ORIGINAL ARTICLE

Veterinary Research Forum. 2020; 11 (4) 409 – 414
doi: 10.30466/vrf.2019.92037.2226
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Histopathological and cytological analyses of endometrium in water buffaloes (*Bubalus bubalis*) to detect estrus and endometritis

Zahra Salemi[1], Annahita Rezaie[2]*, Saad Goorani Nejad[3], Babak Mohammadian[2]

[1] DVM Graduate, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran; [2] Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran; [3] Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.

Article Info

Article history:

Received: 16 August 2018
Accepted: 09 March 2019
Available online: 15 December 2020

Keywords:

Bubalus bubalis
Cytology
Cytomorphometry
Endometritis
Histopathology

Abstract

This study aimed to determine cytological, histopathological and cytomorphometrical characteristics of endometrium in healthy and endometritic uterus in the water buffalo. Fifty eight non-pregnant reproductive systems were collected from slaughterhouse. Efficiency of three methods of sampling including cotton swab, smear, and aspiration were compared for cytologic study. Concurrent histopathologic examination revealed endometritis in 38 uteri including 8 (21.00%) with mild endometritis, 7 (18.42%) with moderate endometritis, 6 (15.90%) with severe endometritis and 17 (44.73%) with chronic endometritis. Cytomorphometrical results showed significant relationship between diameter and area of epithelial nuclei with phases of estrus cycle. Neutrophil and lymphocytes densities in swab and aspiration samples were significantly higher in severe endometritis than normal and chronic endometritis samples. Similarly, lymphocytes density in smear and aspiration methods was significant between normal and moderates, and also severe and chronic endometritis. Cytomorphometric analysis of epithelial nuclei characteristics (diameter and area) in buffalo were performed for the first time and it could be valuable to identify estrus cycle in this species. Aspiration had the most efficiency to identify endometritis in comparison with other methods.

Introduction

Cytology is a commonly used diagnostic method for the evaluation of the intrauterine condition and reproductive status of domestic animals such as camels, dogs, sheep, cows, and mares, and can be easily applied to living animals.[1-6] Endometrial cytologic examination of mares and cows are acceptable diagnostic techniques.[7,8] It is an easy, cheap, low-risk, and reliable method for the diagnosis of clinical and subclinical endometritis.[3]

Endometritis is an inflammation limited to the endometrium, usually occurring due to the seminal fluid or bacterial infection in non-pregnant animals.[9] In cows, postpartum endometritis occurs, to some extent, even after a normal pregnancy and parturition. It results in abortion, reduced fertility per insemination, increased interval between calving, and causing high economic losses.[10-12] Endometritis was identified as one of the most important causes of reproductive failure in cows and buffaloes.[13, 14] The high rate of endometritis in buffaloes may be the main cause of infertility issues in the south of Iran.[15] Such buffalos show longer postpartum anestrous period (six months) with inactive ovaries lacking large follicle and corpus luteum in 72.00% of the cases, and persistent corpora lutea in the remaining 28.00%.[16] It is believed that the effects of uterine infection on ovarian function are mediated by bacterial products or inflammatory mediators acting indirectly on the hypothalamus and/or pituitary gland or directly on the ovary .[17]

Concerning the decreased rate of reproduction in this valuable species and high prevalence of endometritis, the aim of this study was to evaluate cytological, histopathological, and cytomorphometrical characteristics of endometrium in water buffalo and compare the results between healthy uterine and those affected by endometritis (according to histopathological results). Also, three methods of sampling for cytologic study were compared.

***Correspondence:**

Annahita Rezaie. DVM, DVSc
Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran
E-mail: a.rezaie@scu.ac.ir

Materials and Methods

Complete reproductive tracts of 58 female mature non-pregnant water buffalo were collected from a local abattoir and transferred to laboratory freshly after slaughter. The stage of the estrus cycle (follicular and luteal stages) of the animal was estimated by careful observation of the left and right ovaries and based on the presence of growing/large antral follicles or corpus luteum.[18]

Cytology samples were prepared by three methods: 1) Cotton swabs were prepared from uterine fluid discharges. Discharges varied from clear to cloudy and brown, 2) A sample of uterus lavage was prepared by injecting 50.00 mL normal saline into the uterus. The fluid was then collected and transferred into a sterile tube and centrifuged at 1,500 rpm for 6 min. Then, a smear was prepared from the sediment,[19] 3) The direct smear of the uterus was prepared using coverslip and spread on slides. All cytology slides were air-dried, fixed in methanol, and stained with Giemsa. They underwent careful microscopic examination (BH2; Olympus, Norfolk, USA) at 100, 400, and 1000 magnifications to identify the different cellular populations. The cytomorphometric characteristics of endometrial epithelial cells nucleus such as diameter and area were analyzed by Axiovision software (Carl Zeiss, Jena, Germany). At least 10 pictures were taken from the cytology smear slides and 100 nuclei in each sample were measured under 1000 magnification. Only nuclei with complete and identifiable outlines were selected. Neutrophil and lymphocyte counts were calculated in 10 microscopic fields per slide at 400 magnification. Sections of the uterine samples were prepared and fixed in 10.00% buffered formalin and embedded in wax. Sections (5.00 µm thickness) were prepared and processed for Hematoxylin and Eosin (H & E) staining. Based on histopathology, samples were classified into control, and endometritis groups. The control group was normal in microscopic examination. The endometritis group was sub-classified to mild, moderate, severe, and chronic endometritis.[20] Mild endometritis was characterized by hyperemia, edema, and mild infiltration of inflammatory cells. Desquamation of endometrium epithelium, hyperemia, hemorrhage, and moderate infiltration of inflammatory cells such as neutrophils, lymphocytes, and plasma cells regarded as moderate endometritis. Severe endometritis was characterized by diffuse infiltration of inflammatory cells especially neutrophils both in endometrium and glands lumen. The glands were distended and their epitheliums were denuded in some parts. Multiple layers of peri-glandular fibrosis and a large accumulation of lymphocytes and plasma cells were observed in chronic endometritis and the glands were replaced by connective tissue.

Statistical analysis. The data were analyzed in SPSS (version 16.0; IBM, Armonk, USA) using *t*-test, ANOVA, and Tukey's post hoc test. The significance level was considered to be $p < 0.05$.

Results

According to ovary inspection, 51 buffaloes were in estrus cycles including 34 in luteal and 17 in the follicular phase. Four ovaries had follicular cysts and three ovaries were static. Microscopic examination revealed different types of endometritis in 38 uteri, mild endometritis in 8 (21.00%) uteri; moderate endometritis in seven uteri (18.42%); severe endometritis in six uteri (15.9%); chronic endometritis in 17 uteri (44.73%), (Fig. 1).

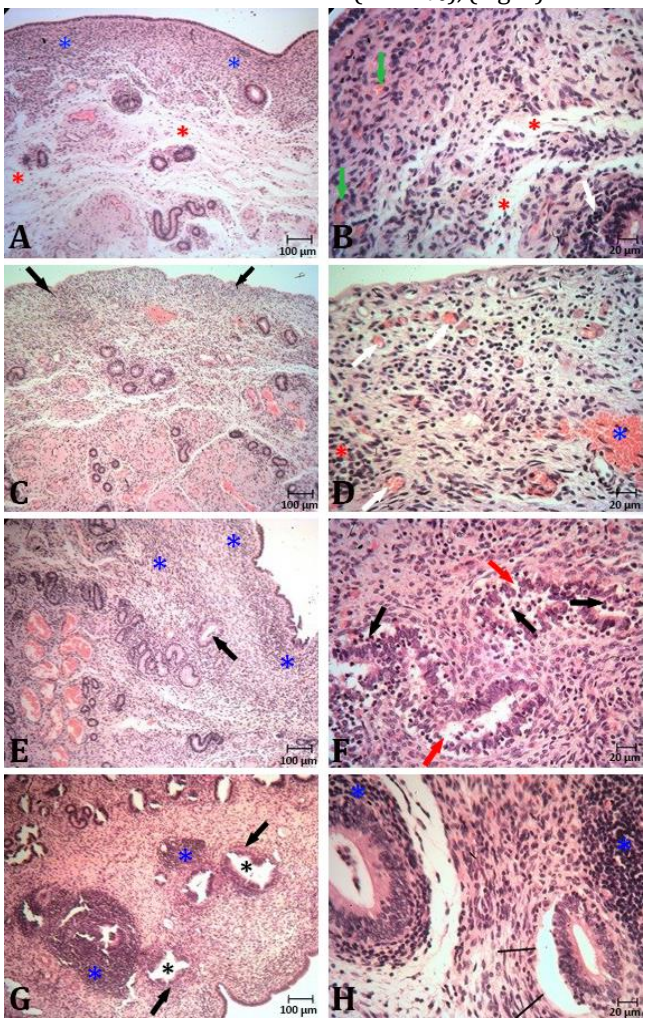

Fig. 1. Endometritis in water buffalo. **A** and **B)** Mild endometritis: Mild infiltration of inflammatory cells (blue asterisk), edema (red asterisk), hyperemia (green arrows) and accumulation of inflammatory cells (white arrow). **C** and **D)** Moderate endometritis: Desquamation of endometrium epithelium (black arrows), hyperemia (white arrows), hemorrhage (blue asterisk), and accumulation of inflammatory cells (red asterisk). **E** and **F)** Severe endometritis: Diffuse accumulation of inflammatory cells in endometrium (blue asterisks) and lumen of glands (black arrow) and desquamation of glands epithelium (red arrows). **G** and **H)** Chronic endometritis: Focal accumulation of inflammatory cells (blue asterisks) and distention of glands (black asterisks) in endometrium, lumen of glands (black arrows). Also, there are multiple layers of periglandular fibrosis (black lines), (H & E).

In the examination of cytological slides, lymphocytes, neutrophils and epithelial cells were observed with round and dark nuclei, multi-lobulated nuclei, and large nuclei with clear cytoplasm, respectively (Fig. 2).

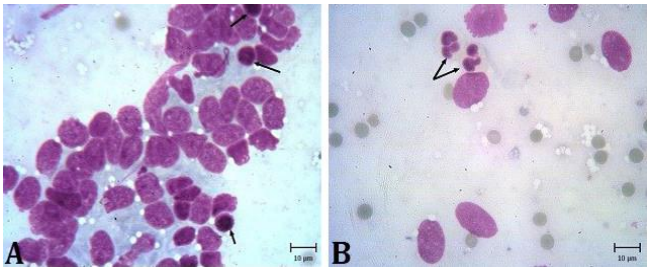

Fig. 2. Photomicrograph of the cytological smear of buffalo uterine (Endometritis group). **A)** Lymphocytes (arrows) between multiple epithelial cells. **B)** Note the shape of neutrophils with segmented nuclei (arrows) and nuclei of epithelial cells (Giemsa staining, Scale bars = 10 μm).

The cytomorphometric characteristics values and cellular counts for each estrus phase, normal or endometritis group, and different types of endometritis are summarized in Tables 1 and 2. A significant relationship was observed in the comparative statistical analysis between the diameter of the epithelia and the estrus phase ($p < 0.001$). A significant relationship was also observed between the area of epithelial cells and the estrus phase ($p < 0.001$). Moreover, the lymphocyte count was higher in the luteal phase compared to that of the follicular phase. Neutrophil count was also higher in the follicular phase compared to that of the luteal phase. No significant difference was observed in the statistical analysis of inflammatory cells between the estrus cycles ($p > 0.05$).

Table 1. Cytomorphometric characteristics value and cellular counts for each estrus phase. Data are presented as mean ± SEM.

Parameters	Luteal phase	Follicular phase
Area (μm²)	62.51 ± 1.24	71.30 ± 1.80
Diameter (μm)	10.64 ±0.12	11.40 ± 0.15
Neutrophils (swab)	55.10 ± 2.74	5.10 ± 3.83
Neutrophils (direct smear)	3.20 ± 0.74	6.94 ± 4.59
Neutrophils (lavage)	6.00 ± 1.88	15.52 ± 8.74
Lymphocytes (swab)	16.26 ± 1.35	15.05 ± 1.54
Lymphocytes (direct smear)	40.00 ± 2.84	36.00 ± 3.39
Lymphocytes (lavage)	57.17 ± 3.88	55.11 ± 6.70

A significant difference was observed between the diameter of the nucleus of epithelial cells in control and endometritis groups. A significant difference was also observed in the nucleus area and between healthy and endometritis uteri ($p < 0.001$). There was a significant difference in lymphocyte count between both direct smear and lavage methods in healthy and infected individuals ($p < 0.001$). A significant difference was observed in the neutrophil count of healthy subjects and those with severe endometritis ($p < 0.001$) and also between those with severe and chronic endometritis using the swab method ($p < 0.05$). Moreover, there were significant differences in the lymphocyte counts using the swab method between the control group and severe endometritis ($p < 0.001$), severe and mild endometritis ($p < 0.001$), severe and moderate endometritis ($p < 0.01$), and chronic endometritis uteri ($p < 0.001$). There were also significant differences in the lymphocyte count of the control group and moderate, severe, and chronic endometritis groups using the direct smear methods ($p < 0.01$). Moreover, there were significant differences in the neutrophil count using the lavage method between control and severe endometritis groups and severe and chronic endometritis groups ($p < 0.05$). Also, there were significant differences in the lymphocyte count of the control group and moderate, severe, and chronic endometritis groups using the lavage method ($p < 0.001$).

Discussion

In the present research, 58 buffalo uteri were examined which 20 were diagnosed with no pathological lesions and 38 uteri had different types of endometritis. Endometritis which is an important cause of infertility is defined as the inflammation of the endometrium without general symptoms. Chethan *et al*. gave an insight on the histopathological changes occurring in riverine buffalo of Indian origin suffering from cytological and purulent endometritis.[21] Similar to what is already known in cows, they considered endometritis is of major concern in buffalo affecting fertility to a great extent resulting in repeat breeding. Being simple and more rapid than histopathology, endometrial cytology was suggested as a

Table 2. Cytomorphometric characteristics values and cellular counts for normal or endometritis groups and different types of endometritis. Data are presented as mean ± SEM.

Parameters	Control group	Chronic endometritis	Severe endometritis	Moderate endometritis	Mild endometritis	Endometritis group
Area (μm²)	67.59 ± 1.84	61.74 ± 1.70	61.69 ± 5.60	61.65 ± 1.20	66.49 ± 3.49	62.72 ± 1.37
Diameter (μm)	11.10 ± 0.15	10.59 ± 0.15	10.39 ± 0.54	10.55 ± 0.13	11.02 ± 0.30	10.64 ± 0.12
Neutrophils (swab)	0.90 ± 0.44	0.64 ± 0.28	28.66 ± 12.99	2.70 ± 1.50	10.10 ± 8.01	7.44 ± 2.97
Neutrophils (direct smear)	1.45 ± 0.69	2.47 ± 0.77	11.83 ± 6.94	8.71 ± 2.10	11.75 ± 9.64	7.05 ± 2.33
Neutrophils (lavage)	4.95 ± 2.48	2.88 ± 0.53	41.83 ± 24.88	11.57 ± 2.28	18.03 ± 2.12	14.68 ± 5.61
Lymphocytes (swab)	14.55 ± 1.35	11.76 ± 0.87	28.83 ± 3.10	17.85 ± 3.30	16.25 ± 1.04	16.52 ± 1.29
Lymphocytes (direct smear)	25.00 ± 2.37	48.88 ± 2.30	46.50 ± 7.23	51.71 ± 5.04	36.75 ± 4.22	46.47 ± 2.08
Lymphocytes (lavage)	36.30 ± 2.46	68.41 ± 3.50	83.00 ± 16.60	76.28 ± 6.20	12.86 ± 8.25	70.02 ± 4.21

practical but reliable diagnostic method to detect subclinical endometritis at the field level.[21] In a study on the uterine pathological abnormalities of buffaloes, endometritis had the highest rate.[13] The rate of endometritis in this species was reported by other researchers.[21-23] In the present study, we used the finding from pathology to classify the samples into healthy or mild, moderate, severe, and chronic endometritis according to the well-recognized criteria. Chronic (44.73%) and severe (15.90%) endometritis had the highest and the lowest incidence, respectively, which is similar to a previous report on buffaloes.[13]

The examination of uterine cytology was carried out with three different methods. Diameter and area of endometrial epithelial cells nucleus were measured in the uterus direct smear. The size of epithelial cells was increased in the follicular phase which includes proestrus and estrus stages while the diameter and area of the nucleus were decreased in the luteal phase including diestrus and metestrus phases. A significant difference was observed in the comparative statistical analysis in the diameter and area of the nucleus of cells between phases of the estrous cycle ($p < 0.001$). Groppetti *et al.* compared the condition of the uterus by cytology and histology in dogs. According to their results, the ratio of different inflammatory cells and the characteristics of the nucleus of epithelial cells in cytology samples were different in all stages of the reproductive cycle and also between healthy and diseased uteri.[4] In their research, computerized nuclear morphometry with 6 different parameters was indicative of its usefulness for the assessment of the estrous stage. Based on their results, the nucleus area in proestrus, estrus, and ultimately anestrus increased which is in consistent with the results of the present research.[4]

The diameter and area of epithelial cells in samples with endometritis were decreased in comparison with healthy samples and a significant difference was observed in diameter and area of the cellular nucleus between healthy subjects and those with endometritis ($p < 0.001$). Groppetti *et al.* reported the minimum area, perimeter, and diameter in pyometra – a type of pathological uterus damage. They also considered the adverse effects of uterine damage on nuclear morphology to be severe in comparison with the physiological conditions of the uterus and introduced nuclear profiles such as area, diameter, and shape as a useful indicator in the diagnosis of uterine pathology.[4]

Neutrophil and lymphocyte counts were different in different estrus phases and had an inverse ratio. They also were increased in follicular and luteal phases, respectively, although this difference was insignificant. Ahmadi *et al*. stated that neutrophil count in camels in the follicular phase was less than the luteal phase, and was similar to cows.[2] The aforementioned results were inconsistent with the finding of the present research. Their findings were contrary to physiological reality because neutrophils are the first line of defense against invading organisms that enter the uterus during the estrous phase. Oliveira *et al.* reported that the lymphocyte population was composed primarily of B-cells, T- cells, and NK cells in both pregnant and cyclic endometrium in cows. They noted that B-lymphocytes were widely distributed throughout the endometrium, localizing in the stroma, the luminal and glandular epithelium, and in the myometrium and the B-lymphocyte population was relatively larger compared to the populations of CD4+, CD8+, and NK cells detected.[24]

A significant difference was observed between the neutrophil count of healthy cases and those with severe endometritis and also between chronic and severe endometritis using the swab method. This fact indicated the presence of high neutrophil count in severe endometritis. Therefore, this method may be used to separate healthy animals and those with chronic endometritis. Moreover, there was a significant statistical difference in the lymphocyte count of severe endometritis and control subjects and also severe endometritis and its different types using the swab method. Using swab for sampling is an easy method, however, it has some disadvantages. Cell damage observed in the obtained smears using this method could be the result of cell adhesion to the cotton fibers, the pressures exerted for the collection of endometrial cells, and swab rotation on the slide.[25] However, some of these effects were eliminated by moistening the slide and swab with saline and smoothly rotating the swab on the slide.

A significant statistical difference was also observed between the neutrophil counts of examined groups using the direct smear method. There was a significant statistical difference in lymphocyte count in this method between control group and moderate, severe, and chronic endometritis but no difference was observed between other groups. Cytobrush is another sampling method that is conducted using a special endometrium sampling brush. This brush has nylon fibers at the tip of the handle fibers perpendicular to it. These fibers are firm and hard. Cell fragmentation is usually observed in cytobrush. According to results obtained from mare cytology, in comparison with multinucleated inflammatory cells, lymphocyte count was higher in the same mares using the cytobrush method compared to other smears. Therefore, cell fragmentation was among the disadvantages reported for this method, and the firm and hard fibers of these brushes were stated to be damaging.[26] Gahlot *et al.* indicated that the relationship of cytobrush cytology with uterine fluid cytology was significant in buffaloes.[14] The direct smear method was used in this research and neutrophil count is most likely decreased for the same reasons. In other words, this decrease can be due to the damage inflicted on neutrophil nuclei when it is dragged and its parts are separated, making the diagnosis difficult and decreasing their count.

There was a significant difference between the neutrophil count of control and severe endometritis and also those with severe and chronic endometritis using the third method, the lavage method. A significant statistical difference was observed in the lymphocyte count of control group and those with moderate, severe, and chronic endometritis. In general, it could be concluded that the lavage method has shown better results compared to the two other methods. The lavage method allows for endometrial sampling from a wider area.[27] However, this method has disadvantages. The lavage method requires variable times from sample collection to slide preparation for microscopic examination.[28] Another disadvantage of the lavage method is that after the collection of the uterine fluid, it requires centrifugation of cell suspension which may not be available at the field level. Another reason is cellular damage during the centrifuge process. Although the rate of 410 rpm for 10 minutes is known to be an appropriate rate for cells, there is a small risk of some minor cellular damages.[28] The remaining fluid in the uterus after lavage can cause inflammation of the endometrium.[27]

In this study, 58 buffalo uteri were examined histopathologically and cytologically. Nuclear morphometric analysis (diameter and area) was conducted on buffaloes for the first time which showed a significant relationship with the estrous cycle. As a result, it may be valuable in the identification of the reproductive cycle stage in this species. Moreover, the observation of a significant relationship between nuclear characteristics and being healthy or infected is valuable in the diagnosis and differentiation of the pathological conditions of the uterus. The consistency of the cytological findings of swab, direct smear, and uterine lavage methods with those of uterine pathology was also evaluated in this research. The swab method had a lower cell count compared to smears prepared using direct smear and lavage methods. Moreover, the direct smear method showed higher cell count compared to lavage and swab methods. This method is of greater value for researching uterine morphometry. Despite its disadvantages, the lavage method was the most efficient and showed higher diagnostic value for the varieties of endometritis compared to the other two methods.

Acknowledgments

The authors wish to express their gratitude to the research council of the Shahid Chamran University of Ahvaz for their financial supports and thank Miss and Mr. Behdarvand for technical assistance.

Conflicts of interest

The authors declare that they do not have any conflict of interest.

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