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Comparative study on the relationship between cytobrush cytology and histopathological examinations on endometrium of slaughtered cows without clinical symptom

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Abstract

Cytobrush cytology of the endometrium has been developed as a practical procedure to diagnose subclinical endometritis in cows and polymorphonuclear neutrophils (PMNs) were used as an indicator of inflammation. However, mononuclear cells, i.e., lymphocytes, macrophages, and plasma cells, are also important for diagnosing chronic inflammation. Therefore, we investigated whether cytobrush cytology based on the percentages of inflammatory cells can evaluate the infiltration of these cells in the endometrium of cows without clinical symptom by comparing cytobrush and histopathological samples collected from slaughtered cows within two years of parturition. Eighteen out of 39 cows (46.2%) were defined as histopathologically inflammation positive. The percentages of PMNs (PMN%) and lymphocytes in cytological samples were higher in positive cows than in negative cows ($P < 0.01$). In positive cows, PMN% was higher in superficial region than in deep region ($P < 0.01$) and the percentages of lymphocytes was higher in the stratum compactum than in cytological sample and the epithelial region ($P < 0.05$). These results indicated that the increase observed in PMN% in cytological samples related to the presence of inflammation in endometrium because PMNs infiltrated mainly into the surface of the endometrium. The increase of lymphocytes in cytobrush cytology indicated chronic endometritis; therefore, histopathological examination is recommended for correct diagnosis because more lymphocytes infiltrated into stratum compactum than epithelial region.

Key Words: Cytobrush cytology, Endometritis, Histopathology

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Introduction

Postpartum cows are at risk of reproductive tract disorders, such as metritis, clinical endometritis, and subclinical endometritis (SE). Endometritis is histopathologically characterized by the disruption of the surface epithelium, the infiltration of inflammatory cells, vascular congestion, and stromal edema and also by varying degrees of the accumulation of lymphocytes and plasma cells in the superficial layers[3,30,41,43]. These alterations in the endometrium may have detrimental effects on the secretion of cytokines from endometrial cells, such as granulocyte-macrophage colony-stimulating factor[9,34] and endometrial epidermal growth factor[31], which regulate endometrial and embryonic functions during early pregnancy and are associated with subfertility in cattle[18,19,36]. Thus, it is important to diagnose endometritis precisely, in particular, SE is defined as inflammation of the endometrium in the absence of the signs of clinical endometritis, such as a purulent or mucopurulent vaginal discharge in postpartum cows[43,44], and reduces reproductive performance[16,25,38].

Histopathological examinations by endometrial biopsy enable veterinarians to examine various inflammatory cells and alterations in the uterine endometrium and also diagnose SE in cows[6]; however, they are complex and time-consuming[4,6,44]. Cytobrush cytology of the endometrium has been developed as a practical procedure to diagnose SE in cows based on fertility[17]. Many researchers have focused on polymorphonuclear neutrophils (PMNs) as an indicator of inflammation in the endometrium by cytobrush cytology[2,17,24] because they are major structural components of acute inflammation in the endometrium[11]. Furthermore, previous studies reported positive relationships between the percentage of PMNs (PMN%) detected in smears collected by a cytobrush and those in endometrial histopathological samples collected by endometrial biopsy[13,33]. However, mononuclear cells, i.e., lymphocytes, macrophages, and plasma cells, are also important for diagnosing chronic inflammation in cattle[11,41]. Therefore, not only PMN%, but also the percentage of mononuclear cells may contribute to a more accurate diagnosis of SE. The detection of plasma cells within inflammatory infiltrates in the endometrium is a standard diagnostic method for chronic endometritis[8,15,45], and macrophages are observed in chronic endometritis in women[46]. In the endometrium of women, plasma cells are often detected in the functional layer near the basal layer (the superficial layer of the stratum compactum), while lymphocytes are mainly present in the basal layer (the deep region of the stratum compactum)[21]. However, the distribution of mononuclear cells in bovine endometrium remains unclear and the importance of lymphocytes, macrophages, and plasma cells in cytological samples for the diagnosis of SE has not yet been assessed in cows. Furthermore, the distribution of inflammatory cells, including PMNs and mononuclear cells, in the deep region of the endometrium has not yet been elucidated.

The objectives of the present study were to investigate whether the proportions of each inflammatory cell (PMNs, lymphocytes, macrophages, and plasma cells) in cytological samples reflect inflammation in endometrium by examining histopathological features and distribution of inflammatory cells in the endometrium. In addition, we divided the stratum compactum in endometrium into two regions (superficial and deep regions) to examine the relationship between distributions of inflammatory cells and the results of cytobrush cytology precisely. We also investigated the relationship between bacterial isolation from the cytobrush and the percentage of inflammatory cells in cytological and histopathological samples.

Materials and Methods

Animals

Animal handling and experimental procedures were performed according to the Guidelines for

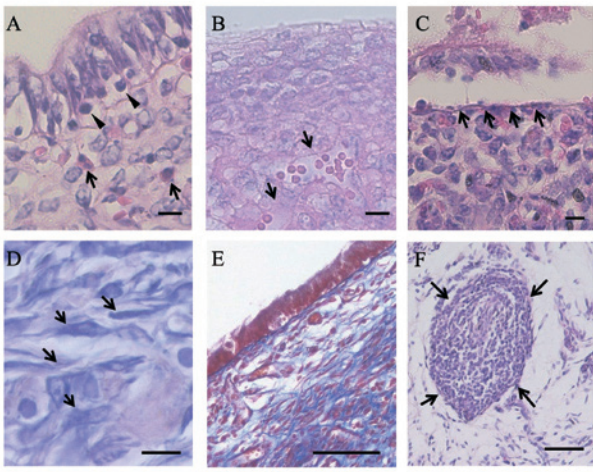

Fig. 1. Histopathological features of the endometrium with subclinical endometritis.
(A) Arrows indicate polymorphonuclear neutrophils (PMNs) and arrowheads show lymphocytes. Hematoxylin-eosin (HE) stain. Bar = 10 µm.
(B) Arrows indicate an increased number of capillaries. HE stain. Bar = 10 µm
(C) Arrows indicate desquamated epithelial cells of the endometrium. HE stain. Bar = 10 µm.
(D) Arrows indicate fibrin deposits. Phosphotungstic acid hematoxylin stain. Bar = 10 µm.
(E) Tissue fibrosis with collagen (stained blue) observed in the stratum compactum. Masson's trichrome stain. Bar = 100 µm.
(F) Arrows indicate the aggregation of lymphocytes. HE stain. Bar = 100 µm.

the Proper Conduct of Animal Experiments by the Science Council of Japan. We conducted experiments between September 2017 and December 2018 at a local slaughterhouse in Shimane prefecture, Japan. Immediately after slaughter, viscera were extirpated from a cow, and the uterus was removed intactly by cleaving vagina and placed in a refrigerated container. To exclude cows with clinical endometritis, those with vaginal mucus containing white or off-white mucopurulent materials[44,48] were not used in the present study. Holstein cows (n = 36, age: 6.3 ± 2.4 years old) and Japanese Black cows (n = 3, age: 15.1 ± 1.5 years old) were used in experiments and their body condition scores[12,20] were between 2.5 and 3.75 and ranging between 38 days and two years after parturition (8 cows at 38-99 days postpartum, 20 cows at 108-346 days, and 11 cows at 366-709 days postpartum). Approximately 40% of cows (n = 17) had clinical disorders, such as motor disorders (hip dislocation, arthritis, and myositis), respiratory diseases, and mastitis.

Sample collection

We stored uteri at 4°C and collected all samples within 3 h of slaughter. Endometrial cytology smears and histopathological samples were collected at the median part of the right uterine horn, the left uterine horn, and the uterine body. After making dorsal incisions with surgical scissors from the uterine body to the right and left uterine horns, smears for cytology were obtained using a cytobrush (Metribrush, Fujihira Industry Co., Ltd., Tokyo, Japan) that was rolled gently 360° twice in a clockwise direction. Cytology samples obtained by the cytobrush were prepared on a glass slide. Histopathological samples were obtained from tissues near the site of cytobrush sampling. An approximately 20 × 20 mm full-thickness endometrium specimen was excised using a scalpel and surgical scissors from each uterine region.

Staining and evaluation of histopathological and cytology samples

Histological samples were fixed for 5-7 days with Tissue-Tek Ufix (Sakura Finetech, Tokyo, Japan) and then processed in paraffin wax. Tissue sections (thickness of 3 µm) were conventionally stained with hematoxylin-eosin (HE) and mounted on glass slides. Slides were examined under a light microscope. We defined cows as histopathological feature positive on endometrium if any of the following features were observed on histopathological samples, i.e., the marked infiltration of inflammatory cells (PMNs, lymphocytes, macrophages, and plasma cells) in the endometrium (≥ 20 inflammatory cells per five high power fields), an increased number of capillaries (angiogenesis) in stratum compactum, desquamated epithelial cells of the endometrium, fibrin deposits, fibrosis, and aggregation of lymphocytes (Figs. 1A-F)[3, 28, 29, 41]. To distinguish fibrin deposition and fibrosis from

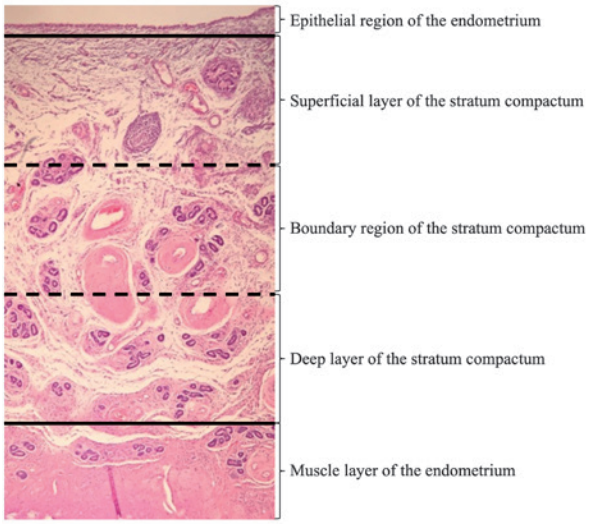

Fig. 2. Histopathological regions of the endometrium in cows. Inflammatory cells in the epithelial region and the stratum compactum (superficial and deep layers) were evaluated in HE-stained slides.

The boundary region of the stratum compactum was not used to evaluate the distribution of inflammatory cells.

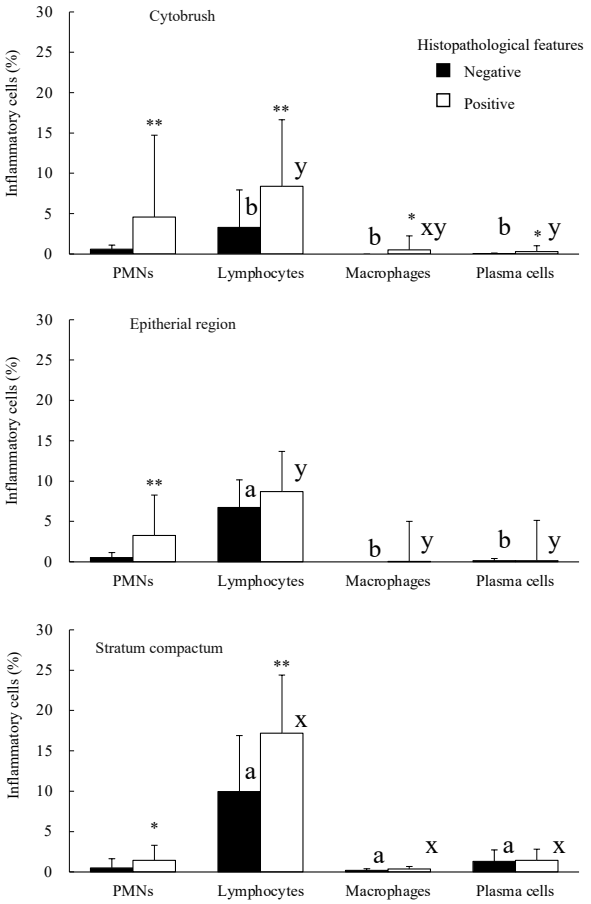

Fig. 3. Percentages of polymorphonuclear neutrophils (PMNs), lymphocytes, macrophages, and plasma cells in cytological and histopathological (epithelial region and stratum compactum) samples of histopathological feature negative and positive cows.

The highest values of each inflammatory cell observed in the uterine regions were used as representative values in individual cows.

Asterisks indicate a significant difference between histopathological feature negative (n = 21) and positive (n = 18) cows (*$P < 0.05$ and **$P < 0.01$).

[ab and xy] Different letters indicate a significant difference between samples ($P < 0.05$).

eosinophilic structures on HE-stained slides, we also conducted phosphotungstic acid hematoxylin and Masson's Trichrome staining, respectively. The proportion of inflammatory cells (PMNs, lymphocytes, macrophages, and plasma cells) based on all nucleated cells (including epithelial cells and stromal cells) was evaluated in the epithelial region by counting 500 cells and in the stratum compactum by counting 500 cells (summation of 250 cells each in the superficial and deep regions) at ×400 magnification (Fig. 2) of the endometrium[33,40]. To minimize the deflection of inflammatory cells, the regions of the stratum compactum with aggregated lymphocytes were excluded from cell counting. We also assessed differences in the percentages of PMNs, lymphocytes, macrophages, and plasma cells between the superficial and deep regions of the stratum compactum.

Cytological samples were fixed and stained with the Hemacolor system (Merck, Tokyo, Japan). Cytology slides were then dried and mounted with Eukitt (O. Kindler GmbH & Co., Freiburg, Germany). By counting 200 cells at ×400 magnification under a light microscope, the proportion of inflammatory cells (PMNs, lymphocytes, macrophages, and plasma cells) based on all nucleated cells (including epithelial cells and stromal cells) was evaluated in cytological samples[1,16,40,42].

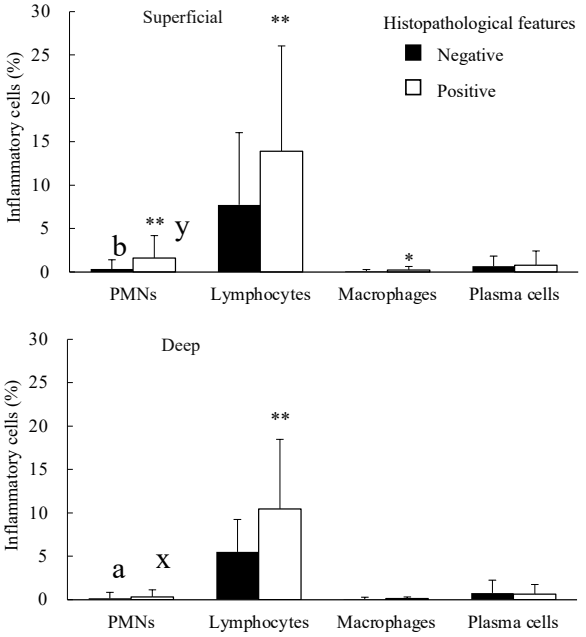

Fig. 4. Distribution of inflammatory cells between superficial and deep regions of the stratum compactum in the endometrium of histopathological feature negative (n = 21) and positive (n = 18) cows.

The average of each inflammatory cell observed in the uterine body, left uterine horn, and right uterine horn was used as a representative value in individual cows.

Asterisks indicate a significant difference between histopathological feature negative and positive cows (*$P < 0.05$ and **$P < 0.01$).

[ab] Different letters indicate a significant difference between regions ($P < 0.05$).

Table 1. Intraclass correlation coefficients of polymorphonuclear neutrophils (PMNs), lymphocytes, macrophages, and plasma cells between the uterine body, left uterine horn, and right uterine horn in cytobrush and histopathological (the epithelial region and stratum compactum) samples.

Inflammatory cells	Intraclass correlation coefficients in each sample		
	Cytobrush	Epithelial region	Stratum compactum
PMNs	0.739	0.737	0.806
Lymphocytes	0.410	0.466	0.370
Macrophages	0.319	0.327	0.001
Plasma cells	0.178	0.169	0.312

Bacterial culture

Suspensions (100 µl) of endometrial smears from the cytobrush were cultured aerobically on sheep blood agar at 37°C for 48 h. Colonies on sheep blood agar were identified based on the shape of the colony, Gram staining, hemolysis, and bacterial cellular morphology. Bacterial isolation was defined as positive when three or more of the same colonies were isolated.

Statistical analysis

The percentages of inflammatory cells (the maximum values in all uterine regions were used as the representative value of each cow) in cytological and histopathological (the epithelial region and stratum compactum) samples were compared between with and without histopathological features or between with and without bacterial isolation by the Student's t-test and between cytological and histopathological (the epithelial region and stratum compactum) samples using a one-way ANOVA followed by Tukey-Kramer's multiple comparison test to clarify the relationship between the results of cytobrush cytology, histopathological inflammation and infection on endometrium (Figs. 3 and 7). All pairwise comparisons of the least squares means of the percentages of inflammatory cells (each value from three uterine regions were used for analyses) at the superficial and deep regions of the stratum compactum were conducted to assess the distributions of inflammatory cells followed by Tukey-Kramer's multiple comparison test (Fig. 4). Spearman correlation coefficients of PMN% and the percentage of mononuclear cells (MNC%) were calculated to examine the relationship between cytological and histopathological samples among uterine regions (Figs. 5 and 6). Prior to the Student's *t*-test and one-way ANOVA, the percentages of cells in each sample were transformed by empirical logistic transformation to normalize data. The intraclass correlation coefficients[22,23] of the percentages of inflammatory cells in each uterine region (the uterine body, left uterine horn, and right uterine horn) were evaluated to clarify the effects of the sampling site in the uterus (Table 1). Differences of $P <$

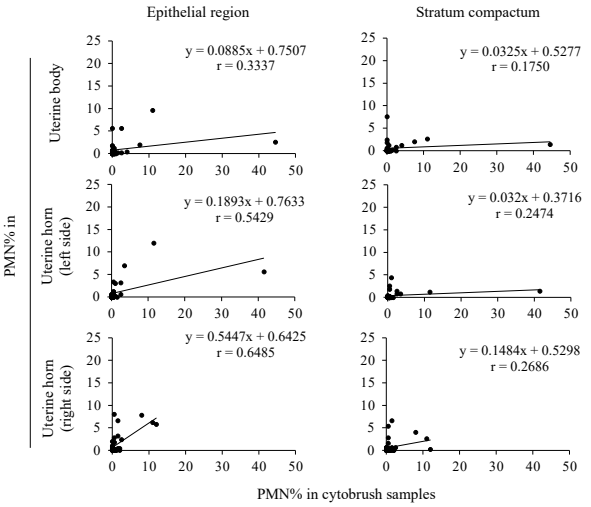

Fig. 5. Relationships between the percentages of polymorphonuclear neutrophils (PMN%) in cytological and histopathological samples (epithelial region and stratum compactum) in the uterine body and left and right uterine horns.

The lines indicate a linear regression between PMN% in each sample.

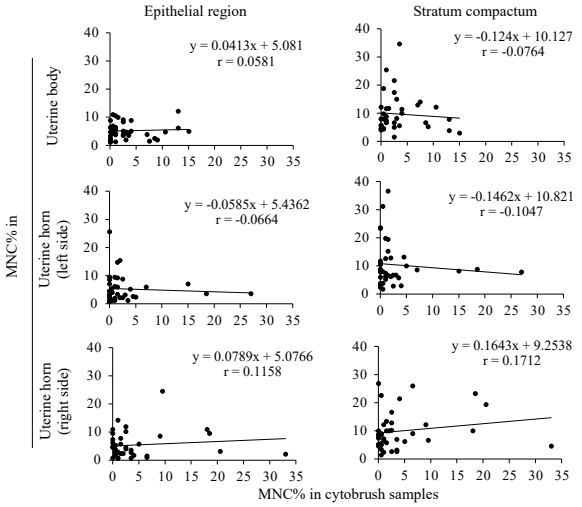

Fig. 6. Relationships between the percentages of mononuclear cells (MNC%) in cytological and histopathological samples (epithelial region and stratum compactum) in the uterine body and left and right uterine horns.

The lines indicate a linear regression between the MNC% including lymphocytes, plasma cells, and macrophages in each sample.

0.05 were considered to be significant. Statistical analyses were performed using Excel for Mac (Microsoft Corporation, Seattle, USA), Ekuseru-Toukei 2008 software for Windows (Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results

Eighteen out of 39 cows (46.2%) were defined as histopathological feature positive; marked infiltration of inflammatory cells 23.1% (9/39), increased number of capillaries 12.8% (5/39), abruption of the epithelium 10.3% (4/39), fibrin deposition 5.1% (2/39), fibrosis 12.8% (5/39), and lymphocyte aggregation 28.2% (11/39).

The relationships between the percentages of each inflammatory cell in cytological and histopathological (the epithelial region and stratum compactum) samples in cows with and without histopathological features are shown in Fig. 3. The percentages of PMNs, lymphocytes, macrophages, and plasma cells in cytological samples were higher in positive cows than in negative cows ($P < 0.05$). The percentages of PMNs in the epithelial region were higher in positive cows than in negative cows ($P < 0.01$). The percentages of PMNs and lymphocytes were higher in positive cows than in negative cows in the stratum compactum ($P < 0.05$). The percentages of lymphocytes in negative cows were higher in the epithelial region and the stratum compactum than in cytological samples ($P < 0.05$), and that in positive cows was higher in the stratum compactum than in cytological sample and the epithelial region ($P < 0.05$). Macrophages and plasma cells were rarely detected in any samples, and lymphocytes were the main mononuclear inflammatory cell.

The percentages of inflammatory cells in the stratum compactum of histopathological samples were shown in Fig. 4. At the superficial region of stratum compactum, PMN% was higher in positive cows than in negative cows ($P < 0.01$). In addition, PMN% in negative and positive cows were higher in superficial region than in deep region ($P < 0.01$). The percentages of lymphocytes at the superficial and deep regions of the stratum compactum were

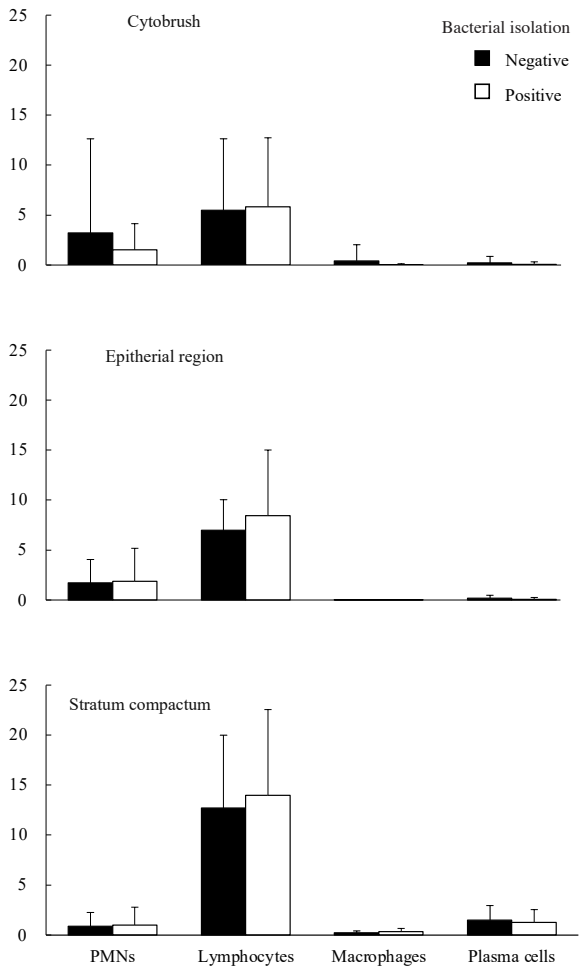

Fig. 7. Percentages of inflammatory cells (PMNs, lymphocytes, macrophages, and plasma cells) in cytological and histopathological (epithelial region and stratum compactum) samples of bacterial isolation negative (n = 21) and positive (n = 18) cows.

The highest values of each inflammatory cell observed in the uterine regions were used as representative values in individual cows.

higher in positive than in negative cows ($P < 0.01$). As shown in Fig. 5, Spearman correlation coefficients of PMN% between cytological and histopathological (epithelial region) samples and between cytological and histopathological (stratum compactum) samples at uterine body were lower than those at uterine horns (left and right side). Spearman correlation coefficients of the percentages of mononuclear cells (total of lymphocytes, macrophages, and plasma cells) in all uterine regions between cytological and histopathological samples (epithelial region and stratum compactum) were low in all uterine regions (Fig. 6) compared to those of PMN%.

Agreements in the percentages of inflammatory cells between the uterine body, left uterine horn, and right uterine horn in cytological and histopathological (the epithelial region and stratum compactum) samples were evaluated by intraclass correlation coefficients (Table 1). The intraclass correlation coefficients of PMN% (0.737-0.806) were higher than those of lymphocytes, macrophages, and plasma cells in cytological samples, the epithelial region, and stratum compactum (0.001-0.466).

Bacteria were isolated from 18 out of 39 cows (46.2%; 6/8, 6/20, and 6/11 cows at 38-99, 108-346, and 366-709 days postpartum, respectively) and the percentages in histopathological feature positive (55.6%, 10/18) and negative cows (38.1%, 8/21) were similar ($P > 0.05$). In addition, no significant differences were observed in the percentages of any inflammatory cells in cytological and histopathological (epithelial region and stratum compactum) samples between cows with and without bacterial isolation (Fig. 7).

Discussion

Cytobrush cytology in cows has been developed based on fertility[16] on farm and accepted as a diagnostic method for endometritis in recent years by whose less-invasiveness and rapidity compared with histopathology[2,17,24]. However, there is little information about correlation of the proportion of inflammatory cells in cytological sample with histopathological changes on endometrium in cows. In the present study, PMN% in cytological sample and histopathological sample of epithelial region and stratum compactum increased in cows with histopathological features. By dividing the stratum compactum into the superficial and deep regions, we also found that the presence of endometrial inflammation increased infiltration of PMNs on

the superficial region of stratum compactum. These results suggest that PMNs infiltrate into mainly the surface of the endometrium, and increase observed in PMN% in cytological samples reflect the uterine acute inflammation since PMNs migrate to the tissue in response to injury or infection at acute inflammation phase in general in mammals[39]. However, veterinarians need to consider false positives and false negatives by cytobrush cytology when diagnosing SE based on PMN% in clinical practice. In the present study, the correlation coefficients between cytological and histopathological (epithelial region and stratum compactum) samples were lower at uterine body compared with uterine horns. It may be caused by the higher value of PMN% on uterine body in a cow in the present study. These results indicate the limitations of cytobrush cytology as a diagnostic method for endometritis because cytobrush collects superficial cells only and debris in the uterine luminal contents derived from other sites in the uterus and/or cervix. Also it was reported that debris in the uterine lumen included neutrophils and epithelial cells in mares[7] and cows[17] and that subclinical cervicitis in cows without endometritis[10,24] may cause contamination of inflammatory cells from uterine cervix on endometrium of uterine body. From the results, we recommend to collect samples from uterine horns to avoid the contamination of inflammatory cells in cytological samples, although cytological samples were collected from uterine body in many previous studies[2,17,26] and clinical practices[16].

Our present study firstly showed that lymphocytes, which were main components of chronic inflammation in endometrium of cows[11], infiltrated mainly in the stratum compactum when cows became SE because the higher percentage of lymphocytes was observed in stratum compactum than in epithelial region and the percentages of lymphocytes in epithelial region were similar between normal and SE cows. In addition, lymphocytes mainly infiltrated into stratum compactum entirely in cows because the percentages of lymphocytes were similar between superficial and deep regions of stratum compactum in SE cows in the present study, although they were mainly present in the basal layer (the deep region of the stratum compactum) in woman[21]. Due to this localization, the correlation coefficients were quite low in the percentage of lymphocytes between cytological and histopathological samples in the present study. It means that the diagnosis of SE by cytobrush cytology solely based on the percentage of lymphocytes is challenging. However, lymphocytes infiltration means chronic inflammation in endometrium of cows[11]. Therefore, the confirmation of chronic inflammation by histopathological examination is recommended when lymphocytes are frequently observed in cytological samples.

Differences in the percentages of inflammatory cells among sampling sites in uteri have been overlooked in clinical practice. Pascottini *et al.*[32] reported the uneven distribution of PMNs and mononuclear cells among uterine regions. In the present study, the intraclass correlation coefficients were lower in the percentage of lymphocytes than in PMN% in cytological and histopathological samples, suggesting that localization of lymphocytes in endometrium varied compared to PMNs among uterine regions. Thus, sample collections from multiple uterine regions are recommended to use the percentage of lymphocytes in cytological sample as a diagnostic criterion.

Relatively large numbers of plasma cells and macrophages were observed in stratum compactum in the present study compared to epithelial region of endometrium, but those numbers were few compared with that of lymphocytes. In addition, difficulties are associated with using plasma cells and macrophages as a tool to diagnose SE in cows because they are rarely collected by cytobrush cytology. It is important to note that plasma cells were observed in some of cows without inflammation in the present study in spite of the fact that the detection of plasma cells in the endometrium is a standard diagnostic method for chronic endometritis in women[8,15]. Further studies

are necessary to use the presence of plasma cells and macrophage as a diagnostic criterion for SE in cows.

In the present study, we performed aerobic culture only to isolate aerobic or facultative anaerobe bacteria because they are the majority of SE pathogens[43,48]. In addition, isolation of bacteria by anaerobic culture from endometrial swab is not always reliable by its difficulty of sample collection[5]. In a clinical study including cows with clinical endometritis, the endometrium of infected cows had a higher number of inflammatory cells in cytological[14] and histopathological[47] samples than those of normal cows, although it was reported that there was only slight agreement between bacterial infection and PMN% or gross vaginal discharge score[27]. In addition, in previous studies excluding clinical endometritis, there was a weak relationship between bacterial isolation and PMN% by cytobrush cytology[35,37] and histopathology[13]. In the present study excluding cows with vaginal mucus containing white or off-white mucopurulent materials, PMN% and the percentages of mononuclear cells were similar between bacterial culture negative and positive cows in cytological and histopathological (epithelial region and stratum compactum) samples, presumably due to the large dispersions of the percentages of inflammatory cells in cows with and without bacterial isolation. These results suggest that SE and bacterial infection are not always concurrent. In further large-scale study, the relationship between bacterial infection and the distribution of inflammatory cells should be conducted.

In conclusion, the increase of PMN% observed in cytological samples indicates the presence of SE because PMNs infiltrate mainly into the surface of the endometrium. However, cytobrush sample should be collected from the uterine horns not uterine body for avoiding contamination. The increase of lymphocytes in cytobrush cytology may indicate chronic endometritis; however, cytobrush cytology could not detect the increase of lymphocytes correctly because they were infiltrating mainly into stratum compactum. Therefore, histopathological examination is recommended for confirmation of chronic SE. In addition, cytological and histological samples need to be collected from multiple uterine regions to observe the increase of lymphocytes because the percentage of lymphocytes in endometrium varies among uterine regions.

Conflicts of interest

The authors declare no conflicts of interest.

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