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Study on Alterations in the Distribution of Epithelial and Inflammatory Cells at the External Os Region of the Uterine Cervix in the Different Stages of River Buffaloes' Gestational Period

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Abstract: The changes in the kinds and percentages of the epithelial and inflammatory cells of the external os region of the uterine cervices of the river buffaloes during three different stages of pregnancy were studied by microscopic evaluation of mucus smears which had been prepared by wet swab sampling of mucus from the external os of the pregnant buffaloes' uterine cervices. The mucus smears were stained by Giemsa's staining method. The present study revealed evidence that changes in the percentages of the vacuolated and unvacuolated epithelial cells, lymphocytes, eosinophils and basophils were not significant statistically, but changes in the numbers and percentages of neutrophils in the three different stages of pregnancy period were statistically significant. We also found that changes in the number and percentage of monocytes (macrophages) between the second and third stage of pregnancy was not significant, but the difference between the first stage of pregnancy and the two other stages was statistically significant. We concluded that the numbers and percentages of neutrophils and monocytes (macrophages) increase significantly as the pregnancy progresses.

Key words: External os of the uterine cervix, epithelial cells, inflammatory cells, pregnancy, river buffalo

INTRODUCTION

Cervix or the uterine neck is the downest part of the uterus (Lesson *et al*., 1988) and is thick-walled, muscular and rich in elastic fibers. The mucosa-submucosa forms high folds with secondary and tertiary folds. In cows, 4 large circular and 15-25 longitudinal primary folds, each with many secondary and tertiary folds, are present. Uterine glands do not extend into the cervix and the glandular elements present in the cervix are mostly mucinogenous (Dellman and Eurell, 1998).

The cervical canal has various prominences. In ruminants these are in the form of transverse or spirally interlocking ridges known as annular rings which develop to varying degrees in the different species. They are especially prominent in the cow, where they fit into each other to close the cervix securely. Mucus discharged from the cervix is expelled from the vulva (Hafez and Hafez, 2000). The cervix varies in length from 2-3 cm in the heifer to approximately 10 cm in the mature cow (Ball and Peters, 2004).

In most species, the epithelium is the simple columnar type with many mucigenous cells. Increasing quantities of mucus are secreted during estrus and pregnancy and much of the mucus passes to vagina. In pregnancy, the mucus thickens to form the cervical seal. Intraepithelial and simple tubular glands may be present in ruminants. The properia-submucosa consists of dense irregular connective tissue, which becomes edematous and assumes a loose areolar structure during estrus. The tunica muscularis consists of inner circular and outer longitudinal smooth muscle layers. Elastic fibers are prominent in the circular layers. Thickening and infolding of the circular layer occurs in the region of the circular folds or prominences in the small ruminants. In mares and cows, the thickened circular layer forms the body of the intravaginal portion of the cervix. The tunica serosa of the cervix consists of loose connective tissue. A longitudinal duct of the epoophoron (Gartner's duct) may be present in this layer on one or both sides (Dellman and Eurell, 1998).

The connective tissue of the cervical stroma is made of ground substance, fibrous constituents and cellular elements. Gross changes in the biochemical composition of the cervix during pregnancy indicate that the cervix during pregnancy is preparing for a change in its

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functional properties by alterations in the parameters that regulate the physical properties of connective tissue matrices. Morphologically, these pregnancy-related changes do not become apparent until quite late during pregnancy, when tissue breakdown and destruction of the collagen network become apparent.

During the course of pregnancy, the cervix may show as much as eightfold increase in mass. The enhanced growth and the decreased concentration of the matrix components may be a consequence of several factors, including increased vascularization and increased concentrations of glycoproteins.

Cervical softening and ripening are not due exclusively to enzymatic activity involving only matrix degradation. The dynamic nature of the cervix at the time of parturition may provide an anabolic basis by which a new matrix with altered physical properties is produced. Cervical mucus consists of macromolecules of mucin of epithelial origin which are composed of glycoproteins (particularly of silomucinous type) that contain about 25% amino acids and 75% carbohydrates.

The secretion of cervical mucus is stimulated by ovarian estrogen and inhibited by progesterone (Hafez and Hafez, 2000).

Successful labor and delivery requires both coordinated uterine contractions and extensive remodeling of the cervix. In human, sheep, guinea pig and rat pregnancy, cervical remodeling can be divided into two phases. The first phase, termed cervical softening, is a gradual process that begins in the first trimester of human pregnancy and by day 12 in the rat. The second phase, termed cervical ripening, begins in the day(s) before onset of parturition (Leppert, 1995). Because 85% of the cervix is connective tissue, remodeling requires extensive alterations of the connective-tissue matrix (Timmons and Mahendroo, 2006). The changes in connective tissue during pregnancy and parturition in part reflect alterations in Glycosaminoglycan (GAG) and proteoglycan compositions of the cervix (Dowing and Sherwood, 1986; Osmers *et al*., 1993) and increased collagen turnover (Breeveld *et al*., 2003).

In addition to the influence of proteoglycans on cervical collagen structure, several studies have reported a decline in collagen concentration as a result of increased activity of collagenases and other proteolytic enzymes. These proteases are present in cervical fibroblasts as well as polymorphonuclear leucocytes and macrophages. Infiltration of inflammatory cells into the cervical tissue is one of the main histological features observed in the cervix immediately after birth or in the late stages of normal cervix ripening. Infection of the lower reproductive tract in human subjects is frequently associated with premature cervical ripening and labor (Timmons and Mahendroo, 2006).

These observations have led to the currently accepted model of cervical ripening, in which the influx of inflammatory cells is a major regulatory event in the initiation of cervical ripening during normal parturition (Timmons and Mahendroo, 2006). Inflammatory cells, such as neutrophils, macrophages and eosinophils, are proposed to play an important role in the synthesis of cytokins and proteolytic enzymes that regulate the ripening process (Mackler *et al*., 1999). These cells are recruited to affected tissue from circulating blood. The invasion of the inflammatory cells into the cervical stroma is negatively regulated by progesterone. There are increased numbers of neutrophils within the cervical stroma late in pregnancy, as reported in the rodent and other species (Timmons and Mahendroo, 2006).

The high estrogen concentrations that occur at estrus and parturition cause changes in the numbers and proportions of circulating white blood cells, with a relative neutrophilia and a 'shift to the left'. Moreover, at estrus, the blood supply to the uterus is increased under the influence of estrogen, whilst at parturition there is a massive blood supply to the gravid uterus. This increased blood supply, coupled with the migration of white cells from the circulation to the uterine lumen, enables vigorous and active phagocytosis of bacteria to occur. Estrogens also cause an increase in the quantity and nature of vaginal mucus, which also plays an important role in defense of the uterus against bacteria by providing a protective physical barrier and by flushing and diluting the bacterial contamination (Noakes *et al*., 2001).

Estrogen and progesterone contribute to the increase in the cervical cell content during late pregnancy by both promoting proliferation and inhibiting apoptosis of cervical cells. Studies conducted with ovariectomized nonpregnant rats demonstrated that estrogen alone promotes marked cervical growth. Estrogen may contribute to the accumulation of cervical cells during pregnancy by increasing the rate of cell proliferation and by decreasing the rate of apoptosis (Lee and Sherwood, 2005). Mitogenic actions of estrogen on the cervix and uterus in nonpregnant mice have been reported (Quarmby and Korach, 1984). A study by Berman *et al*. (1998) demonstrated an inverse correlation between serum estrogen levels and the percentage of cells undergoing apoptosis in the vagina of cyclic rats. Moreover, bilateral ovariectomy resulted in an increase in the rate of vaginal cells, which was reversed following estrogen-replacement treatment (Berman *et al*., 1998). In rats, serum

concentrations of estrogen steadily increase during the second half of pregnancy and estrogen likely plays an important role in regulating the rates of proliferation and apoptosis of cervical cells as pregnancy progresses.

Limited evidence indicates that the elevated serum levels of progesterone during the second half of pregnancy also regulate cervical growth. These limited findings in pregnant rats indicate that progesterone may restrain cervical growth, perhaps by inhibiting the accumulation of cervical cells. However, as in the case with estrogen, endogenous progesterone has not been reported to influence either cell proliferation or apoptosis of cervical cells during pregnancy in any species (Lee and Sherwood, 2005).

A study provides evidence that each of the steroids, estrogen and progesterone contribute substantively to an increase in cell number during late pregnancy by both promoting cell proliferation and inhibiting apoptosis. These hormonal effects are most pronounced during late pregnancy, when the rate of cervical growth is most profound (Zhao *et al*., 2001).

An increasing finding with respect to progesterone's actions is that the hormone exerts inconsistent actions on apoptosis of cervical cells during pregnancy. Progesterone promotes apoptosis of cervical cells during midpregnancy, but inhibits apoptosis of cervical stromal cells during late pregnancy. The mechanism(s) that explains progesterone's apposite role is not known (Lee and Sherwood, 2005). Receptors for estrogen (Romani *et al*., 2002; Nephew *et al*., 2000) and progesterone (Ohta *et al*., 1993) have been reported to be present in epithelial cells and subepithelial stromal cells in rodent reproductive tract (Lee and Sherwood, 2005).

Some studies have been conducted about the changes in the vaginal mucosal wall of cows and river buffaloes during different stages of the estrus cycle and pregnancy period, but to our knowledge no study has been done about the changes and percentages of the epithelial and inflammatory cells in the uterine cervical external os region of river buffaloes during different stages of pregnancy. In the present study, we have tried to evaluate these probable changes.

MATERIALS AND METHODS

The present study was conducted on 45 pregnant river buffaloes that were in different stages of their gestation period. The selected animals were between 6-7 years old and were multiparous. The gestation period was divided into three equal stages and 15 pregnant buffaloes were put in each group of the three stages according to their similar pregnancy stage. In other words, in stage 1 were those animals that were in the first third of their pregnancy period, in stage 2 were those animals that were in the second third of their pregnancy period and in stage 3 were those animals that were in the last third of their pregnancy period.

To take swab samples from the external os of the uterine cervix, each pregnant buffalo was restrained in a box and after doing caudal epidural anesthesia and washing and disinfecting the perineal and vulval regions, a sterile glass speculum was inserted into the vagina, so that the external os of the uterine cervix became apparent. Sample taking was performed by rotating the swab in the external os of the cervix and then smears were prepared by rotating the swab sample on the microscope slides. We took two swab samples from the external os of each buffalo's cervix and each swab was rotated on five glass slides, therefore we provided ten mucus smears from each case and a total of 150 samples for each stage of pregnancy. The slides were dried in the environment air, fixed by methyl alcohol and stained by Giemsa's staining method.

We also took a microbial swab sample from the external os of each buffalo's cervix and sent it to the microbiology Laboratory of the College of Veterinary Medicine of Urmia University to perform bacteriologic cultures to study the numbers and types of the present bacteria in the external os of the cervix in order to know whether the presence of the inflammatory cells in the region of the external os of the cervix is due to the stage of pregnancy or to the presence of an inflammatory or infective problem. In addition, we also took a blood smear from each case in order to perform differential blood count in the laboratory of hematology to study the results in association with the microbiologic results.

The stained mucus smears were studied microscopically by magnification 1000× in order to determine the percentages of the inflammatory and epithelial cells of the external os of the cervix in the different stages of buffaloes' gestation period. The cells present on one randomized microscopic field of each slide with the area of 0.1 millimeter square of smears were counted.

Data were analyzed using one-way ANOVA to determine if there were any significant differences in the percentages of inflammatory and epithelial cells of the external os of the uterine cervix between the three stages of the buffaloes' gestation period. The level of significant was set at $p \leq 0.05$.

RESULTS

Mean±SEM of each cell type including the unvacuolated epithelial cells, the vacuolated epithelial cells, lymphocytes, monocytes (macrophages), neutrophils, eosinophils and basophils was calculated in the three stages of buffaloes' gestation period (Table 1) and the differences were analyzed.

Mean±SEM of the unvacuolated and vacuolated epithelial cells and lymphocytes of the external os of the pregnant buffaloes' uterine cervices were not significantly different in the three stages of the buffaloes' gestation period ($p>0.05$).

Mean±SEM of monocytes (macrophages) between the second and third stages of pregnancy was not significant statistically ($p>0.05$), but the difference between the first stage of pregnancy and the two other stages was statistically significant ($p\leq0.05$). In other words, the number and percentages of monocytes increase significantly in the second and third stages of gestation period of buffaloes, when they were compared with the first stage.

Mean±SEM of neutrophils in the first, second and third stages of the gestation period were 4.76±1.63, 7.81±2.28 and 16.72±5.13, respectively. The percentage of neutrophils in the third stage of pregnancy was more than the percentage of them in the second stage. Also, the percentage of this kind of cell in the second stage of pregnancy was more than its percentage in the first stage. The differences in the percentages of neutrophils between the three stages of pregnancy were statistically significant ($p\leq0.05$).

Mean±SEM of eosinophils and basophils of the external os secretions of the uterine cervices of pregnant buffaloes was not different significantly among the three stages of the gestation period ($p>0.05$).

The present study, which is the first one to examine the changes in the numbers and types of the epithelial and inflammatory cells of the external os secretions of the uterine cervices of pregnant buffaloes during different stages of the gestation period, provides evidence that the number of the unvacuolated epithelial cells are more than the other cell types in this area and the percentage of them in the first stage of the gestation period is more than that in the second and third stages of the gestation period, in other words, the number of the unvacuolated epithelial cells increases as the pregnancy progresses. Also, this study provides evidence that in the first stage of pregnancy the percentage of lymphocytes is more than the other cell types except the unvacuolated epithelial cells.

Table 1: Mean±SEM of different cell types in the external os secretions of river buffaloes' uterine cervix in the three different stages of pregnancy. Epc: Non-vacuolated epithelial cells, Epv: Vacuolated epithelial cells, L: Lymphocytes, M: Monocytes (macrophages), N: Neutrophils, Eos: Eosinophils, Bas: Basophiols

Type of cell	Stage 1	Stage 2	Stage 3
Epc	71.0093±2.34031	68.5520±2.76661	61.5200±4.91270
Epv	7.1407±1.55128	8.4620±2.48918	6.9480±1.77628
L	12.3307±2.61414	6.9760±1.65333	8.3107±1.89169
M	1.9473±1.14949	10.7047±1.70799	9.3347±2.40884
N	4.7600±1.63862	7.8147±2.28690	16.7293±5.13876
Eos	.0000±.0000	.0000±.0000	.0000±.0000
Bas	.0000±.0000	.0000±.0000	.0000±.0000

The present study also provides evidence that the percentage of neutrophils increases as the pregnancy progresses, so that its percentage is lowest in the first stage and highest in the third stage of the gestation period. The results of the CBC counts and the microbial cultures were in the normal ranges and no abnormality was observed.

DISCUSSION

In the present study we found that the percentage of the unvacuolated epithelial cells in the external os of the uterine cervices of pregnant buffaloes in the first stage of pregnancy was more than those of the second and third stages. In other words, the percentage of the unvacuolated epithelial cells decreases as the pregnancy progresses, but this decrease is not significant statistically. Therefore, the amount of the unvacuolated epithelial cells of the external os of the uterine cervices of the pregnant buffaloes remain unchanged during the gestation period, which indicates the steady effects of progesterone on this area. On the other hand, the percentage of the epithelial cells of the external os of the uterine cervix was more than the percentages of the other cell types in the three stages of the gestation period. These findings are consistent with those that Ahmadi *et al.* (2000) described previously, since in both the pregnancy period and the luteal phase of the estrus cycle the external os of the uterine cervix is under the influence of progesterone and the progesterone concentrations in plasma and milk during the first few days of pregnancy increase in a similar manner to that occurring in the early luteal phase of the nonpregnant animal (Ball and Peters, 2004).

Although the percentage of the vacuolated epithelial cells of the external os of the cervix in the second stage of the gestation period was more than those of the other two stages, these differences were not significant statistically. In a study, blocking the actions of estrogen and progesterone with subcutaneous injections of the

estrogen antagonist ICI 182,780 (ICI) and the progesterone antagonist RU480, respectively, to rats at 3-day intervals during the second half of pregnancy decreased the rates of proliferation and increased the rates of apoptosis of both cervical epithelial and stromal cells during late pregnancy. However, blocking the actions of progesterone had the apposite effects on apoptosis of both cervical epithelial and stromal cells during the middle of pregnancy. Therefore, estrogen and progesterone contribute to the increase in the cervical cell content during late pregnancy by both promoting proliferation and inhibiting apoptosis of cervical cells. On the other hand, the elevated serum levels of progesterone during the second half of pregnancy may also regulate cervical growth (Lee and Sherwood, 2005). A recent study demonstrated that the administration of the progesterone antagonist RU480 into ovariectomised pregnant rats on day 22 resulted in a tendency for increased cervical wet weight (Zhao and Sherwood, 2004).

In the present study the differences in the percentages of lymphocytes between the three stages of pregnancy were not statistically significant, although the number of lymphocytes in the first stage of pregnancy was more than those in the other stages. Also the number of lymphocytes in the second stage of pregnancy was less than those in the other stages. On the other hand, the most abundant cells in the first stage of pregnancy were the unvacuolated epithelial cells and lymphocytes, respectively.

The percentages of monocytes (macrophages) of the external os of the uterine cervix increased markedly in the second and third stages of pregnancy in comparison with the first stage of pregnancy and this increase suggests a statistically significant difference between the second and third stages of pregnancy in one hand and the first stage of pregnancy in the other hand. This finding is inconsistent with one study in which it was reported that tissue macrophages were not increased within the cervix until after birth (Timmons and Mahendroo, 2006) but other studies have reported a decline in collagen concentration as a result of increased activity of collagenases and other proteolytic enzymes. These proteases are present in cervical fibroblasts as well as polymorphonuclear leukocytes and macrophages (Hertelendy and Zakar, 2004; Junqueria *et al*., 1980; Rajabi *et al*., 1991; Sato *et al*., 1991). Infiltration of the inflammatory cells into the cervical tissue is one of the main histological features observed in the cervix immediately after birth or in the late stages of normal cervical ripening (Knudsen *et al*., 1997; Osman *et al*., 2003).

In the present study we also found out that the number of neutrophils in the external os of the cervix is increased as the pregnancy progresses. Its number is peaked in the third stage of pregnancy which indicates a role of these cells in postpartum remodeling of the cervix rather than in the initiation of cervical ripening at parturition (Timmons and Mahendroo, 2006).

Inflammatory cells, such as neutrophils and eosinophils, are proposed to play an important role in the synthesis of cytokines and proteolytic enzymes that regulate the ripening process (Mackler *et al*., 1999). These cells are recruited to affected tissue from circulating blood (Timmons and Mahendroo, 2006). Several studies reported increased number of neutrophils within the cervical stroma late in pregnancy in the rodents and other species (Timmons and Mahendroo, 2006; Bokstorm *et al*., 1997; Marodny *et al*., 1997). The invasion of neutrophils and monocytes into the cervical stroma is negatively regulated by progesterone. The ability of the progesterone receptor antagonist to partially restore migration of these cells confirms this fact (Timmons and Mahendroo, 2006).

In the present study it was shown that the presence of eosinophils and basophils in the region of the uterine cervices of pregnant buffaloes is negligible. This finding is in agreement with the result of a study in which it is reported that eosinophils activity is not increased within the cervix until shortly after birth (Timmons and Mahendroo, 2006).

CONCLUSION

This study indicated that the number of the unvacuolated and vacuolated epithelial cells, lymphocytes, eosinophils and basophils do not change significantly in the external os of the pregnant buffaloes' uterine cervices in the different stages of gestation period, but the numbers of neutrophils and macrophages increase as the pregnancy progresses. We propose further studies in this subject in the different months of the pregnancy period of buffaloes and the days immediately after parturition in order to obtain better results regarding the numbers of the epithelial and inflammatory cells of the external os of pregnant buffaloes' uterine cervices.

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