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Seksüel Siklus Döngülerinin Proöstrus, Metöstrus-Diöstrus Ve Anöstrus Dönemlerinde Kangal Veya Kangal Melezi Dişi Köpeklerin Uterus Dokusunda Muc1 Ve Muc16 Ekspresyonlarının Araştırılması

Investigation Of Muc1 And Muc16 Expressions In Uterus Tissue Of Kangal Or Kangal Cross-Bred Bitches In Proestrus, Metestrus-Diestrus And Anestrus Periods Of Sexual Cycles

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Öz

Bu çalışmada, seksüel siklus döngülerinin proöstrus, metoöstrus-diöstrus ve anöstrus dönemlerinde Kangal veya Kangal melezi dişi köpeklerin uterus dokusunda MUC1 ve MUC16 ekspresyonları immünohistokimyasal yöntemle incelendi. Çalışmada 3-6 yaş arası sağlıklı ve sahipli Kangal veya Kangal melezi 15 köpeğin uterus dokuları kullanıldı. Köpekler seksüel siklus dönemlerine göre proöstrus (n=5), metöstrus-diöstrus (n=3) ve anöstrus (n=7) olarak 3 gruba ayrıldı. Sonuç olarak, MUC1'in köpek uterusunda hem kornu hem de korpus uteri epitel hücrelerinde, kript epitel hücrelerinde, uterus bezi epitel hücrelerinde, stratum vaskülaris, miyometriyum ve perimetriyum tabakalarından eksprese edildiği tespit edildi. Östrus siklusları karşılaştırıldığında metöstrus-diöstrus döneminde ekspresyonun diğer dönemlere göre daha fazla arttığı saptandı. MUC16'nın ekspresyonu, metöstrus-diöstrus döneminde kornu uteri'de gözlendi. Bu çalışmada MUC1 ve MUC16'nın köpeklerin uterus dokusundan eksprese edildiği ve salınımlarının hormonlardan etkilendiği tespit edildi.

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Abstract

In this study, MUC1 and MUC16 expressions in uterus tissues of Kangal or Kangal cross-bred bitchs in proestrus, metestrus-diestrus and anestrus sexual periods were investigated by immunohistochemical method. Uterus tissues of 15 healthy and owned Kangal or Kangal cross-bred bitches aged 3-6 years were used in the study. The biches were divided into 3 groups according to sexual cycle periods as proestrus (n=5), metestrus-diestrus (n=3) and anestrus (n=7). As a result, MUC1 was found to be expressed in both cornu and corpus uteri epithelial cells, crypt epithelial cells, uterus gland epithelial cells, stratum vascularis, myometrium and perimetrium layers in uterus of bitches. When estrus cycles were compared, it was found that expression increased more in the metestrus-diestrus period compared to other periods. Expression of MUC16 was observed in the cornu uteri during the metestrus-diestrus period. In this study, MUC1 and MUC16 were determined to be expressed in the uterus tissue of bitches and their expressions were found to be affected by hormones.

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1. INTRODUCTION

The estrus cycle is characterised by morphological changes occurring in the ovary, uterus and vagina (Goldman et al., 2007). In mammals, the uterus is a complex and dynamic organ with very important functions as a habitat from implantation of the embryo during pregnancy until birth. During the sexual cycle, many structural differences are observed in the entire uterine, especially in the endometrium, with the effect of sex hormones (Dekel et al., 2010). These structural differences play a crucial role in the regulation and maintenance of implantation and pregnancy (Nikas et al., 2000).

Mucus, which has a slimy and viscous structure, is released by gland epithelial cells and goblet cells. Mucus consists of water, various salts, lipids and glycoproteins. Mucus also contains mucins (MUC) (Bansil et al., 1995). Mucins are glycoprotein aggregates consisting of high amounts of carbohydrates, released from the apical surface of epithelial cells in many systems (Rachagani et al., 2009), especially the apical surface of polarised epithelial cells in the tracheobranchial, gastrointestinal, reproductive system and eyes (Yin and Lloyd, 2001). It was first found on the apical surface of the uterus epithelium in 1980 (Mullins et al., 1980) in a study with pigs. In addition, both in vivo (Braga and Gendler, 1993) and in vitro (Pimental et al., 1996) studies reported MUC1 expression in the mouse uterus tissue.

Although the secretion of MUCs varies according to the organ, their main functions are to protect against bacteria, drugs, toxic substances, digestive enzymes and acids (Hattrup and Gendler, 2008). In addition to these functions, it also has very important functions, especially in the reproductive system, such as protection against pathogens (Argueso et al., 2003), implantation and healthy realisation and maintenance of pregnancy (Meseguer et al., 2001).

MUC1 expression in uterus tissue was first reported in human endometrium (Arklie et al., 1981). In later studies, MUC1 expression was reported in many species such as mouse (McGuckin et al., 1998), rodent (Hewetson and Chilton, 1997), pig (Stenner and Crawford, 1999) and baboon (Ilekis et al., 1997). It was reported that MUC1 played an important role in embryo implantation (Hewetson and Chilton, 1997), was affected by estrogen and progesterone hormones and these hormones increase MUC1 expression (McGuckin et al., 1998). MUC16, similar to MUC1, has protective functions against pathogens by forming a tissue barrier specific to the organ in which it is located and facilitating blastocyst attachment in pregnancy (Argueso et al., 2003).

In this study, it was aimed to investigate the release of MUC1 and MUC16, which were

known to have protective and supportive effects in the uterus tissues of Kangal or Kangal cross-bred bitches in proestrus, metestrus-diestrus and anestrus sexual cycle periods and whose expression was reported in the reproductive system of many species, by immunohistochemical method.

2. MATERIALS AND METHODS

2.1. Ethical Approval

All experiments were approved by Kafkas University Ethics Committee for animal experiments (Approval no: KAU-HADYEK/2016-009).

2.2. Animal Material

In the study, 15 healthy Kangal or Kangal cross-bred and owned bitches aged 3-6 years, which were brought to Kafkas University Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology for ovariohysterectomy (OH), were used. The bitches were routinely examined for pregnancy before the operation. Detailed clinical examinations were carried out in order to determine that the bitches that were found to be non-pregnant were healthy and OH was performed after fasting for at least 12 hours before the operation.

Vaginal cytology was performed to determine the sexual cycle periods of the bitches before the operation. Cytological samples were taken from the vagina tissue with the help of speculum and two preparations were prepared. After the preparations were stained with giemsa stain used in the laboratory, the cell profile was examined under light microscope and the cycle periods of the dogs were determined. The bitches were divided into 3 groups according to sexual cycle periods as proestrus (n=5), metestrus-diestrus (n=3) and anestrus (n=7).

Before the operation, the bitches were anaesthetised by subcutaneous injection of atropine at a dose of 0.04 mg/kg, followed by intramuscular administration of xylazine at a dose of 12 mg/kg and deep intramuscular administration of ketamine HCl at a dose of 10-20 mg/kg. The uterus tissue along the median line of the operation was removed completely after appropriate ligatures were placed. After this procedure, the peritoneum, muscles and skin were sutured separately and the area was bandaged. Antibiotic treatment was applied for 7 days to prevent complications.

2.3. Histological Procedure

The uterus tissues were fixed in 10% formalin for 48 hours. After fixation, the samples were processed for routine histological protocols and embedded in paraffin. The tissue sections were taken at thickness of 5 µm from the paraffin blocks prepared and stained with Crossman's triple staining for histological examination (Crossman, 1937).

2.4. Immunohistochemical Procedure

The streptavidin biotin peroxidase complex method was applied in the uterus tissue. Sections of 4-5 µm thickness were collected on adhesive slides. The sections were processed in citrate buffer solution (ph 6.0) for 10 min in a microwave oven at 700 watts. Then, tissues kept on hold in 3% hydrogen peroxide (H2O2) for 15 min. The blocking solution A was dripped to prevent the nonspecific binding by IHC Kit. Sections were incubated with MUC1 primary antibody (ab104978, Abcam, Cambridge, MA 02139-1517 USA, 1/250 dilution), MUC16 primary antibody (ab133419, 1/500 dilution) were applied on the sections in a humid environment at the ambient temperature for 1 h. Seconder antibody and after streptavidin was dripped on the sections for 30 min. The 3,3'-Diaminobenzidine tetrahydrochloride (DAB) used as chromogen for 10 min then Mayer's hemotoxilen was used for the background staining. Rabbit serum without primer antibody served as the negative control. Sections were evaluated using research microscope (Olympus BX51, Tokyo, Japan). Evaluation of immunoreactivity of MUC1, and MUC16 were scored. Immunoreactive cells were categorized as having negative, mild, moderate, and intensive.

3. RESULTS AND DISCUSSION

As a result of vaginal cytology performed to determine the sexual cycle periods of the bitches before the operation, dense erythrocytes and nucleated or non-nucleated superficial cells were observed during the proestrus period (Figure 1a). Increased neutrophil granulocytes, parabasal cells were found in the metestrus-diestrus stage (Figure 1b) and parabasal and intermedier cells were found in the anestrus stage (Figure 1c).

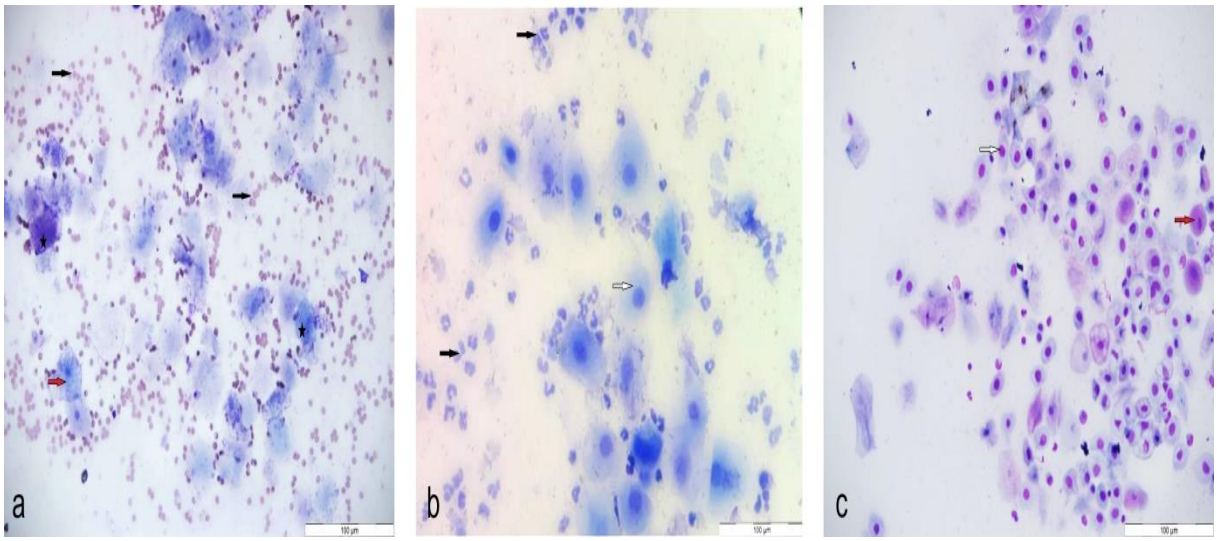

Figure 1. Vaginal cytology in bitchs. a: proestrus: erythrocytes (black arrows), nucleated superficial cell (red arrow) and non-nucleated superficial cells (stars), b: metestrus-diestrus: neutrophil granulocytes (black arrows), parabasal cell (white arrow) c: anestrus: parabasal cell (white arrow) and intermedier cells (red arrow). Giemsa staining.

The estrus cycle in the bitches consists of proestrus, estrus, metestrus-diestrus and anestrus periods. Hormonal changes in the estrus cycle also cause morphological changes in the structure of the uterus (Goldman et al., 2007). The endometrial stroma gains an oedematous appearance in proestrus period when sexual desire begins due to the increase in estrogen hormone. In cross-sections, the lumen looks like an X. In the endometrium, surface epithelial cells proliferate and form superficial recesses into the stroma to create crypts. Increased capillary hyperaemia is associated with extravasation of erythrocytes into the lumen (Van Cruchten et al., 2004). In the study, proliferation of surface epithelial cells, glandular epithelial cells and crypts were detected in the endometrium of both the cornu uteri and corpus uteri in the proestrus period. Thickening of the endometrium due to increased proliferation was observed. In addition, hyperaemic appearance was also noted in the blood vessels. Thickening due to increased muscle cell proliferation was observed in the myometrium. At this stage, the mucosal layers were extremely oedematous and thick (Figure 2a, 2b).

In the metestrus-diestrus period, progesterone hormone expression is at the highest level. Especially in early diestrus, the endometrium and myometrium reach the highest thickness and cellular density (Galabova et al., 2003). At this stage the female accepts the

male. At the beginning of this stage, mucous gland epithelium and surface epithelium acquire high characteristics. The number and secretion of basal glands increases. At the same time, the thickness of the endometrium increases due to increased vascularisation (Wick and Kress, 2002; Vermeirsch, 2001). In the metestrus-diestrus period of this study, the crypts and glands of uterus in the endometrium layer of the cornu and corpus uteri were observed to be extremely enlarged and voluminous. Thickening of the endometrium layer was detected with increasing size. Similarly, the thickness of the layers of the myometrium and perimetrium was also significantly increased. In the stratum vascularis layer, hyperaemia of the blood vessels was observed and their width was extremely increased (Figure 2c, 2d).

If fecundation does not occur, the progesterone level drops rapidly and the anestrus period begins. At this stage, epithelial cells start to take cubic shape. The number of crypt and secretory epithelial cells and the secretions of the uterus glands are reduced (Barrau et al., 1975). The endometrium and myometrium are completely atrophic. All compartments of uterus result in reduced cellular cytoplasm and high nuclear density (Rehm et al., 2007). In the study, it was observed that the growth and enlargement seen in the metestrus-diestrus phase in the anestrus phase gradually weakened in this phase. It was found that the lumen was more enlarged in the anestrus stage and the thickness of the endometrium and myometrium layers decreased due to atrophy. The epithelial layer was extremely thin and the crypts and uterus glands were reduced in size. In addition to the reduction of hyperaemia in the blood vessels, a narrowing of their width was also noted (Figure 2e, 2f).

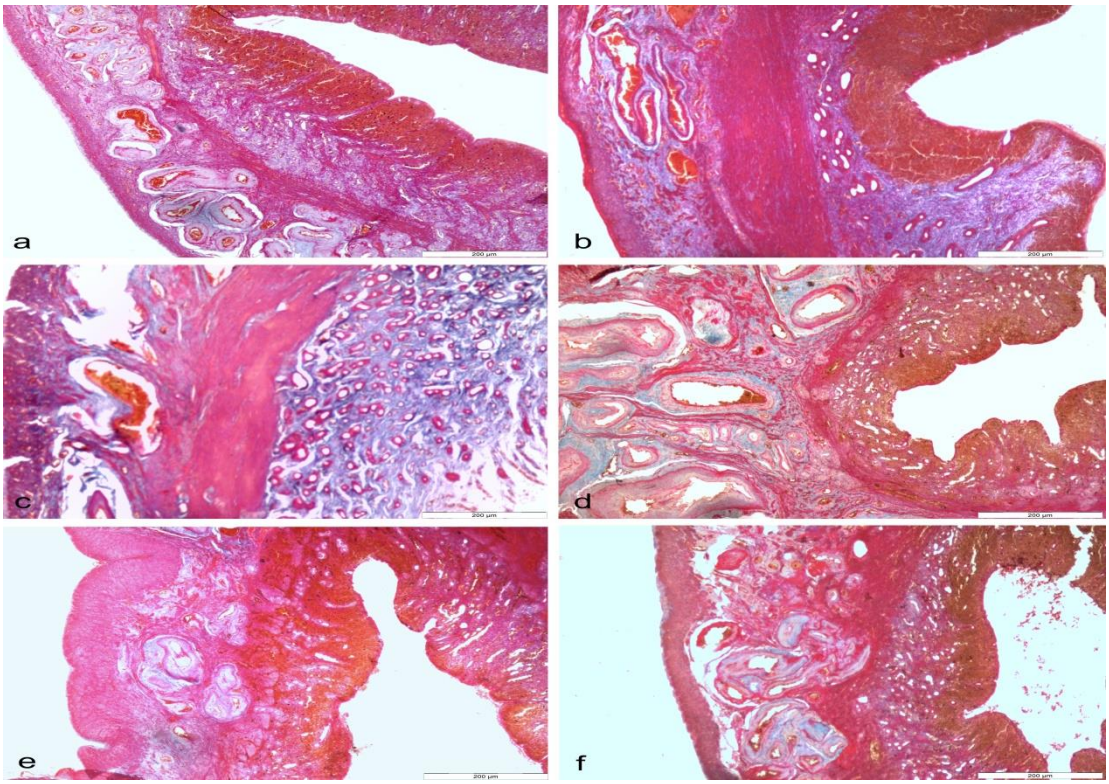

Figure 2. The histological structure of the corpus and cornu uteri in sexuel cycle of bitches. corpus uteri (a) and cornu uteri (b) in proestrus; corpus uteri (c) and cornu uteri (d) in metestrus-diestrus; corpus uteri (e) and cornu uteri (f) in anestrus. Crossman's Triple Staining.

Mucins with glycoprotein content are very important for embryo implantation (Meseguer et al., 2001). In addition, it was reported that mucins also play a protective role by acting as a barrier against enzymatic and microbial attacks (Jentoft N., 1990). It was reported that MUC1 is expressed from the cervix epithelium as well as the uterine epithelium and facilitates the movement of sperm and controls bacterial passage. Deficiency or loss of MUC1 secretion from the surface epithelium of the endometrium has been shown to impair implantation in the uterus of mice (Surveyor et al., 1995) and pigs (Bowen et al., 1996). It was reported that implantation of the embryo into the uterus was not possible when mucins were removed enzymatically from the apical surface of the uterine epithelium (De Souza et al., 1999).

MUC1 expression was reported to be regulated by estrogen, progesterone and glucocorticoids (Dharmaraj et al., 2010). It was reported that the expression of MUC1 is increased in mice during proestrus and estrus periods by hormonal effect (Arklie et al., 1981; Meseguer et al., 2001). In our study, it was found that the release of MUC1, which was

detected in the proestrus period, increased in the metestrus-diestrus period. Moderate MUC1 expression was also detected in the surface epithelial cells, crypt epithelial cells, uterus gland epithelial cells, myometrium and perimetrium layers of the endometrium layer of the corpus uteri during the proestrus period (Figure 3a). In the cornu uteri, moderate MUC1 expression was found in the crypt epithelial cells, uterus gland epithelial cells, myometrium and perimetrium, and intensive MUC1 expression was found in blood vessels in the stratum vascularis (Figure 3d). Intensive MUC1 expression was observed in surface epithelial cells of the corpus uteri, crypt epithelial cells, uterus gland epithelial cells, myometrium, stratum vascularis and perimetrium during the metestrus-dioestrus phase (Figure 3b). It was found that MUC1 expression was intensive in the stroma of the endometrium of the cornu uteri near the lumen, crypt epithelial cells and uterus gland epithelial cells, and moderate expression was noted in the blood vessels of the stratum vasculare (Figure 3e). In the anestrus period, moderate MUC1 expression was observed in the stroma cells and crypt epithelial cells close to the lumen of the endometrium in the corpus uteri, while no reaction was observed in other layers (Figure 3c). In the cornu uteri, a mild reaction was observed in the crypt epithelial cells in the endometrium, while MUC1 expression was not observed in the other layers and blood vessels in the stratum vasculare (Figure 3f).

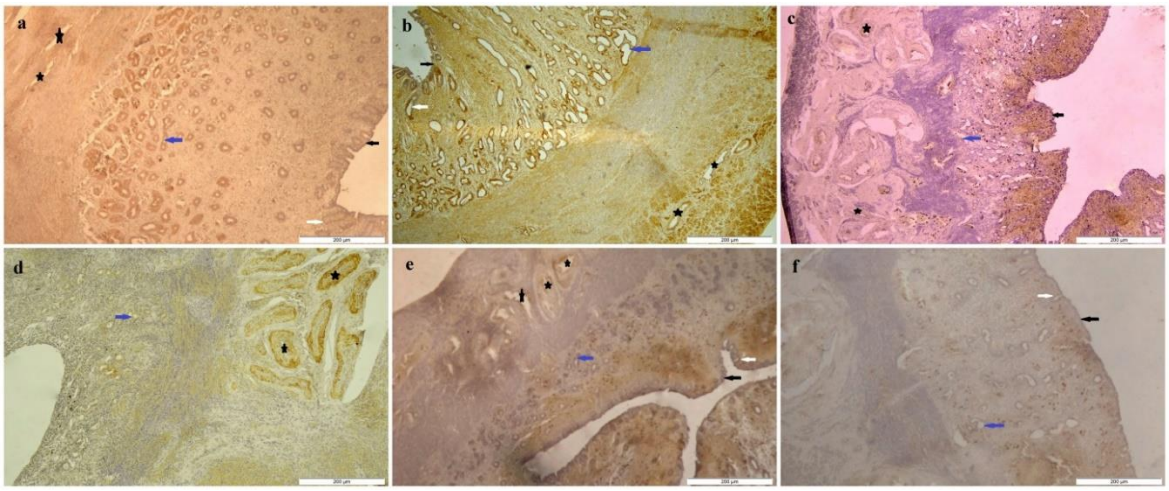

Figure 3. Expression of MUC1 in the bitch uterus during sexual cycle. corpus uteri (a) and cornu uteri (d) in proestrus; corpus uteri (b) and cornu uteri (e) in metestrus-diestrus; corpus uteri (c) and cornu uteri (f) in anestrus. Uterus epithelial cells (black arrow), crypt epithelial cells (white arrow), uterus gland epithelial cell (blue arrow), blood vessel (stars). Immunohistochemical Staining.

MUC16, also known as CA125 antigen, is the largest of the mucins known to date with a molecular weight of 2.5 MDa and a length of 22,152 amino acids (Perez et al., 2008). MUC16 is involved in barrier formation on the surface of epithelial cells (Dharmaraj et al., 2010). In human endometrium, MUC16 was reported to be expressed on the surface of luminal epithelium and glandular epithelium throughout the menstrual cycle (Gipson, 2008). In gynaecological practice, MUC16 serum levels are used as a tumour marker in ovarian cancer (Babic et al., 2017). In addition to the insufficiency of literature data on the relationship between MUC16 expression and reproductive outcome, it was reported that it acted as a chemical barrier to protect the epithelium against harmful environmental conditions and pathogens in the tissue where it was located (Felder et al., 2014; Haridas et al., 2014). Muc-16 expression was detected only in the cornu uteri during the metoestrus-dioestrus period. Similar to other cycle periods, MUC16 expression was absent in the corpus uteri (Figure 4a). MUC16 expression was found to be intensive in the endometrium of the cornu uteri and mild in the blood vessels in the stratum vasculare (Figure 4b).

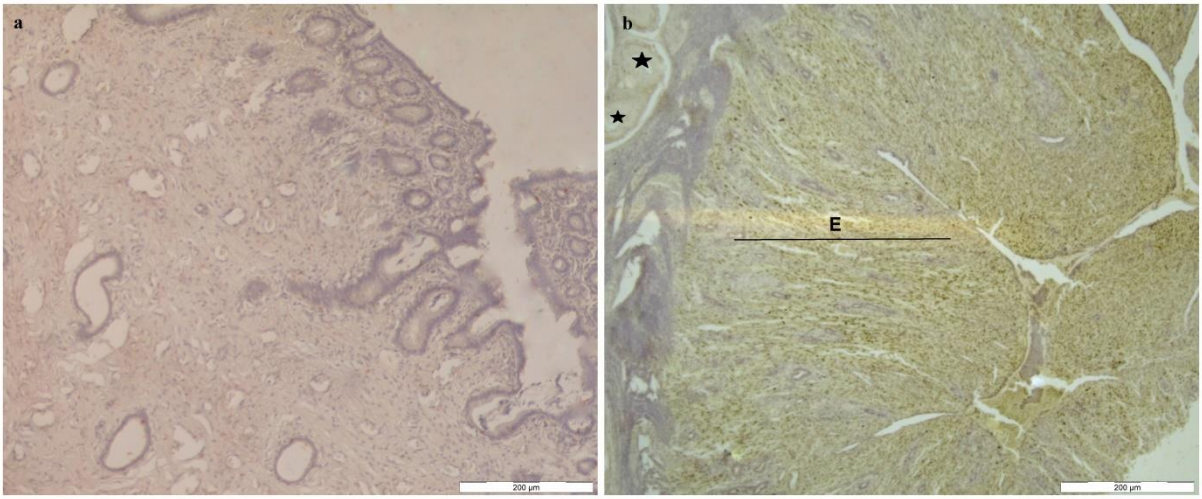

Figure 4. Expression of MUC16 in the bitch uterus in metestrus-diestrus. corpus uteri (a) and cornu uteri (b). Endometrium (E), blood vessels (stars). Immunohistochemical Staining.

The absence or persistence of pregnancy requires a combination of several reasons to be considered. In this study, immunohistochemical expressions of MUC1 and MUC16 were analysed in estrus cycles of bitches and it was observed that MUC1 was released in all sexual cycle periods. MUC1 was found to be expressed in the surface epithelium of both cornu and corpus uteri, crypt and uterus gland epithelium, stratum vasculare, myometrium and

perimetrium layers in bitches uterus tissue. It was noted that its secretion increased during metestrus and decreased during anestrus. MUC16 was found to be expressed in the cornu uteri during the metestrus-diestrus period. In the literature, the importance of mucins in facilitating implantation, protecting against bacterial damage and ensuring healthy continuity of pregnancy is mentioned. In our study, the increase in the release of MUC1 and MUC16, especially in the metestrus period, observed that mucins were affected by hormones. Our results also demonstrated the importance of mucins in implantation and pregnancy.

We believe that our study may provide a different perspective in addition to the existing criteria in the evaluation of complications occurring during pregnancy in different animal species, especially in veterinary medicine.

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Conflict Of Interest

The authors report no conflicts of interest.

Author's Contributions

The authors declare that they have contributed equally to the article.

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