Comparative histomorphological characteristics of the endometrium of young and aged mares in estrus and diestrus

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Abstract. The histomorphometric indices (height of the lining (LE) and glandular (GE) epithelium, diameters and density of glands) in endometrial samples (n=21) of young gynecologically healthy mares of 3–8 yo (n = 4) and older problem mares of 12–19 yo (n=7) were studied in estrus and diestrus of one cycle. The height of the LE and GE in aged mares was slightly higher than in young ones, but the difference was not significant. It was found significant differences between groups in diameters and density of glands, more obvious in stratum spongiosum (SS) then in stratum compactum (SC) of endometrium. It has been shown that diameter of endometrial glands in older mares was two to three times larger than in young mares in both estrus (55.9–22.5 μm, p<0.05) and diestrus (62.5–20.1 μm, p <0.01), respectively. The density of glands in SS in young mares in diestrus significantly (p<0.01) increased, compared with this in estrus (9.8–17.5 units/field), while in older mares it almost did not change (11.1–11.3 units/field). The difference of histomorphometric indices in estrus and diestrus can serve as indirect characteristic of an elasticity of an endometrial tissue probably connected with clearance of the uterus. Therefore the double biopsy carried out in estrus and diestrus can be useful for endometrium function assessment in mares.

1. Introduction
Evaluation of a condition of mares’ reproductive system is an important and obligatory aspect in horse breeding technology. Rectal, vaginal, ultrasonic examinations, cytological, bacteriological analyses are the main diagnostic methods. However in some cases (subclinical or chronic endometritis, endometrosis, lymphatic lacunas, angiosis) only the endometrial biopsy allows to reveal pathologies and degree of the destructive process in the uterus and to predict infertility in mares [1, 2, 3, 4, 5, 6, 7]. However nowadays in Russia the histological analysis of endometrium isn’t among routine diagnostic methods in horse reproduction. Therefore the true reason of a subfertility and infertility of mares often remains not found out.

E. Seaborn in 1925 was one of the first researchers who described histological structural changes of endometrium in mares (postmortem) [8]. According to R.M. Kenney (1978), the mares’ endometrium consists of the lining epithelium (LE) and lamina propria, which has two layers: stratum compactum (SC) and stratum spongiosum (SS). Lamina propria contains the glands and vessels (arterioles, venules, lymphatics) connected by a collagen matrix. Glands are covered by a glandular epithelium (GE), with
the end opened into the uterus lumen and with branches going deeply into the stratum spongiosum [3, 9].

Many authors studied the relationship between fertility and specific pathology in endometrium of a mare [10, 3, 4, 11, 6, 12]. R.M. Kenney provided the bases and the main principals of histological evaluation of mares’ endometrium and the guidance for prognoses of its reproductive potency by analyses of biopsy samples. Later he improved this system with P.A. Doig [3, 4]. Three categories of destructive changes in endometrium of mares (from mild (I) to moderate (IIA, IIb) and severe (III) that highly correlate with decrease in fertility were found out [4]. The degree of inflammation, fibrosis and atrophy of endometrium were considered to be the main characteristics. Later this system became "the gold standard" for diagnostic of mare’s endometrium condition [7]. Some researchers find this classification incomplete without important histopathological data (angiosis, angiopathy, endometrial maldifferentiation) [2, 13, 14].

Indications to histological evaluation of endometrium in broodmares are: numerous cases of barrenness, embryonic death and pregnancy loss in reproductive history of a mare, disorders or absence of normal estrus cycles during physiologic breeding season and other pathologies.

Number of polymorphonuclear leukocytes - neutrophils (PMN-cells) in cytological samples and the positive bacteriological analysis are the main signs of an acute endometritis in mares along with other clinical signs (fluid accumulation within the uterus, discharge from the vulva, etc.). However at a subclinical endometritis neutrophils can be absent in samples [1]. And in a chronic stage of a disease lymphocytes may present in cytological and biopsy samples [3, 4]. Therefore at diagnostics of the subclinical endometritis "the best (gold) standard" is the endometrial biopsy and identification of PMN-cells in a compact layer of endometrium in histologic samples [15, 3, 4, 5]. According to J.M. Nielsen (2005), more exact diagnosis is received in the result of bacteriological and histological analysis of endometrium biopsy sample in comparison with routine cytological and bacteriological analysis of swabs from the surface of endometrium [16].

Fibrosis of endometrium is considered to be one of the main reasons of mares subfertility [17]. According to C. Hoffmann et al. (2009), an expression of receptors of steroid hormones is decreased in fibrous stromal cells compared with an intact stroma that indicate their dedifferentiation [2]. However there were not found significant differences in the content of periglandular collagen in histological samples of endometrium of the mares susceptible to a post-breeding endometritis and resistant to it [18] and also no any relationship between type of a collagen and fibrosis of endometrium revealed [19].

S.W. Ricketts and S. Alonso (1991) reported close connection between the severity of a chronic degenerative disease of endometrium (endometrosis) and an age of mares [6]. M. Hanada et al. (2014) also established that aging plays an important role in a pathogenesis of endometrosis in mares. Disorders of arterial blood supply and venous outflow because of angiosis or angiopathy of uterus vessels lead to a decrease in a lymphatic drainage and closely connected with the beginning and progressing of fibrosis and atrophy of endometrium that can cause of a hypofunction of a myometrium in the peri-implantation or puerperal period [20].

Results of the researches by M. Hanada et al. (2012) showed that any part of an uterus, except of a caudal part of an uterus body where density of glands and thickness of endometrium were considerably lowered [21], are suitable for a routine biopsy of endometrium. There weren’t found essential differences in number of PMN-cells in the cytological and histological samples taken from different sites of the basis and tip of horns of the uterus [22]. It was also reported that the biopsy procedure even at repeated examinations had no negative effect on next pregnancies in ginecologically healthy mares [23].

The choice of the most suitable phase for endometrium biopsy procedure in connection with seasonality of reproduction function in mares was a subject of the studies by R. Killisch et al. (2017). Authors concluded that for assessment of the functional morphology of endometrium the diagnostic biopsy has to be carried out in breeding season, since the end of spring till the beginning of an autumn transition period [24].

The histological structure of endometrium changed under the influence of hormones in different phases of estrus cycle. So, the diffuse hyperplasia and stromal oedema of endometrium was observed in
estrus [25, 3, 4]. Some alterations in the LE and GE cells are noted, in particular, change of its height in estrus and diestrus. Therefore histomorphometrical indices are considered very important as the useful addition in assessment of mare endometrium [25, 26]. G. D.Mansour et al. (2015) showed by histochemical methods that cells of the LE have higher proliferation during estrus, while GE cells during diestrus [27]. At the same time the expression of a number of enzymes participating in the process of metabolism of a glycogen raises in a diestrus and early pregnancy [28]. To identify an inflammatory process in endometrium, the criteria of which is the quantity of PMNs in the sample, it is recommended to get the material for a cytological and histological research in the phase of estrus [15]. However, according to R.M. Kenney and P.A. Doig (1986), histologic structure of endometrium is least variable during diestrus [4]. W. Overbeck also considered the phase of a diestrus more suitable for the histological analysis at diagnostics of a subclinical endometritis [5].

The aim of this study was to assess and compare histomorphometric indices (the height of LE and GE, diameter and density of endometrial glands in SC and SS of mare endometrium) in estrus and diestrus in young and aged mares. We hypothesized that the indices difference between two phases of estrus cycle can serve as a reference point for assessing endometrial function in young and old mares.

2. Methods and materials

The work was carried out on the basis of the experimental stable of “The All-Russian Research Institute for Horse Breeding” (Ryazan) and private horse-breeding farms of the central region of Russia. Mares of draft and riding breeds were included in the experiment. The biopsy samples of endometrium (n=21) were taken from barren mares (n=11) in estrus (day 2-3) and diestrus (day 8-10 after ovulation) in one cycle in the period of breeding season. Mares were distributed in two groups by age: 1) 3-8 years (n=4) and 2) 12-19 years (n=7). We couldn't get biopsy sample from one aged mare in estrus because it was mating in this period.

The reproductive tract of all mares was examined by rectal palpation, ultrasonic exam (Ultrasonic Scanner Honda HS 2000, 7.5 MHz probe), vaginoscopic and cytologic diagnostics before carrying out a biopsy. By results of examination, all animals in the group of young mares, were referred to the category of clinically and gynecologically healthy with normal regular cycle. In the group of aged mares three mares had signs of endometritis (fluid accumulation in the uterus, > 8-10 PMNs in the field of cytologic sample). All aged mares had cases of barrenness in the previous reproductive history, one mare had an embryonic death and four mares - abortions.

For biopsy procedure Jackson Uterine Biopsy Forceps (J-116, Jorgensen Laboratories, Inc.) with 4 mm x 28 mm cutting area and 60 cm length were used. Sampling of endometrium was performed under rectal control in the uterine horn-body junction or bifurcation area. The samples were fixed in 10% neutral formalin solution and sent to a pathology-anatomic department at the City Hospital No.11 (Ryazan). Histological sections were stained with Hematoxylin-Eosine. The histological sections have been analyzed under binocular optical microscope "OptitechXSP-128-30" with 400x magnification. For measurement of structural elements it was used a micrometer eyepiece WF10XDIN/18 mm. The height of LE and GE was measured from the basement membrane to the apical edge of the cell. The diameter of glands (the width of gland lumen) in SC and SS were determined as a half-sum distance between apical edges of the opposite epithelial cells in two perpendicular directions (figure 1). The density of glands and other average values were counted in ten randomly selected fields in each sample. All the histomorphometric indices were calculated separately in SC and SS (figure 2).

The obtained data were analyzed by usage of the Statistica 6.0 program and presented as means ± standard error. Significance of differences between compared values were determined using Student's t-test.
3. Results

Results of histologic research of endometrium of young and aged mares in different phases of the estrus cycle are presented in Table 1.

Table 1. Histomorphometric indices of endometrium in young and aged mares in estrus and diestrus

| Age of mares, years | Mares, n | Height of the LEa, μm | Height of the GEb, μm | Diameter of endometrial glands, μm | Density of endometrial glands, units per field |
|---------------------|----------|-----------------------|----------------------|-----------------------------------|-----------------------------------------------|
|                     |          | SCc                   | SSd                  | SC                  | SS                  |
| Estrus              |          |                       |                      |                     |                     |
| 3–8                 | 4        | 12.3±0.2              | 12.4±1.0             | 11.5±1.5            | 20.8±2.6            | 22.5±3.5e            | 19.9±1.4g            | 9.8±1.2c            |
| 12-19               | 6        | 15.4±1.5              | 13.5±0.9             | 17.0±2.7            | 23.8±3.5            | 55.9±11.0f           | 10.6±1.2h           | 11.1±0.8            |
| Diestrus            |          |                       |                      |                     |                     |
| 3–8                 | 4        | 14.4±1.3              | 14.1±0.5             | 14.0±1.4            | 20.0±3.2            | 20.1±3.0i            | 20.1±1.9k            | 17.5±1.8m           |
| 12-19               | 7        | 15.7±1.5              | 14.9±1.5             | 15.8±1.6            | 34.6±6.0            | 62.5±9.9j            | 11.3±1.4l            | 11.3±1.7n           |

*LEpering Epithelium
bGlandular Epithelium
cStratum Compactum
dStratum Spongiosum

\( p<0.05; \) \( \text{e}p<0.001; \) \( \text{i}p<0.01; \) \( \text{k}p<0.01; \) \( \text{m}p<0.05; \) \( \text{m}o p<0.01 \)

**Estrus.** LE of young mares consisted of single row of prismatic cells. GE was presented by cuboidal or prismatic cells with basal location of nuclei. In SC layer there were observed rare, tubular, roundish glands, mainly empty, and a few single glands with an eosinophilic secret. There were noted thin-walled full-blooded vessels. In SS layer glands were distributed evenly, had extended form with an uneven apical edge.

In aged mares endometrium had a lobular appearance. LE consisted of columnar cells. In SC there were observed tortuous glands with the nuclei extended along a basal membrane and an uneven apical edge.
edge. Diameter of glands in SS was twice larger, than in SC that evidences their stretching. In the most of aged mares the periglandular fibrosis was expressed (1-3 fibrous layers around glands) and glands organized in nests were present (figure 5). In mares with signs of endometrium inflammation there were found more than 10-15 neutrophils and a few lymphocytes per field in SC. Also it was found cystic transformation of some glands.

**Diestrus.** In endometrium samples of young mares in diestrus there were noted nuclei located at different levels and vacuolization of cytoplasm in LE cells were present. This was described by R.M. Kenny as a physiological sign of the estrus period [3]. Glands were small, tubular and tortuous. GE is of a single row, from low to high cylindrical. Nuclei were located basally, cytoplasm was vacuolized. The glands were mainly narrowed, and a secret was visible in separate glands. Apical edge of glands was irregular, characteristic of glands with merocrine type of secretion. The shape of glands were different: round, extended, tortuous. Separate glands of a star-shaped form differed in higher epithelium. Vessels were full-blooded, lying mainly in SS.

During diestrus in aged mares a single-row LE was presented by cells with nuclei located at one level. Endometrium stroma was compact of lobular structure. Individual large irregular glands covered by a low single-row epithelium were located inside segments, some of them were cystously dilated with an eosinophilic secret inside. Two-three layers of fibrin were found around glands. In tortuous glands the epithelium was more elongated. The even apical edge of GE cells demonstrated lack of secretory activity [29]. Vessels were high full-blooded, and hemorrhagic impregnation was noticed in some places of stroma.

**4. Discussion**

The analysis of the obtained data showed that in young mares in estrus the height of LE (14.4-12.3 μm) and GE both in SC (14.1-12.4 μm) and in SS (14.0-11.5 μm) decreased, compared with diestrus, that demonstrated secretory function of epithelium. In older mares in diestrus and in estrus height change of LE (15.7-15.4 μm) and GE in SC (14.9-13.5 μm) had the same character as in young mares, but with smaller difference. And in SS in aged mares it was observed the opposite dynamics (15.8 - 17.0 μm in diestrus and estrus, respectively), that may be due to delay of process of a secret excretion and its stasis in glands of a deeper layer of endometrium.

In aged and young mares in identical phases of estrus cycle diameter and number of glands in different layers of endometrium significantly differ (figures 3 and 4). More essential distinctions are observed in SS layer of endometrium. In young mares in diestrus, because of the absence of oedema, density of glands increases both in SC (19.9-20.1 units/field) and in SS (9.8-17.5 units/field), and diameter of glands slightly decreases (20.8-20.0 μm and 22.5-20.1 μm, respectively). In aged mares the average diameter of endometrial glands in diestrus is higher, than in estrus, in SS it is two-three times more (55.9-62.5 μm), than in young mares (22.5-20.1 μm) in estrus (p <0.05), and in diestrus as well (p <0.01). In SC diameter of glands in both groups had no significant differences in two phases of a cycle.

Density of glands in SC at young mares in estrus (p <0.001), and in diestrus (p <0.01) is twice more than in older mares. Density of glands in SC of young mares in diestrus significantly (p <0.01) increases, in comparison with estrus (9.8 – 17.5 units/field). While in older mares it almost doesn't change (11.1-11.3 units/field).
Figure 3. The young mare (4 yo). The density of glands in estrus (a) and diestrus (b). 400x

Figure 4. The aged mare (14 yo). The density of glands in estrus (a) and diestrus (b). 400x

Figure 5. The aged mare (16 yo). Estrus. Periglandular fibrosis: G- glandula, V – vessel, PG – periglandular fibrosis. 400x

Histomorphometric indicators (height of the LE and GE, diameters of glands) received in our researches agree with the data of M. Herrera et al. (2018) for mares in estrus phase. The authors reported increase in these indices in susceptible (SM) to post-breeding endometritis in comparison with the
resistant (RM) to it mares [26], which is similar to our results in groups of older and young mares. However the density of glands in this study was more in susceptible mares, then in resistant (24.8–20.5 glands/field, respectively). Also the authors didn't differentiate SC and SS in endometrium, so our results confirm their data, mainly, referring to a SS of endometrium.

The increase in the number of glands and the decrease in their diameter during diestrus is associated with a decrease in estrous edema and contraction of the uterus, which directly depends on the progestin-estrogen regulation [3]. It was shown that the destructive processes in endometrium, provoked by formation of the fibrous tissue, lead to change of expression of progesterone and estrogen receptors in affected areas of stroma and glandular epithelium. Therefore they become independent of mechanisms of hormonal control of the uterus and have specific dynamics of cell differentiation [2, 11]. Disorders in endometrium, including age-related one (decrease of total number of glands, formation of fibrotic and dilated glands, glandular “nesting”) affecting the elasticity of the tissue in older mares, indicate a poor drainage of the uterus, decrease in a trophicity of an endometrium and an uterus clearance. Also the protective function of the uterus and its ability to resist to infection reduces [29]. Such pathologies can be the reasons of a subfertility, embryonic death, abortions and stillbirth in mares.

5. Conclusion

Results of our researches confirmed that histomorphometric indices of endometrium in mares uterus change, depending on the estrus cycle stage [3, 4]. However this process has different intensity in young healthy mares and aged mares with subfertility problems. So in young mares LE in the period of estrus was lower than in diestrus whereas height of the LE in older mares remained invariable throughout the estrus cycle. The most essential distinctions are observed in a SS of endometrium. Increase in number of glands in SS layer in the period of diestrus in comparison with estrus, is a physiological indicator, which evidences contraction and compression of endometrium due to absence of estrus oedema. This fact should be considered at interpretation of results of the histologic analysis of the endometrial samples obtained from mares in various phases of the estrus cycle. Older mares have diameter of glands in a SS of endometrium significantly (2-3 times more) larger, and the density of glands in SC lower than in young mares both in estrus and in diestrus. At the same time in aged mares the difference in density of glands in both layers of endometrium (SC and SS) in estrus and diestrus is leveled whereas in young mares it significantly increases in SS in a diestrus. The difference of histomorphometric indices in estrus and diestrus can serve as indirect characteristic of an elasticity of an endometrial tissue probably connected with clearance of the uterus. Therefore the double biopsy carried out in an estrus and diestrus can be useful for endometrium function assessment in mares. The obtained data need to be confirmed on larger material.

References

[1] Amorim M, Gartley C J, Foster R A, Hill A, Scholtz E L and Hayes A 2016 J. Equine Vet. Sci. 44 54–61
[2] Hoffmann C, Ellenberger C, Mattos R C, Aupperle H, Dhein S, Steif B and Schoon H A 2009 Anim. Reprod. Sci. 111 (2–4) 261–78
[3] Kenney R M 1978 J. Am Vet. Med. Assoc. 172 241–62
[4] Kenney R M and Doig P A D 1986 Current Therapy in Theriogenology : Diagnosis, Treatment and Prevention of Reproductive Diseases in Small and large Animals (Morrow DA: Publishing WB Saunders) pp 723–9
[5] Overbeck W, Witte T S and Heuwieser W 2011 Theriogenology 75(7) 1311–8
[6] Ricketts S W and Alonso S 1991 Equine Vet. J. 23(1) 185–8
[7] Snider T A, Sepoy C and Holyoak G R 2011 Theriogenology 75(9) 1567–81
[8] Seaborn E 1925 Anat. Rec. 30 277–87
[9] Love C C, McKinnon A O, Squires E L, Vaala W E and Varner D D 2011 Equine Reproduction 2 Ed (Blackwell Publishing Ltd.) pp 1929–39
[10] Concha-Bermejillo A and Kennedy P C 1982 J. Am. Vet. Med. Assoc. 181 680–1
[11] Lehmann J, Ellenberger C, Hoffmann C, Bazer F W, Klug J, Allen W R, Sieme H and Schoon H A 2011 Theriogenology 76(7) 1326–36
[12] Walter I, Handler J, Reifinger M and Aurich C 2001 Reproduction 121 581–6
[13] Schoon D, Schoon H-A and Klug E 1999 Pferdeheilkunde 15(6) 541–6
[14] Schoon H-A and Schoon D 2003 Pferdeheilkunde 19(6) 698–701
[15] Kozdrowski R, Sikora M, Buczkowska J, Nowak M, Raś A and Dziecioł M 2015 Animal Reprod. Sci. 154 56–62
[16] Nielsen J M 2005 Theriogenology 64 (2) 510–8
[17] Lebedev S, Gavrish I A, Gubaydullina I Z 2019 Different chrome sources influence on morphbiochemical indicators and activity of digestive enzymes in wistar rats Sel’skokhozyaistvennaya biologiya [Agricultural Biology] 54 (2) 304-315
[18] Perez-Martin C C, Vizuete G, Borge C and Galisteo J J 2018 Acta Veter. Hungarica. 66(3) 462–73
[19] Lunelli D, Cirio S M, Leite S C, Camargo C E and Kozicki L E 2013 Bioscience and Biotechnology 4 599–605
[20] Hanada M, Maeda Y and Oikawa M 2014 J. Equine Sci. 25(2) 45–52
[21] Hanada, M, Maeda Y and Oikawa M 2012 J. Equine Sci. 23(3) 35–40
[22] Overbeck W, Jäger K, Schoon H-A and Witte TS 2013 Theriogenology 79(9) 1262–8
[23] Watson ED and Sertich PL 1992 J. Am. Vet. Med. Assoc. 201 438–40
[24] Killisch R, Böttcher D, Theuß T and Schoon H-A 2017 Reprod. Domestic Animals 52(6) 1011–8
[25] Mansour G D, Ferreira A M R, Tavares F and Henry M 2004 Revista Brasileira de Ciência Veterinária 11(1/2) 44–8
[26] Herrera M, Herrera J M, Cantatore S, Aguilar J, Felipe A and Fumuso E 2018 Anat. Histol. Embryol. 47(2) 153–8
[27] Kvan O, Gavrish I, Lebedev S, Korotkova A, Miroshnikova E, Bykov A, Serdaeva V, Davydova N. 2018 Effect of probiotics on the basis of Bacillus subtilis and Bifidobacteriumlongum on the biochemical parameters of the animal organism. Environmental Science and Pollution Research 25(3) 2175-2183
[28] Bramer S A, Macedo A and Klein C 2017 Reproductive Biology and Endocrinology 15 4
[29] Troedsson M H T, Marcos J D M and Irwin K M L 1993 Am. J. Vet. Res. 4 570–2