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Comparison of Cervical and Uterine Cytology Between Different Classification of Postpartum Endometritis and Bacterial Isolates in Holstein Dairy Cows

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Abstract: The aim of this study was to compare cervical and uterine cytology of the postpartum endometritis and bacterial isolate groups in Holstein dairy cows. Four hundred two postpartum dairy cows from 13 commercial dairy herds were examined once between 21 and 35 days postpartum and 86 Holstein cows with postpartum endometritis were sampled. Endometritis was diagnosed by external observation, rectal palpation, vaginal exam, ultrasonography and cervical and uterine cytological examinations. Bacterial swabs were collected using a transcervical double-guarded swab. In total, cows were classified by clinical signs severity, ovarian status and bacterial culture results. The neutrophil percentage in cervical mucosa and uterine fluid of the cows affected by *Arcanobacterium pyogenes* and clinical signs of purulent discharge (E3) were significantly ($p<0.05$) higher than other groups. The large vacuolated epithelial cells percentage in cervical mucosa were higher significantly ($p<0.05$) than that percentage in uterine fluid. The result of this study couldn't show any significant differences between neutrophils percentages of cervical mucosa and uterine fluid smear in cows with three classifications. Therefore, cervical sample is practical and applicable in all commercial herds. In conclusion, the cytological evaluation of cervical smear at fresh cows is suitable for diagnosis of subclinical endometritis, planning for treatment and prognosis of fertility after voluntary waiting period of dairy cows.

Key words: Endometritis, *Arcanobacterium pyogenes*, uterus, dairy cow, cytology, neutrophil

INTRODUCTION

A significant proportion of reproduction problems can be attributed to varying degrees of endometritis. Uterine infection described as acute metritis or chronic endometritis is the most important reproductive disorder of dairy cows (Jana *et al*., 2007). Endometritis is a common reproductive disorder in female domestic animals with consequences ranging from no effect on reproductive performance to permanent sterility. It affects the general health of animals and adversely affects their reproductive performance (Amiridis *et al*., 2003). Reproductive tract abnormalities especially in the uterus and ovaries of cows often results in infertility. Metritis and endometritis are prevalent in dairy herds and contribute to increased days to first breeding, decreased conception rate and pregnancy rate and increased culling (LeBlanc *et al*., 2002). The reported lactational incidence of endometritis varies from 7.5 to 61.6% (Curtis *et al*., 1985; Markusfeld, 1987; Gilbert *et al*., 2005).

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However, these figures should be interpreted with care, since the definition of endometritis used in different publications is inconsistent (Földi *et al*., 2006). Subclinical endometritis, based on uterine cytological examination, is also prevalent in dairy cows and has a profound negative impact on reproductive performance (Hammon *et al*., 2006).

Endometritis has been diagnosed by observation of purulent material on the escutcheon or tail, detection of an enlarged uterine horn or cervix following transrectal palpation of the uterus, detection of purulent material in the vagina following insertion of a gloved hand or visualisation of the cervical os and vagina by vaginoscopy (LeBlanc *et al*., 2002; Sheldon *et al*., 2002). Cow's in which intravaginal purulent material was detected upon vaginoscopy have poorer reproductive performance than those not having such a discharge (McDougall, 2001; LeBlanc *et al*., 2002). Cows with no clinically evident signs of endometritis, but with presence of intrauterine fluid upon transrectal ultrasonography or inflammatory cells in smears or uterine flushings, also have reduced reproductive performance (Kasimanickam *et al*., 2004; Gilbert *et al*., 2005).

Cytological methods have been used to determination of the subclinical endometritis in the in cows (Kasimanickam *et al*., 2004; Raab *et al*., 2002; Ahmadi *et al*., 1998). There were significant negative correlations between neutrophil percentage in cervical mucosa and days after parturition (Ahmadi *et al*., 2001). Ahmadi *et al*. (2006a) reported that the cytological evaluation of cervical smear at fresh cow is suitable for diagnosis of subclinical endometritis, planning for treatment and prognosis of fertility after voluntary waiting period of dairy cows. There was no significant difference between the cervical and uterine smears reproductive tracts in normal and endometritis affected slaughtered cows (Ahmadi *et al*., 2005).

There was no enough information about cytological comparison of cervix and uterus in postpartum cows. The aim of this study was to compare cervical and uterine cytology between different classification of postpartum endometritis and bacterial isolates in Holstein dairy cows.

MATERIALS AND METHODS

Animals

During autumn of 2005 to summer of 2006 this study was carried out in 13 big commercial dairy herds in 5 provinces of Iran. Four hundred two postpartum Holstein dairy cows were examined once between 21 and 35 days postpartum. In total, 89 clinical endometritis affected cows were selected. Cows in all herds were calved in calving boxes hygienically and kept in individual boxes for at least 10 days after parturition. Corn silage, alfalfa hay and concentrates as a total mixed ration were used. None of the cows received any intrauterine or reproductive hormonal therapy at least 10 days before sampling for this study.

Clinical Examination

At first, cows were inspected for the presence of fresh discharge on the vulva, perineum, or tail. If discharge was not visible externally, cows were examined vaginally. The cow's vulva was thoroughly cleaned with a dry paper towel and a clean, lubricated, gloved hand was inserted through the vulva. In each cow, the lateral, dorsal and ventral walls of the vagina were palpated and the mucus contents of the vagina withdrawn manually for examination (Sheldon *et al*., 2002). The vaginal mucus was assessed for color and proportion of pus. Endometritis was classified in three categories: clear mucus with flakes of pus (E1), mucopurulent discharge or fluctuating contents in the uterus (E2) and purulent discharge with or without palpable contents in the uterus (E3) (Drillich *et al*., 2005).

Uterine Swab Collection and Bacteriological Culture

For each animal, a transcervical guarded swab was collected from the uterine body (Noakes *et al*., 1989). The swab comprised a long copper wire bearing a cotton wool tip sheathed in a metal guard tube

(8 mm external diameter; 58 cm long) and was wrapped and sterilized by autoclaving at 121°C for 15 min. The guard tube was covered by a sterile plastic sheath to prevent contamination of the swab during the cervix insertion.

After restraining the animal and securing its tail, the perineal region was washed and cleaned. The cervix was grasped per-rectum and the sterilized catheter was passed through the cervix into the uterine body. Then, the inner rod of the catheter was pushed forward to expose the swab to the endometrium and was rotated against the uterine wall and then withdrawn within the catheter. To avoid contamination, catheter was cleaned with alcohol. Swabs were cultured immediately on sheep blood agar and MacConkey agar (MERCK) and incubated at 37°C for 48 h. The same culture on sheep blood agar (MERCK) was incubated anaerobically for up to 7 days. Standard biochemical tests were used for the isolation and identification of the isolates (Quinn *et al*., 1994). The result of bacteriological culture was classified in three groups: A - no growth, B - positive bacterial growth without *A. pyogenes* and C - positive bacterial growth with *A. pyogenes*.

Cervical and Uterine Cytology

Cytological samples were obtained from the discharge of cervical mucus and uterine fluid. Cervical aspirated samples were collected by gentle suction from the cervical external os with a plastic uterine pipette and aspirated by suction with a 50 mL syringe (Ahmadi *et al*., 2006a). Uterine swabs which were collected for bacteriological culture were also used for cytological examination. Once the samples had been taken, the swabs were rolled on glass slides.

Thin smears were prepared for cytological examination by smearing a drop of cervical mucus on a clean slide. The smears were then allowed to dry at room temperature for 30-35 min. Slides were transported to the laboratory and examined within two hours of collection. A differential cell count of each smear was done on Giemsa-stained slides. Hundred to 200 cells were counted in each of 20 microscopic fields (x900). Recorded cell types were epithelial, large vacuolated epithelial, neutrophils, lymphocytes and macrophages (Jain, 1986).

Blood Sampling and P4 Assays

Blood samples were collected from the coccygeal vein or artery into evacuated tubes and transported on ice to the laboratory. Serum was separated by centrifugation at 2500 x g for 10 min and stored frozen at -20°C until required. Plasma progesterone concentration was measured by radioimmunoassay (Spectria® Progesterone RIA, Espoo, Finland) with a sensitivity of 0.1 ng mL^{-1} and intra- and inter-assay coefficients of variation of 10.2 and 6.5%, respectively.

Statistical Analysis

Data were analyzed by using SAS software, version 9.1. Means of cervical and uterine cells of endometritic cows with different discharges, bacterial finding and ovarian status were compared with t-test and One way ANOVA. Duncan multiple range test used as a posthoc test for determination of significant difference between groups (SAS, 1991).

RESULTS

The neutrophils percentage in cervical mucosa and uterine fluid of the *Arcanobacterium pyogenes* culture group were significantly ($p<0.05$) higher than no growth and positive culture groups. There were significantly ($p<0.05$) differences between epithelial cells percentage in cervical mucosa between *Arcanobacterium pyogenes* and positive culture groups. No growth and positive culture groups showed lower epithelial cells percentage than *Arcanobacterium pyogenes* group in uterine smear. Also

macrophage cells percentage in cervical mucosa of the cows affected to *Arcanobacterium pyogenes* were significantly ($p<0.05$) higher than cows in other groups. The large vacuolated epithelial cells (LVEP) percentage in cervical mucosa in cows contaminated by *Arcanobacterium pyogenes* and no growth were higher significantly ($p<0.05$) than that percentage in uterine fluid (Table 1).

The neutrophil percentage in cervical mucosa of the cows affected to endometritis group E3 were significantly ($p<0.05$) higher than groups E2 and E1. Also there was significantly ($p<0.05$) differences between neutrophil percentage of uterine fluid in groups E3 and E1. There were significantly ($p<0.05$) differences between epithelial cells percentage in cervical and uterine smear of groups E3 and E1. Also macrophage cells percentage in cervical mucosa of the cows affected to endometritis group E2 were significantly ($p<0.05$) higher than groups E1. Lymphocyte cells percentage in uterine fluid of the cows affected to endometritis group E3 were significantly ($p<0.05$) higher than groups E2. The large vacuolated epithelial cells (LVEP) percentage of cervical mucosa in cows affected to endometritis groups E1, E2 and E3 were higher significantly ($p<0.05$) than that percentage in uterine fluid. Also macrophage cells percentage in cervical mucosa of the cows affected to endometritis group E1 were significantly ($p<0.05$) higher than that percentage in uterine fluid (Table 2). There was no significant difference in neutrophils percentage between cervical mucosa and uterine fluid in all different classification in postpartum dairy cows (Table 1, 2). Except LVEP cells, there was no significant difference between cervical and uterine cells percentage of cows with progesterone above and below of 1 ng mL^{-1} (Table 3).

Table 1: The mean (±SD) of the percentage of cells in the cervical mucosa and uterine fluid of endometritic-affected cows with different results of the bacterial culture

Bacteriology cells	No growth 47	Positive 21	*Arcanobacterium* 18
Neut - C	14.51±20.58^{a}	12.71±17.40^{a}	26.67±22.38^{b}
EPC - C	76.89±19.44^{ab}	80.81±16.82^{a}	66.61±21.26^{b}
LVEP- C	8.19±7.07*	5.24±4.84	5.61±3.77*
Lym - C	0.30±0.80	1.14±3.93	0.78±1.52
Mac - C	0.11±0.31^{a}	0.09±0.44^{a}	0.33±0.48^{b}
Neut - U	14.54±22.70^{a}	23.55±24.04^{a}	45.73±33.41^{b}
EPC - U	80.50±23.93^{a}	69.70±24.94^{a}	51.27±31.95^{b}
LVEP- U	3.50±5.77	3.10±4.67	2.13±3.89
Lym - U	1.19±3.15	3.30±10.81	0.67±1.54
Mac - U	0.26±0.65	0.35±0.99	0.20±0.56

EPC: Epithelial cells, LVEP: Large vacuolated epithelial cells, MAC: Macrophage, Neut: Neutrophils, Lym: Lymphocytes, LH: Lutenizing hormone, C: Cervix, U: Uterus, Values with different superscripts in each row are those that differ significantly ($p<0.05$). *: Indicates significant difference in each column between the same cells percent of cervical mucosa and uterine fluid ($p<0.05$)

Table 2: The mean (±SD) of the percentage of cells in the cervical mucosa and uterine fluid of postpartum cows with different grades of endometritic

	Endometritis classification		
Cells	E1 (57)	E2 (11)	E3 (18)
Neut - C	12.65±18.37^{a}	14.92±10.92^{a}	28.05±26.81^{b}
EPC - C	78.88±17.67^{a}	77.67±11.28^{ab}	65.95±25.26^{b}
LVEP - C	7.76±6.91*	6.92±4.44*	5.00±3.41*
Lym - C	0.64±2.45	0.17±0.58	0.74±1.48
Mac - C	0.07±0.25^{a}*	0.33±0.65^{b}	0.26±0.45^{ab}
Neut - U	16.27±23.86^{a}	27.36±31.91^{ab}	42.70±30.93^{b}
EPC - U	78.30±25.06^{a}	64.91±31.27^{ab}	55.94±30.93^{b}
LVEP - U	3.70±5.79	3.00±4.05	1.06±2.01
Lym - U	1.39±3.48^{ab}	4.55±14.09^{a}	0.23±0.44^{b}
Mac - U	0.34±0.81	0.18±0.60	0.06±0.24

Values with different superscripts in each row are those that differ significantly ($p<0.05$). *: Indicates significant difference in each column between the same cells percent of cervical mucosa and uterine fluid ($p<0.05$)

Table 3: The mean (±SD) of the percentage of cells in the cervical mucosa and uterine fluid of endometritic-affected in different progesterone levels

	Progesterone concentration	
Cells	P4 < 1 ng mL^{-1} (58)	P4 > 1 ng mL^{-1} (28)
Neut - C	18.40±23.35	12.93±13.25
EPC - C	74.29±22.40	78.61±11.99
LVEP- C	6.36±5.92*	8.11±6.44*
Lym - C	0.78±2.55	0.25±0.70
Mac - C	0.17±0.38	0.11±0.42
Neut - U	21.66±26.70	24.52±29.90
EPC - U	73.39±27.02	70.24±29.86
LVEP- U	3.34±4.91	2.72±5.83
Lym - U	1.27±3.42	2.40±9.38
Mac - U	0.34±0.81	0.12±0.44

*: Indicates significant difference in each column between the same cells percent of cervical mucosa and uterine fluid ($p<0.05$)

DISCUSSION

The initial uterine defense against the bacterial infection is phagocytosis by the uterine leucocytes (mainly neutrophils). Neutrophils constitute the first line of an increase in postpartum defense against the invading pathogenic organisms resulting in large neutrophil populations within the uterine lumen (Watson *et al*., 1990; Butt *et al*., 1991; Hussain and Daniel, 1991). Phagocytosis is a primary mechanism involved in the elimination of bacteria from the cow's uterus. The phagocytic activity of leucocytes in the uterine fluid seems to play a crucial role in halting the propagation and establishment of bacterial infection in the uterus immediately after calving and thereafter (Hussain and Daniel, 1992). Effective phagocytosis within the uterine lumen necessitates the mobilization and migration of adequate numbers of neutrophils from the peripheral circulation in response to a chemotactic stimulus produced either directly or indirectly by the bacteria that are present (Hussain and Daniel, 1991; Anderson *et al*., 1985; Lander Chacin *et al*., 1990). Klucinski *et al*. (1990) showed that the inflammatory process in the uterus caused intensive migration of large numbers of neutrophils into the lumen.

Endometrial cytology and ultrasonography were used as diagnostic techniques for identification of subclinical endometritis. Cytological methods can be useful for the diagnosis of clinical and subclinical endometritis of cows (Kasimanickam *et al*., 2004; Ahmadi *et al*., 2006a). Cervical cytology has no side effects on the uterus of cows (Ahmadi *et al*., 2003) and it can be used as a practical and applicable method in dairy herds (Ahmadi *et al*., 2005, 2006a). The result of this study couldn't show any significant differences between neutrophils percentages of cervical mucosa and uterine fluid smear in cows with three classifications of postpartum endometritis. It is accordance with our reports on the slaughtered cows' genital tracts of normal cows (Ahmadi *et al*., 2004) and endometritis affected cows (Ahmadi *et al*., 2005). Also there were significantly differences in neutrophils percentage between different group of bacterial culture results and between endometritis severity classification grades, but the result of this study supported that the increase in neutrophils percentage in uterine fluid was in accordance with increase of neutrophils percentage in cervical mucosa. The result of this study revealed that the contamination of uterus to Arcanobacterium cause increase neutrophils migrant to uterus and severity of endometritis.

The result of this study in all endometritis classification showed that the LVEP percentage in cervical mucosa is higher than in uterine fluid in postpartum period. Ahmadi *et al*. (2005) reported that the LVEP percentages were low and there was no significant difference between percentages of LVEP in cervical mucus and uterine fluid in the slaughtered cows' genital tracts. Ahmadi *et al*. (2006a) reported that the mean of LVEP cells percentage in cervical mucus of normal cows was 4.52±0.15 in 25-30 days of postpartum. The mean (±SEM) of LVEP cells percentage in cervical mucus of

55-60 days of postpartum in cows conceived with 2 or 3 artificial (8.36±0.66) was significantly ($p<0.5$) higher than cows conceived with one inseminations (2.85±0.14). It is reported in dairy cattle the time taken for complete involution ranged from 26 to 52 days but the changes after 20-25 days are generally almost imperceptible (Noakes *et al*., 2000). The cervix also involutes from 15 cm in 2 days postpartum to 5-6 cm at 60 days (Gier and Marion, 1968). It seems that the regeneration of cervical tissue is active about 25-35 days postpartum. The sample collection in study was in this period and all cows affected to endometritis. However there was no enough report about changes in percentages of LVEP, Macrophage and lymphocyte cell. So we recommended further study for illustration of reason significant difference between percentages of LVEP in cervical mucus and uterine fluid.

The results of our study reveal no relation between level of progesterone and cell percentages in postpartum cows. The LVEP cell percentages in cervical mucosa was higher significantly than of uterine fluid in postpartum cows with P4>1 ng mL^{-1} and P4<1 ng mL^{-1}. We reported in previous work that the hormonal changes in different phases of the estrous cycle affect neutrophil migration to cervical mucosa so that neutrophil percentage significantly increased during metestrus in cervical mucosa (Ahmadi *et al*., 2006b). The result of present study didn't reveal the effect of progesterone level on percentage of LVEP cells. Progesterone suppresses uterine immune defenses and predisposes the uterus to nonspecific infections. This occurs most commonly in postpartum animals and postpartum uterine infections may reduce the reproductive performance of livestock (Lewis, 2004). The estrogen action causes decrease in viscosity and maximum ferning of cervical mucus on the day of estrus to produce a string of clear mucus hanging from the vulva (Jainudeen and Hafez, 2000). The study of Subandrio *et al*. (2000) did not support the hypothesis that the influence of the reproductive state of the cow on the resistance of the uterus to infection is mediated by the inherent differences in either peripheral or intrauterine neutrophil function.

Cervical mucosa can be taken without manipulation of cow uterus at commercial herd and there were no side effects on the uterus of cows. Therefore, this method is practical and applicable in all commercial dairy herds. In the other hand it seems evaluation of LVEP cells percentage may be useful for determine voluntary waiting period in dairy industry. In conclusion, it is suitable the cytological evaluation of cervical smear at fresh cows for diagnosis of subclinical endometritis, planning for treatment and prognosis of fertility after voluntary waiting period of dairy cows.

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