

# Vaginal cytology in mares in the different stages of the estral cycle associated with the seasons of the year

## *Citologia vaginal em éguas nas diferentes fases do ciclo estral associadas as estações do ano*

Joice Maria Bazerla Andreta<sup>1</sup> , Luiz Henrique Garcia da Silva<sup>2</sup>, Matheus Runyan Belotto<sup>3</sup> , Cleber Fernando Menegasso Mansano<sup>4</sup> , Halim Atique Netto<sup>5</sup> , Anelise Ribeiro Peres<sup>6\*</sup> 

**ABSTRACT:** The reproductive biotechnologies directly assist in the development of different methods of reproductive evaluation. The objective of this study was to develop a new method to determine the estrous cycle phase in equine females through vaginal cytology throughout the seasons, as well as to establish an estimate of inflammatory cells present in the vagina. Six mares were evaluated at different ages without a defined breed and reproductive activity and were subjected to ultrasound evaluation of the reproductive system and cytological analysis of the vaginal region. In the summer, there was a predominance of keratinized epithelial cells in the estrus and intermediate in the diestrus. In the autumn, there was a predominance of keratinized cells in estrus and parabasal cells in estrus and diestrus. In anestrus (winter), a greater number of parabasal cells was identified in relation to the other cell types. In estrus (spring), there was a predominance of parabasal and intermediate cells. Conversely, in diestrus, parabasals were found in greater numbers. The evaluation of inflammatory cells of the vaginal epithelium of mares showed greater activity in the summer; however, there are no reference values for healthy animals in the literature, and it is necessary to conduct studies on the subject. It is concluded that there is an influence of cyclicity on the vaginal epithelium of the equine species, varying according to the season. Additionally, vaginal cytological evaluation is an important complementary tool in the diagnosis of vaginitis in mares, requiring further research on the subject.

**KEYWORDS:** estrous cycle; equines; cytological examination; vagina.

**RESUMO:** As biotecnologias reprodutivas auxiliam diretamente no desenvolvimento de diferentes métodos de avaliação reprodutiva. O objetivo deste estudo foi desenvolver um novo método para determinar a fase do ciclo estral em fêmeas equinas através da citologia vaginal ao longo das estações, bem como estabelecer uma estimativa das células inflamatórias presentes na vagina. Seis éguas foram avaliadas em diferentes idades, sem raça definida e em atividade reprodutiva, e foram submetidas à avaliação ultrassonográfica do aparelho reprodutor e análise citológica da região vaginal. No verão, houve predomínio de células epiteliais queratinizadas no estro e intermediárias no diestro. No outono, houve predomínio de células queratinizadas no estro e células parabasais no estro e diestro. No anestro (inverno), foi identificado maior número de células parabasais em relação aos demais tipos celulares. No estro (primavera), houve predomínio de células parabasais e intermediárias. Por outro lado, no diestro, os parabasais foram encontrados em maior número. A avaliação das células inflamatórias do epitélio vaginal de éguas mostrou maior atividade no verão; entretanto, não há valores de referência para animais saudáveis na literatura, sendo necessário a realização de estudos sobre o assunto. Conclui-se que há influência da ciclicidade no epitélio vaginal da espécie equina, variando de acordo com a estação do ano. Além disso, a avaliação citológica vaginal é uma importante ferramenta complementar no diagnóstico da vaginite em éguas, necessitando de mais pesquisas sobre o assunto.

**PALAVRAS-CHAVE:** Ciclo estral; Equinos; Citologia; Vagina.

<sup>1</sup>Pós graduanda em Reprodução Animal - Faculdade de Ciências Agrárias e Veterinárias – FCAV/UNESP. Departamento de Patologia, Reprodução e Saúde Única – Jaboticabal, São Paulo, Brasil.

<sup>2</sup>In memoriam

<sup>3</sup>Médico Veterinário Autônomo. Potirendaba, São Paulo, Brasil.

<sup>4</sup>Docente do Programa de Mestrado em Ciências Ambientais, Universidade Brasil – Fernandópolis, São Paulo, Brasil.

<sup>5</sup>Docente do Centro Universitário de Rio Preto – UNIRP. São José do Rio Preto, São Paulo, Brasil.

<sup>6</sup>Médica Veterinária - Divisão de Bem Estar Animal da Secretaria do Meio Ambiente - Prefeitura Municipal de Ribeirão Preto, São Paulo, Brasil.

\*Corresponding author: ane.medvet@hotmail.com

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## INTRODUCTION

Equine farming in Brazil is in constant development, with a herd of 5.58 million heads, occupying 3rd place in the world ranking, according to 2016 (PRODUCTION PECUÁRIA MUNICIPAL, 2016). In addition to generating approximately 610 thousand direct jobs, the equine industry generated approximately BRL 16.5 billion in 2018 (CILO, 2019). Such growth is associated with factors such as the use of these animals in agricultural activities, intensification and dissemination of equestrian sports and the use of reproductive biotechnologies, which directly assist in increasing this herd.

Mares are classified as seasonal polyestric females with a positive photoperiod because their estrous cycle is directly related to the duration of light during the seasons, influencing the hypothalamic-pituitary-gonadal axis. In addition, factors such as age, race, climate, nutrition, body condition score and health status also influence reproductive activity. The reproductive cycle of these animals occurs mainly in the interval between the winter and summer solstice in most countries located in South America, with an average duration of 21 days, which, according to Hafez and Hafez (2004), is divided into follicular and luteal phases.

The reproductive system of the female equine is composed of the vulva, vagina, cervix, uterine body and horns, uterine tubes and ovaries. These structures are subject to the occurrence of infectious and inflammatory diseases. The main factor that affects the vagina is vaginitis, usually associated with the presence of nonspecific and opportunistic etiologic agents, perineal conformation, and failed coaptation of the vulvar rhyme (SANTOS; ALESSI, 2016).

The diagnosis is made by finding clinical signs such as hyperemia of the vulvovaginal mucosa and the presence of exudate and with the aid of complementary exams of exfoliative cytology, allowing an analysis of vaginal cell morphology (SANTOS; ALESSI, 2016).

Cytological analysis is more widely used in the evaluation of uterine pathologies in mares, such as endometritis. However, it is of great diagnostic value as confirmatory evidence in cases of urovaginal and fungal infections (GALHÓS, 2018; AHMADI et al., 2006). It is of utmost importance to develop new studies on these complementary exams, given that the current literature has a deficit on the subject addressed above, to allow exploring the potential of vaginal cytology both for disease evaluation and for obtaining values under normal conditions.

The objective of this study was to develop a new method to determine the estrous cycle phase in equine females by evaluating the different cellular morphologies present in the vagina of mares in reproductive activity throughout the seasons and their different phases of the estrus cycle, as well as to establish an estimate of inflammatory cells in the vagina by sizing the number of cells.

## MATERIALS AND METHODS

Six mares were evaluated from the herd of the Veterinary Hospital located in the city of São José do Rio Preto, SP, Brazil, with different ages (7 - 14 years), mixed breed and reproductive activity. The evaluations occurred in 5 stages. They were subjected to the initial gynecological evaluation (Figure 1), in which the stage of the estrous cycle of each female was classified. Proestrus is the period in which the growth of one or more follicles occurs under the influence of follicle stimulating hormone (FSH). Estrus is the phase defined by the time when the mare is sexually receptive because the follicles secrete estrogen, which is responsible for the behavior of estrus. In the diestrus phase, progesterone is produced by the corpus luteum formed after ovulation of the dominant follicle (HAFEZ; HAFEZ, 2004).

Based on this classification, each female was subjected to evaluations throughout the year, according to the seasons: summer, autumn, winter and spring.



**Figure 1.** Gynecological evaluation in mares. A - Transrectal palpation and ultrasound. B - Evaluation of the external organs of the female reproductive system of mares. C - Internal evaluation in the vagina and sample collection of the vaginal epithelium for cytological analysis with the aid of a speculum.

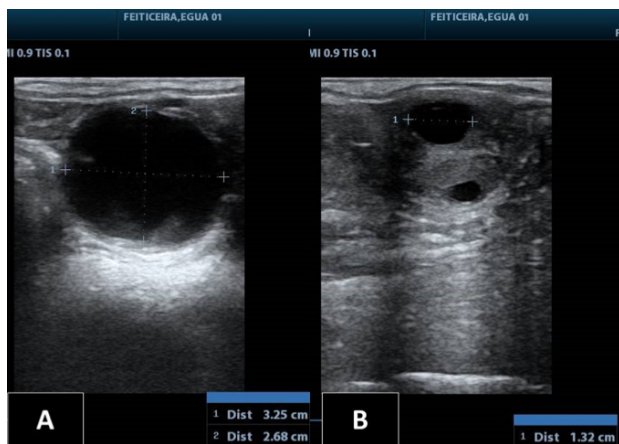
The mares were taken to the containment trunk, where the rectum was emptied, followed by ultrasound (Mindray DP-10 Vet), linear probe, B-mode, transrectally. The phases of the estrous cycle were defined by means of ultrasound examination of the reproductive system, via the transrectal route, and by measuring the diameter of the largest follicle in the ovary. The classification of the estrous cycle phase by follicle diameter was based on the study by Almeida et al. (2001), who evaluated estrus synchronization and follicular dynamics through different hormonal treatments and found that the animals manifested estrus with follicles starting at  $21.34 \pm 0.84$  mm. that animals with follicles up to 20 mm in diameter and absence of corpus luteum were classified in the proestrus phase. Animals with follicles 30 mm in diameter were classified in the estrus phase (it was decided not to collect samples from the beginning of estrus). Animals with the presence of the corpus luteum were classified in the diestrus phase (Figure 2). In the winter season, in which the follicles remained with a diameter of less than 20

mm and thus there was no ovulation (there was no formation of the corpus luteum), the animals were classified into anestrus, which is the period of ovarian inactivity.

After evaluation of the estrous cycle phase, rigorous hygienization of the perineum was performed with 2% chlorhexidine, followed by the collection of the cytological sample, with the aid of a sterile vaginal speculum and cytological brush. After collection, the slides were stained using panotic dye. The collection and processing of material for cytological analyses were performed according to Costa et al. (2009), adapted for the equine species.

At each station, samples were collected for cytology according to the stages of the estrous cycle of each female: proestrus, estrus, diestrus and anestrus. The evaluation of the slides was performed with the aid of microscopy, counting 100 cells per slide, allowing us to quantify the percentage of epithelial and inflammatory cells in each sample.

Statistical analyses were performed using Fisher's LSD method and 95% confidence. The null hypothesis was rejected at 25% when the p-value was less than 25%. As the biological model was used, a higher level of the p value (0.25) was adopted due to the oscillation in this type of reading.



**Figure 2.** Measurement of the diameter of the largest follicle to determine the estrous cycle. A: Stage Estro - Follicle with a diameter of 32.5 mm. B: Proestrus Phase: Follicle with 13.2 mm diameter.

## RESULTS

During the summer, as shown in Table 1, in the estrus phase, there was a significant difference between the superficial cells ( $\bar{X} = 4.25\%$ ) and keratinized cells ( $\bar{X} = 29.0\%$ ), and the keratinized fruits were exhibited in greater quantity in this phase. There was no significant difference between the other cell types in this phase. Conversely, in diestrus, there was a significant difference between the intermediate cells ( $\bar{X} = 29.75\%$ ) and superficial cells ( $\bar{X} = 1.00\%$ ) and between intermediate and keratinized cells ( $\bar{X} = 10.50\%$ ), and the intermediates were present in greater quantity in this phase; there was also a significant difference for keratinized cells ( $\bar{X} = 10.50\%$ ), between the parabasal cells ( $\bar{X} = 22.25\%$ ) and inflammatory cells ( $\bar{X}$

**Table 1.** Different cell types of the vaginal epithelium of mare in the different stages of the estrous cycle throughout the seasons.

Seasons	Summer		Winter	Autumn			Spring		
Estrous cycle phase	Estrus	Diestrus	Anestrus	Proestrus	Estrus	Diestrus	Proestrus	Estrus	Diestrus
	<b>Células (%)</b>								
Basal	15,25 <sup>ab</sup>	12,75 <sup>abc</sup>	10,00 <sup>b</sup>	3,75 <sup>b</sup>	3,75 <sup>b</sup>	5,50 <sup>b</sup>	18,00	7,67 <sup>b</sup>	12,00 <sup>b</sup>
Parabasal	23,50 <sup>ab</sup>	22,25 <sup>ab</sup>	56,50 <sup>a</sup>	22,8 <sup>ab</sup>	34,00 <sup>a</sup>	36,30 <sup>a</sup>	23,00	26,67 <sup>a</sup>	42,7 <sup>a</sup>
Intermediary	21,50 <sup>ab</sup>	29,75 <sup>a</sup>	5,25 <sup>b</sup>	18,50 <sup>ab</sup>	22,00 <sup>ab</sup>	23,50 <sup>ab</sup>	13,67	27,00 <sup>a</sup>	10,67 <sup>b</sup>
Surface	4,25 <sup>b</sup>	1,00 <sup>c</sup>	12,00 <sup>b</sup>	6,00 <sup>b</sup>	5,25 <sup>b</sup>	5,00 <sup>b</sup>	15,00	13,33 <sup>ab</sup>	16,7 <sup>ab</sup>
Keratinized	29,0 <sup>a</sup>	10,50 <sup>bc</sup>	12,75 <sup>b</sup>	32,8 <sup>a</sup>	21,3 <sup>ab</sup>	24,50 <sup>a</sup>	23,3	17,67 <sup>ab</sup>	14,67 <sup>ab</sup>
Inflammatory cells	6,50 <sup>ab</sup>	23,75 <sup>ab</sup>	3,50 <sup>b</sup>	16,25 <sup>ab</sup>	13,75 <sup>ab</sup>	5,25 <sup>b</sup>	7,00	7,67 <sup>b</sup>	3,33 <sup>b</sup>
Standard error	3,32	2,37	1,94	2,77	2,87	2,57	3,41	2,34	3,71
F Value	1,45	3,26	17,43	2,51	2,65	4,44	0,55	2,30	2,21
P Value	0,250	0,029	0,001	0,068	0,050	0,008	0,739	0,110	0,121

Grouping Information Using Fisher's LSD Method and 95% Confidence. Averages that do not share a letter are significantly different. The null hypothesis was rejected at 25% when the p-value was less than 25%.

= 23.75%); there was no significant difference between the basal cells and the other cells found (Figure 3).

During the autumn, in the proestrus phase, there was a significant difference between the keratinized cells ( $\bar{X}$  = 32.80%), superficial ( $\bar{X}$  = 6.0%) and baseline ( $\bar{X}$  = 3.75%), and the number of keratinized cells was higher; there was no significant difference between the other cell types. In the estrus phase, there was a significant difference between the parabasal cells ( $\bar{X}$  = 34.00%), basal cells ( $\bar{X}$  = 3.75%) and superficial cells ( $\bar{X}$  = 5.25%), and the parabasal cells were exhibited in greater numbers; there was no significant difference for the other cell types. In the diestrus phase, there was no significant difference between the parabasal cells ( $\bar{X}$  = 36.30%), keratinized ( $\bar{X}$  = 24.50%) and intermediate ( $\bar{X}$  = 23.50%), and the number of parabasal cells was higher. However, there was a significant difference between the cell types mentioned above (except the group of intermediate cells) and the basal cells ( $\bar{X}$  = 5.50%), inflammatory ( $\bar{X}$  = 5.25%) and superficial ( $\bar{X}$  = 5.00%; Table 1; Figure 3).

In the winter, during the anestrus phase of the mares, there was a significant difference in parabasal cells ( $\bar{X}$  = 56.50%) in relation to the other cell types, being present in greater quantity (Table 1).

During the spring, in the proestrus phase, there was no significant difference between cell types. In the estrus phase, there was no significant difference between the intermediate cells ( $\bar{X}$  = 27.00%), parabasal ( $\bar{X}$  = 26.67%), keratinized ( $\bar{X}$  = 17.67%) and superficial ( $\bar{X}$  = 13.33%). However, there was a significant difference between the cellular groups (parabasal and intermediate) when compared to the basal cells ( $\bar{X}$  = 7.67%) and inflammatory ( $\bar{X}$  = 7.67%). In this phase, there was a predominance of intermediate and parabasal cells. In the

diestrus phase, there was a significant difference between the parabasal cells ( $\bar{X}$  = 42.70%) and basal cells ( $\bar{X}$  = 12.00%), intermediate ( $\bar{X}$  = 10.67%), and inflammatory ( $\bar{X}$  = 3.33%), with a predominance of parabasal cells (Table 1; Figure 3).

The evaluation of the presence of inflammatory cells in the vaginal epithelium, as shown in Table 2, found that there was a significant difference between the phase of diestrus ( $\bar{X}$  = 23.75) in the summer with the estrus phases ( $\bar{X}$  = 6.50) also in the summer, from the diestrus in the fall ( $\bar{X}$  = 5.35), anestrus in the winter, proestrus ( $\bar{X}$  = 3.33), estrus ( $\bar{X}$  = 7.67), and diestrus in the spring ( $\bar{X}$  = 7.00). There was also a significant difference between autumn proestrus ( $\bar{X}$  = 16.25) and winter anestrus ( $\bar{X}$  = 3.50; Figure 4).

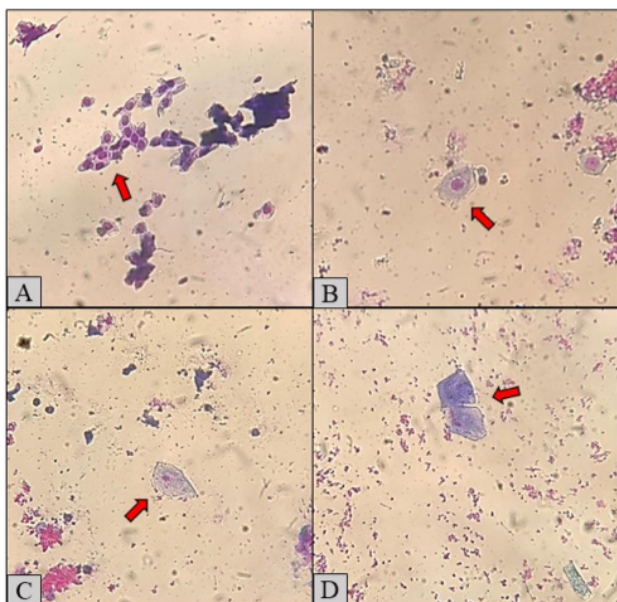
### DISCUSSION

Exfoliative vaginal cytology is an easy and low-cost technique that consists of monitoring the various stages of the estrous

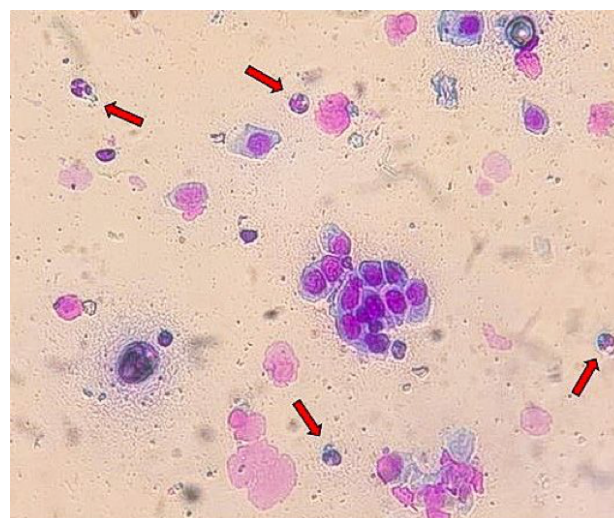
**Table 2.** Quantitative evaluation of inflammatory cells in the different stages of the estrous cycle of mares throughout the seasons.

Inflammatory cells		
Summer	Diestrus	23,75 <sup>a</sup>
	Estrus	6,50 <sup>bc</sup>
Winter	Diestrus	5,25 <sup>bc</sup>
	Estrus	13,75 <sup>abc</sup>
	Proestrus	16,25 <sup>ab</sup>
Autumn	Anestrus	3,50 <sup>c</sup>
Spring	Diestrus	3,33 <sup>bc</sup>
	Estrus	7,67 <sup>bc</sup>
	Proestrus	7,00 <sup>bc</sup>

Grouping Information Using Fisher's LSD Method and 95% Confidence. Averages that do not share a letter are significantly different.



**Figure 3.** Cytology of vaginal epithelium of mares. A - Parabasal epithelial cells. B - Intermediate cell. C - Superficial cell. D - Keratinized cells.



**Figure 4.** Cytology of vaginal epithelium of mares. The arrows indicate inflammatory cells in the cytology of the vaginal epithelium of mares.

cycle. This technique is based on the observation of different vaginal epithelial cells that may vary according to the predominant hormonal stimulus in each phase (KUSTRITZ, 2010).

The mare's estrous cycle has an average duration of 21 days, consisting of 14 days of diestrus and 7 days of estrus. The secretion of LH (luteinizing hormone) gradually increases throughout estrus and reaches its peak on the day following ovulation. FSH (follicle stimulating hormone) secretion occurs approximately every 10 days in the middle of estrus and after estrus. ovulation, during the follicular phase of the estrous cycle, follicular growth occurs with production of estrogen by the follicle, which results in behavioral estrus. After ovulation, the CL (corpus luteum) that secretes progesterone forms, and maximum concentrations of progesterone are reached 6 days after ovulation, characterizing the phase of diestrus (HAFEZ; HAFEZ, 2004; BRINSKO et al., 2011).

Females of equine species are considered seasonally polyestric with a positive photoperiod because they have more than one estrous cycle per year during the months when there is a greater presence of light (HAFEZ; HAFEZ, 2004).

Unlike equine species, there are extensive studies on vaginal cytology in female dogs for monitoring the estrous cycle. Kudalkar et al. (2020) observed that female dogs in the estrous phase, near the time of ovulation, showed a predominance of 80% keratinized cells in vaginal cytology. These results are similar to those of the present study, in which the predominance of keratinized cells in the estrus phase of summer was identified, the season in which estrous activity occurs in the equine species due to the influence of luminosity on the hypothalamic-pituitary-gonadal axis. However, it is noteworthy that, unlike mares, female dogs are nonseasonal monoestric, and due to such characteristics, the estrous cycle of this species is not influenced by the seasons, as in the case of equine females (CONCANNON, 2011).

Due to the geographic position of our region in relation to the equator, solar incidence predominates during the day even in autumn, although to a lesser extent than summer, which explains the fact that some mares evaluated maintained follicular activity in this period. Under these circumstances, the autumn proestrus phase showed the same predominance of keratinized cells. Conversely, in estrus and diestrus, the equine species showed a predominance of parabasal cells, probably because autumn is considered a transition season between the cyclic period of mares and anestrus. This cell type is predominant in the anestrus phase of both the equine species and the canine species (CONCANNON, 2011; SILVA, 2016).

In winter, the low solar incidence acts on the activity of the hypothalamic-pituitary-gonadal axis in a negative way, inhibiting follicular activity and characterizing the anestrus period of equine species. According to Concannon (2011), in the anestrus phase of the bitches, there is a predominance of parabasal cells, in addition to few neutrophils, similar to the results of this study obtained through cytology performed in

the anestrus. However, Silveira et al. (2017) observed in their study that sheep during the anestrus period have a predominance of intermediate cells.

Similar to autumn, spring is also considered a transition season for mares, where incident light tends to increase, causing the animal to leave the anestrus period and begin its follicular activity, reaching its peak in the summer. At this station, the predominance of intermediate cells in the estrus phase and parabasal cells in the diestrus phase was identified. According to Reddy et al. (2011), unlike mares, female dogs showed a decrease in the number of intermediate cells in the estrus phase. In diestrus, the results were similar, in which there was a predominance of parabasal cells in both species.

A study conducted with 11 sheep and 11 goats evaluated the cytology and the presence of cervical mucus after the synchronization of the estrous cycle, identifying that vaginal cytology in goats had 90% accuracy in detecting the time of ovulation, when 70% of the cells were superficial nuclei. However, ewes subjected to the same protocol showed no cell pattern in the evaluation of vaginal cytology (MACHADO; FONSECA, 2019). A similar study conducted by Silveira et al. (2017) evaluated the estrous cycle synchronization of 11 Corriedale crossbred ewes during the anestrus period and identified a predominance of intermediate cells in all phases, where an increase in the presence was expected. of superficial cells in estrus. Similar to mares, goats and sheep are seasonal polyestric species. However, they have a negative photoperiod, in which the absence of luminosity stimulates the hypothalamic-pituitary-gonadal axis.

It is noteworthy that the abovementioned studies were performed through pharmacological control of the estrous cycle, obtaining different results than expected according to the physiology of each species. However, in the present study, mares were evaluated according to the physiological manifestations of each phase without using any medication.

Veber et al. (2016) evaluated the vaginal cytology of four quarter-mares from the region of Campanha/RS, where they identified a greater number of keratinized cells in relation to the other cell types, similar to the results found in the estrus phase in the summer. present study. However, there was no further elucidation of the respective authors regarding the stage of the estrous cycle, as well as the season of the year in which the samples were collected, preventing a more accurate comparison.

Although transrectal ultrasound is the most accurate method for determining the stage of the estrous cycle for equine species, with the possibility of obtaining more detailed information, in addition to obtaining immediate results, the use of vaginal cytology to determine the stage of the estrous cycle may become an important tool to assist in the reproductive management of these animals.

The counting of inflammatory cells through vaginal cytology of the equine species has the advantage of directly assisting in the diagnosis of pathologies of the vagina, as in the case of vaginitis resulting from urovagina, pneumovagina and failures in

coaptation of the vulvar lips by trauma or low condition score. (AMHMADI et al., 2006). However, there is a lack of studies reporting a normal quantification of inflammatory cells in the vaginal epithelium of healthy mares, thus hindering the diagnosis of vaginitis, as well as the evaluation of treatment efficacy.

In female dogs, the presence of erythrocytes and neutrophils is common in the proestrus phase and reduced almost completely in the estrus, and the presence of inflammatory cells returns in diestrus, and there may even be phagocytosis of some cells (Reddy et al., 2011). Similar results were found in this study, in which in the reproductive season (summer), there was a decrease in inflammatory cells in the estrus period and an increase in diestrus. Due to the scarcity of studies in equine species, it was not possible to compare the other phases of the estrous cycle or with the different seasons of the year, and this is the first study that established an evaluation of inflammatory cells found in the vaginal epithelium of healthy mares during the whole year, that is, in all phases of the estrous cycle, in the four seasons of the year. Thus, the importance

of establishing a dimension of the number of inflammatory cells in healthy animals is emphasized, and subsequently, such evaluation associated with clinical signs confirms the diagnosis of vaginitis, since it is a common condition in this species.

## CONCLUSIONS

It is concluded that there is an influence of cyclicity on the vaginal epithelium of the equine species, varying according to the estrous cycle phase and season of the year and that although transrectal ultrasound is the most accurate method for determining the estrous cycle phase for the equine species, the use of vaginal cytology to determine the stage of the estrous cycle may, through further research in the area, become an important tool to assist in the reproductive management of these animals.

Additionally, the cytological evaluation of the vaginal epithelium is an important tool to aid in the diagnosis of vaginitis in mares, requiring further studies on the subject, with a larger number of animals, thus allowing the differentiation in pathological conditions and the comparison of the efficacy of the treatment.

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