions of uteri from pyometra cases were compared with those of normal uteri from diestrus bitches, a lower expression level of ERα in all uterine layers was observed in the pyometra cases, whereas the expression of PR in most tissue layers of canine pyometra uteri tended to be higher than in normal uteri [8–11]. It may be presumed that the changes of ERα and PR expressions in uterine compartments are involved in the pathogenesis of pyometra. Besides the role of sex steroid hormones in uteri, a study in humans showed that cervical dilation was involved with several mechanisms including the expression of sex steroid receptors [13]. However, a recent study proposed that sex steroid receptor expressions involved with cervical dilation occur in cyclic bitches but not in bitches with pyometra [12]. Oxytocin, a neurohypophysial peptide hormone, is mediated by its own oxytocin receptor (OTR), a member of the class I family of G protein-coupled receptors [14]. Oxytocin regulates many female reproductive functions such as myometrial contractions [15], control of the estrous cycle length [16], and cervical dilation [17]. In general, regulation of oxytocin's actions is strongly sex steroid hormone dependent [18,19]. The expressions of oxytocin and sex steroid receptors in the reproductive tract are influenced by the stages of the estrous cycle [12,20] and the various stages of pregnancy [21]. Moreover, oxytocin is involved with cervical dilation during the estrous cycle [22] and parturition [23]. Although the actions of oxytocin and sex steroid hormones in the reproductive system of normal bitches have been investigated, studies in canine pyometra are rare. Therefore, the present study aimed to compare the localization and mRNA expression of OTR, PR, and ERs in each compartment of the reproductive tract (including ovaries, uteri, and cervices) between normal bitches in diestrus and pyometra bitches. Knowledge about factors regulating the pathogenesis and cervical dilation of canine pyometra may facilitate the development of alternative pharmaceutical treatments for modulation of cervical dilation in bitches developing closed-cervix pyometra.

2. Materials and methods

2.1. Animals

A total of 27 bitches, aged 93.78 ± 40.32 months (range, 24–156 months) developing pyometra and undergoing ovariohysterectomy were classified into two groups according to the presence of mucopurulent vaginal discharge (open-cervix pyometra group; OP group, n = 15) and the absence of mucopurulent vaginal discharge (closed-cervix pyometra group; CP group, n = 12) for the investigation of OTR localization by immunohistochemistry (IHC). None of the bitches had received hormonal treatment for pyometra before or during surgery. All pyometra bitches were identified as in the luteal phase using ovarian structures (diameter of corpora lutea >0.5 cm) and serum progesterone levels (15–50.8 ng/mL) [12]. A total of 15 healthy bitches in the luteal phase, aged 70.04 ± 30.63 months (range, 18–108 months) served as a control group. Five samples of each group were randomly chosen for quantitative reverse transcription polymerase chain reaction (RT-qPCR) technique. Moreover, the immunolocalization of sex steroid receptors was performed in six randomly selected bitches from each group. All samples used in this study were collected from client-owned dogs presented to the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University or Prasu-Arthorn Animal Hospital (The Veterinary Teaching Hospital), Faculty of Veterinary Science, Mahidol University. All tissue sampling were reviewed under the Chulalongkorn University Animal Care and Use Committee (CU-ACUC) approved protocol No. 1431006 with owners providing informed consent.

2.2. Sample collection

Samples including ovaries, uteri, and cervices were immediately collected after ovariohysterectomy. Tissue samples were cut into small pieces (5 mm^3 and <30 mg in weight) and frozen in liquid nitrogen for RT-qPCR. For IHC, samples were cut and fixed in 4% (wt/vol) paraformaldehyde in PBS for 48 hours. Afterward, fixed tissues were embedded in a paraffin block which was cut into 4-μm thick sections and placed on gelatin-coated slides for IHC.

2.3. RT-qPCR technique

Frozen samples were pulverized with a sterile mortar and pestle in liquid nitrogen and then homogenized in a mixture of lysis buffer on ice. Total RNA was isolated using a column-based method (RNeasy Mini Kit, QIAGEN Ltd., West Sussex, UK), according to the manufacturer's instructions. The concentration and integrity of total RNA from each sample was evaluated using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Delaware, USA) and a 1% (wt/vol) agarose gel electrophoresis technique with Red safe staining (RedSafe, iNtRON biotechnology, Gyeonggi-do, Korea). To eliminate genomic DNA, isolated RNA was treated with RQ1 (RNA-Qualified) RNase-Free DNase (Promega, Medison, USA). Complementary DNA (cDNA) synthesis was then performed using avian myeloblastosis virus reverse transcriptase (Reverse transcription system, Promega, Medison, USA). An aliquot of generated cDNA was amplified with a pair of primers (forward 5′CGTTCTTC TTCGTGCAGATGT3′ and reverse 5′ACAAAGGTGGATGAGT TGCTCT3′, annealing at 58 °C) derived from canine *OTR* mRNA sequences (GenBank Accession No. NM_0011 98659.1). Primer pairs for the *OTR* gene were designed using sequences published in GenBank by Primer 3 (Version 0.4.0) available online. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and 5S ribosomal RNA (RP5S) were amplified as reference genes (GAPDH, forward 5′ ATTCCACGGCACAGTCAAG3′ and reverse 5′ TACTCAGCACCAGCATCACC3′, annealing at 52 °C; and RP5S, forward 5′ATCATCCATCTGCTCACTGGT3′ and reverse 5′ AGCTCATCTGCTAGGCACTCA3′, annealing at 62 °C). Primer

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