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RESEARCH ARTICLE

Immunolocalization of Oxytocin Receptor in the Bitch Cervix at Different Phase of Estrous Cycle

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The open and closure of cervical canal is an important function in both reproductive physiological and pathological processes. The cervical dilation during the estrous cycle is involved the presence of oxytocin receptor (OTR) in most species. The regulation of cervical dilation during the estrous cycle in bitch has not been exactly reported. This study aimed to investigate the expression of OTR in bitch cervical tissue during various stages of the estrous cycle using immunohistochemical technique. Thirty-one bitches were divided into three groups, follicular, luteal, and inactive phase according to ovarian structures. The results presented as the mean percentage of the positive staining area showed that the OTR expression was mostly found in the cytoplasm of all cervical compartments. The highest percentage of OTR positive staining area was observed in the follicular phase group ($P<0.05$), whereas the lowest OTR expression was observed in the inactive phase group ($P<0.05$). The present study suggested that the OTR expression is influenced by the phase of the estrous cycle and that cervical cells are the target sites of oxytocin during the estrous cycle. Therefore it may also conclude that the action of oxytocin may play a role in the mechanism of the cervical dilation in cyclic bitches through the expression of its receptor.

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INTRODUCTION

The cervix acts as a barrier against ascending infections to the uterus and is important to allow the passage of spermatozoa (Verstegen *et al.*, 2001) as well as maintenance of pregnancy (Badir *et al.*, 2013). Therefore, the open and closure of cervical canal involved both reproductive physiological and pathological processes. During the estrous cycle of normal bitches, the opening of the cervix was observed concurrent with the day of the maximal value of the estradiol and progesterone ratio, before the decline of estrogen preceding the LH peak. The closure of the cervix occurred about five to seven days after the LH peak and two to three days before the first sign of diestrus (Silva *et al.*, 1995). The recent study found that the cervical dilation involved with several mechanisms during various stages of the estrous cycle (Tamada *et al.*, 2012).

Earlier studies demonstrated that the cervical dilation is regulated by ovarian steroids and gonadotrophic hormones through their specific receptors (Leethongdee *et al.*, 2007). According to various stages of the estrous cycle, the difference of estrogen receptor (ER) and progesterone receptors (PR) expression in the cervix have been reported in sheep (Rodriguez-Pinon *et al.*, 2008), rats (Wang *et al.*, 2000), and dogs (Kunkitti *et al.*, 2011). In sheep, estradiol acts on the cervix to regulate the expression of its own receptors and of those for oxytocin (Kershaw *et al.*, 2007; Falchi and Scaramuzzi, 2013). Oxytocin receptor (OTR) is a member of the class I family of G protein-coupled receptors (Kimura *et al.*, 1992), in which the direct interaction of oxytocin with its receptor causes an increase in intracellular calcium (Asselin *et al.*, 1997), and then leads to mobilization of arachidonic acid (Burns *et al.*, 2000). The predominantly final product of this signal transduction is prostaglandin E2 (PGE2) that causes relaxation of cervical smooth muscle tissue and the softening and dispersal of cervical extracellular matrix which resulted in cervical dilation (Kershaw *et al.*, 2007).

Regarding cervical dilation in the bitch, various factors and reproductive hormones were investigated but the mechanism of this physiological event is not exactly understood. The interaction of oxytocin and its receptor may be an important role on the open and closure of cervical canal in bitch during various stages of the cycle. Moreover, the presence of OTR may reflect the function of oxytocin acting via its receptor on target tissues, which differed among specific cervical compartments. Therefore, the present study aimed to determine the cellular localization of OTR in each compartments of cervical tissues during various stages of estrous cycle.

MATERIALS AND METHODS

Animals: The tissue samples were obtained by routine ovariohysterectomy from healthy nulliparous bitches of mixed breeds. None of the bitches received exogenous hormone or being pregnant. The bitches were classified into three groups according to ovarian structures; follicular, luteal, and inactive phase. The ovarian structure of the bitches in the follicular phase (proestrus and estrus stages) showed large follicles (diameter >0.5 cm) and/or corpora hemorrhagica. The serum progesterone levels of bitches in follicular phase were 0.7 to 15 ng/mL. The luteal phase (diestrus stage) was classified when the bitches showed corpora lutea (diameter>0.5 cm) on the ovary and had high serum progesterone levels (15 to 50.8 ng/mL). For the inactive phase group (anestrus stage), the bitches showed quiescent ovaries and had low serum progesterone levels (0.04 to 1.3 ng/mL) (Concannon, 2011; Kunkitti *et al.*, 2011).

Hormonal analyses: Before surgery, the venous blood was collected and the measurements of serum progesterone levels were evaluated following the protocol of the previous study (Kunkitti *et al.*, 2011).

Collection of cervical tissue samples: The cervix was longitudinally cut and fixed in 4% (wt/vol) paraformaldehyde in a phosphate buffer saline (PBS) solution for 48 h., processed histologically, cut into 4 µm thick for immunohistochemistry.

Immunohistochemical detection of OTR: The avidin-biotin peroxidase complex method was performed. A goat polyclonal antibodies (N-19: sc-8103, Santa Cruz Biotechnology, USA) was used as primary antibody, at dilution1:200 with room temperature incubation for 3 h. A secondary antibody (Biotinylated Anti-Goat IgG made in horse, Vector Laboratories, Inc., USA) was added followed by incubation with avidin-biotin complex (Vectastain® ABC Kits, Vector Laboratories, Inc., USA). Finally, the solution of 3, 3′-diaminobenzidine (ImmPACT™ DAB Peroxidase substrate kit, Vector Laboratories, Inc., USA) was added to visualize the immunoreactivity (brown color). All the sections were subsequently counterstained with Mayer's hematoxylin. The negative control was done by omitting the primary antibody and the placental tissue which was known to contain OTR was used as a positive control.

Immunohistochemical evaluation: Regarding the surface epithelium, the cranial part of the cervix that characterized by a simple columnar epithelium was called uterine part while the caudal part of the cervix that characterized by stratified squamous epithelium was called vaginal part. The expression of OTR in the uterine and vaginal part was evaluated under a light microscope by using the image analysis software (Image-Pro® PLUS 6.0 Programming software, Media Cybernetics, Inc.). Three tissue layers (surface epithelium, lamina propria, and smooth muscle cells of the muscular layer) were evaluated separately. Quantification of the immunostaining was performed on five randomly selected fields in each compartment of the cervix. The results of OTR immunostaining were presented as the mean percentage of the positive staining area.

Statistical analysis: Computerized statistical software (IBM SPSS 18.0, Armonk, NY, USA) was used for analyses. Probabilities<0.05 were considered statistical significant. Comparisons of the progesterone levels and the percentage of positive staining area between each phase were undertaken by one-way ANOVA and the Bonferroni correction was used as post-hoc test. Within phase, comparisons between the uterine- and vaginal-parts of cervix were undertaken by Paired T-test. Pearson's correlation coefficient was used to assess the correlation.

RESULTS

Thirty-one tissue samples from different breeds of bitches were classified into three groups; follicular (n=10), luteal (n=13), and inactive phase (n=8).

Progesterone level: The highest level of serum progesterone was measured in luteal phase (29.12±11.55 ng/mL), when compared with follicular phase (6.49±6.2 ng/mL) and inactive phase (1.38±2.49 ng/mL) (P<0.001). However, the serum progesterone levels in the follicular phase were not different from the inactive phase.

Immunolocalization of oxytocin receptor (OTR): The positive OTR immunostaining cells was found as brown staining in the cytoplasm and cell membrane in all cervical tissue compartments (Fig. 1, 2). The comparisons of the percentage of positive OTR immunostaining in each cervical tissue compartment were done between phases (Table 1). In the luteal phase, the percentage of positive OTR immunostaining of the surface epithelium and laminar propria in the uterine part was significantly higher than the vaginal part (P=0.04, and P=0.003 respectively). Others comparisons between the uterine part and the vaginal part were not significant. In addition, positive OTR immunostaining was also found in cervical blood vessel during follicular phase, which was not observed in the other phases of the estrous cycle (Fig. 3). In the negative control section, no specific staining was found in any cervical cells.

Correlation of OTR immunostaining: The significant positive correlation coefficients of OTR immunostaining in each layer of the uterine- and vaginal-parts of cervix are illustrated in Table 2.

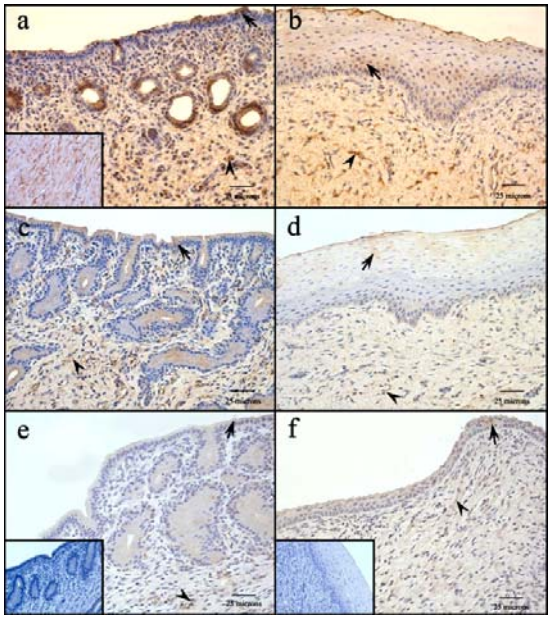

Fig. 1: Immunohistochemical staining of OTR in the surface epithelium and lamina propria of the uterine part (a, c, e) and the vaginal part (b, d, f) of canine cervical tissues at different phases of the estrous cycle; follicular phase (a, b), luteal phase (c, d), and inactive phase (e, f). The positive control is shown as an insert in (a). The negative controls of uterine part and vaginal part are shown as an insert in (e) and (f), respectively. Black arrow and arrowhead show, respectively, positive staining area in surface epithelium and in lamina propria.

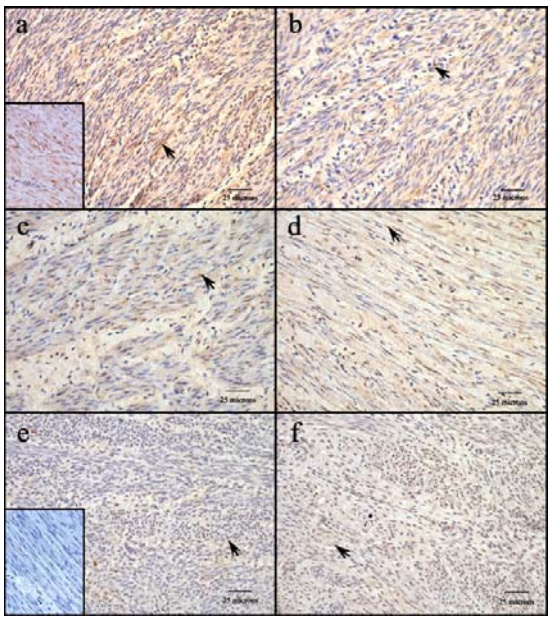

Fig. 2: Immunohistochemical staining of the OTR in the muscular layer of the uterine (a, c, e) and the vaginal part (b, d, f) of canine cervical tissues at different phase of the estrous cycle; follicular phase (a, b), luteal phase (c, d), and inactive phase (e, f). The positive control is shown as an insert in (a). The negative control is shown as an insert in (e). Black arrow shows positive staining area.

DISCUSSION

Canine cervix has an important function in fetal and maternal uterine protection as well as it also acts as a passage way for spermatozoa during insemination.

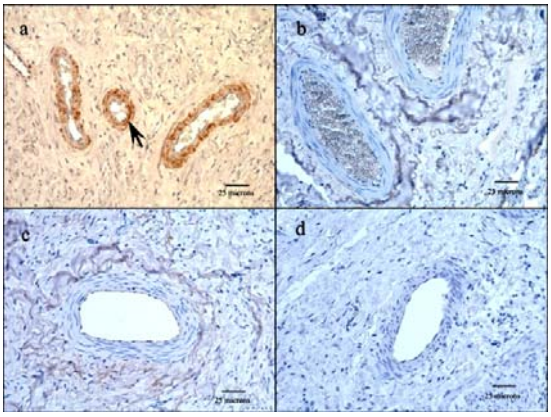

Fig. 3: Immunohistochemical staining of the OTR in the smooth muscle of cervical blood vessel at different phase of the estrous cycle; follicular phase (a), luteal phase (b) and inactive phase (c). The negative control is shown in (d). Black arrow shows positive staining area.

However, the information about the mechanism of cervical opening or closure in bitches is still limited (Goericke-Pesch *et al.*, 2010). The recent study suggested that the open and closure of the canine cervical canal involved with several mechanisms (Tamada *et al.*, 2012) in example, inflammatory cells infiltration and the involvement of inflammatory mediator (Calder, 1994; Gee, 2009). In bitches, previous study suggested that the stage of the estrous cycle has the effects on the expressions of estrogen and progesterone receptors, and it may involve in cervical dilation (Kunkitti *et al.*, 2011). In sheep, it was found that cervical dilation during estrus was probably regulated by cervical prostaglandin E2 (PGE2) synthesis and extracellular matrix remodeling through hyaluronan synthesis (Kershaw-Young *et al.*, 2009). Furthermore, the recent studies in cows and sheep suggested that oxytocin, and its receptor could be associated with cervical ripening via the expression of ovarian steroid hormones (Fuchs *et al.*, 1996; Falchi and Scaramuzzi, 2013).

In the present study, OTR positive staining was found in all cervical tissue compartments which may suggest that all parts of the canine cervical tissue are the target sites of oxytocin acting via its own receptor. Cervical epithelium had many functions such as proliferation, differentiation, and production of essential substance for cervical function such as prostaglandins (PGs) (Timmons *et al.*, 2007). In bovine uterus, the in vitro study suggested that the epithelial cells of the endometrium are the target sites of ovarian steroid hormone and oxytocin for the PG synthesis (Asselin *et al.*, 1996). In lamina propria or stroma, it consists of cervical glands and vascular structures (Goericke-Pesch *et al.*, 2010). Interestingly, OTR positive staining was observed in the endothelium and smooth muscle cells of cervical blood vessels during follicular phase in this study, while it was not found in the other phases. The studies in human and rat demonstrated the expression of OTR in endothelial cells from large vessels (Thibonnier *et al.*, 1999; Jankowski *et al.*, 2000). The study in human revealed that the OTR in the endothelial cells from umbilical vein, aorta, and pulmonary artery was structurally identical to uterine OTR and could activate vasodilation pathway and

Table 1: Percentages of positive immunostaining for OTR in the uterine- and vaginal-parts of the bitch cervix during various phases of the estrous cycle

Compartment		Phase			P-valve
Part	Layer	Follicular	Luteal	Inactive	
Uterine	SE	18.64±4.81[a]	14.12±3.75[b]	5.68±2.60[c]	<0.001
	LP	19.35±5.85[a]	15.22±5.07[a]	5.35±2.11[b]	<0.001
	M	18.13±5.54[a]	11.35±3.88[b]	1.82±1.30[c]	<0.001
Vaginal	SE	15.98±2.83[a]	9.96±4.80[b]	4.88±3.33[c]	<0.001
	LP	15.87±5.32[a]	7.93±6.21[b]	3.84±2.02[b]	<0.001
	M	19.59±6.76[a]	11.16±3.48[b]	2.36±1.69[c]	<0.001

Values (mean±SD) with different superscripts in a row differ significantly. SE: surface epithelium; LP: lamina propria and M: muscular layer.

Table 2: Correlation coefficient (*r*) of the OTR immunostaining between different cervical compartments

Pair of correlation	R	*P*-valve
Uterine-SE and Uterine-LP	0.80	<0.001
Uterine-SE and Uterine-M	0.73	<0.001
Uterine-SE and Vaginal-SE	0.60	0.001
Uterine-SE and Vaginal-LP	0.59	0.002
Uterine-SE and Vaginal-M	0.83	<0.001
Uterine-LP and Uterine-M	0.87	<0.001
Uterine-LP and Vaginal-SE	0.55	0.003
Uterine-LP and Vaginal-LP	0.66	<0.001
Uterine-LP and Vaginal-M	0.84	<0.001
Uterine-M and Vaginal-SE	0.65	<0.001
Uterine-M and Vaginal-LP	0.70	<0.001
Uterine-M and Vaginal-M	0.86	<0.001
Vaginal-SE and Vaginal-LP	0.80	<0.001
Vaginal-SE and Vaginal-M	0.70	<0.001
Vaginal-LP and Vaginal-M	0.73	<0.001

SE: surface epithelium; LP: lamina propria and M: muscular layer.

modulated cell proliferation *in vitro* (Thibonnier *et al.*, 1999). Moreover, earlier study showed the decreasing sensitivity of uterine artery smooth muscle cells to oxytocin during pregnant (Vedernikov *et al.*, 2006). From the study in human, the expression of OTR in microvascular endothelial cells of the myometrium indicates the role of oxytocin in regulating the vascular tone (Weston *et al.*, 2003). Therefore, the results of the present study supported the role of oxytocin acting through OTR in regulating the vascular tone of cervical tissue as it was shown by positive staining in the cervical blood vessels during follicular phase. Regarding the tunica muscularis, it was suggested that the classical role of oxytocin the contractility of the myometrium during parturition. However, during normal estrous cycle oxytocin may be responsible for the transport of spermatozoa or oocytes, or the movement of secretory products by uterine smooth muscle contraction via oxytocin receptor (Einspanier *et al.*, 1998).

The present study demonstrated that the highest positive OTR immunostaining was shown during the follicular phase, when estrogen is dominant and the dilation of the cervix was noticed. The highest expression of OTR may act together with estrogen and its receptors in order to mediate cervical dilation (Silva *et al.*, 1995). However, the oxytocin concentration in the circulation was not different between different phases of the estrous cycle (Olsson *et al.*, 2003). Therefore, the expression of OTR rather than the level of the circulation oxytocin may involve in cervical dilation in cyclic bitches via influence of ovarian steroid hormone, especially estrogen as described before by Falchi and Scaramuzzi (Falchi and Scaramuzzi, 2013). Moreover, previous studies have suggested the role of estrogen in the up-regulation of myometrial OTR expression whereas progesterone could inhibit OTR expression (Ivell and Walther, 1999). The present study was in agreement with those studies by showing the high level of OTR when estrogen was high and lower OTR when the level of progesterone increased.

When comparing with other species, it was clear that the pattern of OTR expressions in the canine cervix were similar to previous studies in sheep and cows, which demonstrated the association of the OTR expression with cervical dilation (Fuchs *et al.*, 1996; Falchi and Scaramuzzi, 2013). Falchi and Scaramuzzi (Falchi and Scaramuzzi, 2013) proposed that estradiol acts on the cervix to regulate the expression of OTR during estrus. When oxytocin binds to its receptor, it activates a G protein that leads to an increase of PGE2 production via cyclooxygenase-2 action (Fuchs *et al.*, 1996; Falchi and Scaramuzzi, 2013). When PGE2 binds to PGE receptors (EP) subtypes 2 and 4 (EP2 and EP4) on smooth muscle and fibroblast cells in the cervix, it stimulates the relaxation of cervical smooth muscle and hyaluronan-like glycosaminoglycan synthesis (Narumiya *et al.*, 1999). This high level of cervical hyaluronan increases water absorption, caused collagen fibers and bundles to separate and disperse, thereby resulted in cervical dilation (Kershaw-Young *et al.*, 2009). However, the recent study in bitch suggested that mRNA and protein expressions of the enzymes involved in PGE2 synthesis and PGE2 receptors are not influenced by hormonal status during the estrous cycle (Linharattanaruksa *et al.*, 2013). Thus, further investigations in bitches on the details of PGE2 synthesis during estrous cycle are required in order to clarify the certain mechanism of canine cervical dilation through the expression of OTR.

Regarding different parts of the cervix, the OTR expression did not differ along the longitudinal axis in most of the cervical tissue compartments but varied between phases of the estrous cycle. In cyclic ewes, the staining pattern of OTR expression was not different when comparing between vaginal part and uterine part of the cervix, whereas the distribution of OTR along the cranio-caudal axis of the bovine cervix varied significantly in tissues at estrus (Fuchs *et al.*, 1996; Falchi and Scaramuzzi, 2013). The OTR staining patterns of the uterine- and vaginal-parts of cervix may be species-specific manner.

Conclusion: the results from the present study suggested that the bitch cervical cells were the target sites of oxytocin acting through its receptor during the estrous cycle. The OTR expression was influenced by the stages of the estrous cycle. The highest OTR expression in follicular phase when the cervical canal was dilated may indicate that the expression of OTR is involved with the cervical dilation mechanism in bitches during the estrous cycle. Further investigations on the expression of OTR in pyometra and dystocia bitches are needed in order to better clarify the mechanisms of canine cervical dilation in the pathological status.

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Author's contribution: SM designed the experiment, prepared the manuscript and discussion. NP performed an experiment and discussion. SS interpreted and discussed data and laboratory consulted. PK collected samples and data. WM performed statistical analysis and discussion. All author approved the manuscript.

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