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Uterine myofibroblasts in mares

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Summary

The purpose of the study was to investigate the histological and immunohistochemical characteristics of fibroblasts and myofibroblasts surrounding normal, fibrotic and dilated equine uterine glands.

In our study 28 samples from mares of different ages and with signs of endometrial lesions were investigated for the presence of fibroblasts and myofibroblasts. Fibroblasts and myofibroblasts were identified using immunohistochemistry. Fibroblasts showed a positive reaction to vimentin, while myofibroblasts were positive for vimentin, α-smooth muscle actin and the human muscle actin, HHF35. Myofibroblasts were found in all stages (according to Kenney and Doing 1986) of endometrium lesions, more often in IIb and III categories, and also around non-dilated glands, regardless of the level of fibrotic changes. We think that the occurrence of myofibroblasts can be due to long-term inflammation or other stress factors.

Keywords: endometrosis, myofibroblasts, fibroblasts, mares

Cyclic activity of the mare depends on hormones and releasing factors. During every cycle there are physiological changes in the endometrium. These changes include epithelial ones from cuboidal to columnar changes of superficial endometrium and of endometrial glands, oedema of the endometrium, convolution of the glands, physiological dilatation of the glands, etc (9, 10). During anoestrus all processes reverse and the endometrium micro-morphological structures return to a resting state. Bacterial illnesses, infections and various other lesions of the endometrium affect these physiological changes and leave irreversible changes of the endometrial structure.

Chronic degenerative endometrial changes involving severe alterations in the uterine glands and connective tissue have been called „endometrosis". It is one of the most frequent reasons for infertility in the mare and can be diagnosed only by histological examination of a uterine biopsy sample (7, 9, 10). The histological changes associated with endometrosis include, amongst others, formation of dilated glands or glandular nests and fibrosis. The pathogenesis of this disease is still unknown, but some authors associated it with the age of the affected animal and/or number of foals (5, 7, 13, 14), and with continuous chemical, physical and inflammatory insults as has been mentioned in human medicine (7, 19).

It is likely that the equine endometrium undergoes cyclic remodelling of the connective tissue extracellular matrix as observed in other animals species (18). The main role in this remodelling is played by fibroblasts; they are also involved in the pathogenesis of fibrotic processes. These fibroblasts express fibronectin, laminin, collagen type IV and tenascin (17, 18). It has been noticed, however, that the fibroblasts in the equine uterus also express α-smooth muscle actin, tropomyosin and desmin, which are typical for smooth muscle cells (myocytes). These fibroblastic cells that express a range of muscle differentiation factors were identified as myofibroblasts (6, 7, 12, 20) and a few attempts to study myofibroblasts in the equine uterus have been described (7, 18).

The aim of the present study was to investigate histological and immunohistochemical characteristics of fibroblasts and myofibroblasts surrounding normal, fibrotic and dilated equine uterine glands.

Material and methods

Uterine biopsy samples (n = 23) and samples from horses at necropsy (n = 5) were collected from 28 mares (7-23 years of age). Clinically and, where possible, at necropsy reproductive organs were grossly examined, evaluating the uterus, intrauterine and extrauterine blood vessels and ovaries. Stages of the oestrus cycle were determined

according to changes in the ovaries and uterus. Single uterine biopsy samples were obtained per animal and three samples per animal were taken during necropsy.

Uterine biopsy samples were fixed in Bouin's solution for 12-24 h, necropsy samples were fixed in 4% neutral formaldehyde for 24 h at room temperature. After fixation all samples were dehydrated and embedded in paraffin wax (Paraplast) using automated embedding equipment. Serial sections were cut at 4 μm, placed on poly-l-lysine-coated glass slides and dried at 53°C overnight.

Sections were stained with different methods. Hematoxylin & eosin stained samples were categorized for the grade of endometrosis according to Kenney and Doig (1986). Fibroblasts and myofibroblasts were identified using immunohistochemistry. The sections were rehydrated and endogenous peroxidase activity was inhibited by 1% H_2O_2 in methanol after which they were rinsed in alcohol and distilled water. After rinsing with PBS, slides were treated with normal horse serum diluted 1 : 10 for 15 min. before incubation with primary antibody for 60 min. The used antibodies and their concentrations are shown in the table (tab. 1). After incubation, slides were rinsed in PBS and incubated with the secondary antibody: Horse anti-Mouse/ /Biotin 1 : 125 (Vector laboratories, Burlingame, CA, USA) for 30 min. The sections were rinsed with PBS, incubated with ABC/PO complex (Vector) for 45 min. and rinsed in PBS. Visualisation was performed with DAB substrate (10 mg 3'3-diaminobenzidine in 50 ml of 0.05 mol Tris buffer (pH 7.8) (Sigma, St. Louis, MO, USA) and 0.033% H_2O_2 for 10 min. at room temperature. Nuclei were stained with Mayer's hematoxylin (Merck, Germany). The slides were dehydrated and mounted with Eukitt (Kindler GmBh&Co, Freiburg, Germany). Control sections were treated with PBS instead of primary antibody. All incubations were performed at room temperature in a humid chamber.

The samples used in this study were classified in 5 groups according to the Kenney and Doig system (1986) (tab. 2). Two samples from healthy mares were used as controls.

Tab. 1. Primary antibodies used in immunohistochemistry

Antibody	Type of antibody	Dilution	Source	Control tissue
Vimentin	Monoclonal	1 : 150	Biogenex, San Ramon, CA, USA	Endothelial cells
α-smooth muscle actin	Monoclonal	1 : 1000	Biogenex, San Ramon, CA, USA	Smooth muscles
Human muscle actin (clone HHF35)	Monoclonal	1 : 300	Dako Cytomation, Denmark	Aorta

Tab. 2. Results of the histological findings

Categories	Number of mares (n = 28)	Endometrosis (n = 10)*	Endometritis (n = 22)*
I	2	–	–
IIa	12	1	11
IIa-b	5	3	5
IIb	6	4	4
III	3	2	2

Explanation: * the number of mares does not correspond with the number of lesions as 5 cases were positive for endometrosis and endometritis within the same mare, but were counted separately

Results and discussion

Of the 28 mares with clinically diagnosed reproductive disorders (abortion, delay in oestrus, infertility, etc.), endometrosis was diagnosed in 10 cases, endometritis in 22. There were 5 cases where endometrosis occurred together with endometritis and two mares (control) were sound. The majority of samples (43%) were classified as category IIa (tab. 1).

Fibroblastic rings were microscopically found around normal and dilated glands, including nest formation, fibrosis, dilatation of the endometrium glands and dilated lymphatics (lymphatic lacunae). Although the accumulation of fibroblasts mostly surrounded dilated glands, some non-dilated glands were also found with 2, 3 or 4 circles of fibroblasts. In cases of endometritis, diffuse fields and/or single foci of mono- and polymorph nuclear leukocytes were observed locally.

As mentioned earlier, immunohistochemically-identified myofibroblasts showed features of fibroblasts and of myocytes. They easily were recognised in the endometrium. Vimentin appeared to represent the major intermediate filament in these cells. This intermediate type cytoskeleton component is known from a variety of mesenchymal or mesenchym-derived non-muscle cell types, including endothelial cells, all fibroblastic cells, macrophages, neuroblastomas, etc. (3). The positive reaction was due to endometrium fibroblasts in the matrix and around fibrotic, dilated glands, as well as in endothelial vessels. Positive reaction with this antibody was also found in the fibroblasts surrounding non-dilated fibrotic glands and surrounding the glands with nest formations. In a few cases, positive staining of the endometrium superficial epithelial and glandular cells was found, but this might represent a false positive cross-reaction.

The next step was to identify which of these fibroblasts show signs of myocytes and can be identified as myofibroblasts. For this reason two antibodies were used: α-smooth muscle actin which binds to smooth muscle cells and myoepithelial cells and anti-human muscle actin, HHF, which reacts with myofibroblasts. Those cells that showed a positive reaction for vimentin, α-smooth muscle actin and human muscle actin, HHF35, finally were identified as myofibroblasts. All other cells that were positive only for vimentin were identified as fibroblasts.

Myofibroblasts positive for α-smooth muscle actin were found around fibrotic glands with few fibrotic circles and in the nest formations (fig. 1). Positive reactions were seen in some cases found around non-

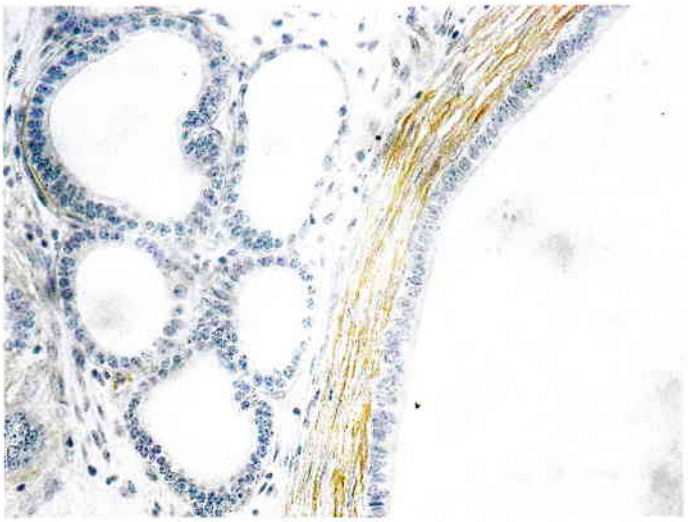

Fig. 1. Dilated uterine glands with several circles of positive myofibroblasts. Anti-α-smooth muscle actin, 10 × 40

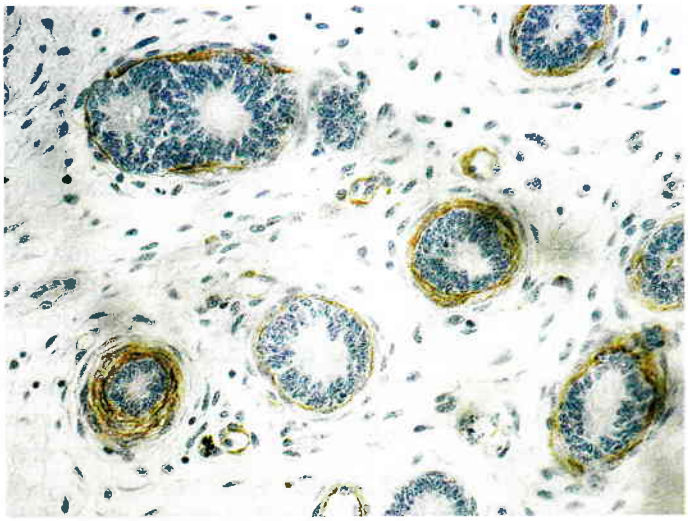

Fig. 2. Non-dilated uterine glands surrounded by a few myofibroblastic rings. Anti-α-smooth muscle actin, 10 × 40

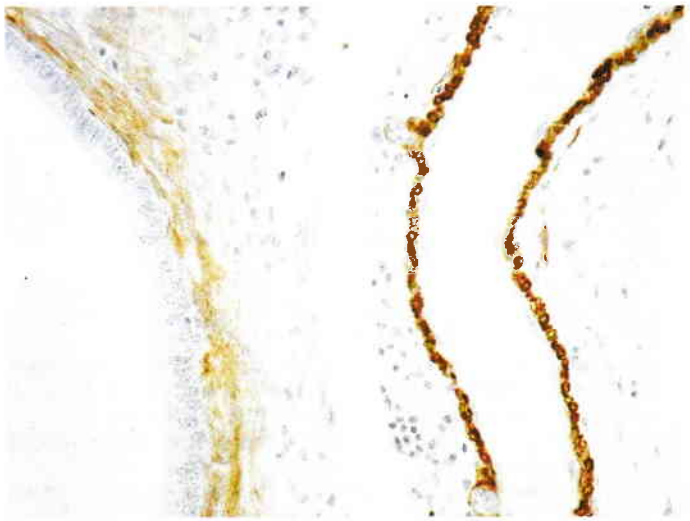

Fig. 3. The same dilated uterine gland as Fig. 1, positive to antibody against human muscle actin (on the left hand side); and endothelial cells positive to the antibody (on the right hand side). Vimentin

-dilated (fig. 2), non-fibrotic glands and in some cases – around non-fibrotic dilated glands. In the case of glands with few circles of fibroblastic cells around them, the positive reaction usually was in the nearest circle to the gland, also such reactions were more often in the deeper stratum spongiosum layer. Similar results were obtained after staining with the anti-human muscle actin, HHF35, though reaction was weaker (fig. 3).

Myofibroblasts were identified in 58% of cases, from which 43% of them indicated signs of endometrosis and 14% were without endometrosis. Myofibroblasts in cases without or with minimal signs of endometrosis were found around dilated glands and/or non-dilated glands.

Fibroblasts in the uterus are the major connective tissue cells. They are responsible for the synthesis of collagen fibrils and extracellular matrix. Fibroblasts are active during reparative processes, for example as myofibroblasts in wound contraction and the formation of granulation tissue and of scars. In the normal endometrium these cells are found in all connective tissue but in the development of endometrosis, fibroblasts start to form circles around the glands. An indicator of endometrosis can be a dilated gland, but fibroblastic rings can also be found around normal, non-dilated glands. According to the classification of Kenney's and Doig's (1986), the severity of the lesion depends on the number of fibroblastic circles around the glands: the more circles are around glands, the more severe the prognosis (9). Earlier it was thought that these circles are formed by fibroblasts. The immunohistochemical and electron microscopy results reveal that same of these cells had differentiated to myofibroblasts what is in accordance with findings of others (7, 18).

Moreover, myofibroblasts exist in many normal tissues or organs as specific stable cells with the potential to differentiate into fibrogenic action (15, 17, 19). They are not common in the healthy uterus, but rather frequent in tumours (18). These findings were unexpected, because further development of endometrosis to benign or malignant tumours is not known in horses. It is possible that this myofibroblastic reversal depends on reactive processes as a form of the uterus' adaptation, in these cases helping to extrude accumulated secretions from the dilated glands. However, the role of myofibroblasts around non-dilated glands is not known. It has been described that this reversal is associated with high activity of periglandular stromal cells in cases of fibrosis (11, 18).

Our study also examined the repartition of myofibroblasts due to endometrial lesions (endometritis and endometrosis) which were categorised according Kenney and Doig (tab. 1). In cases of mild and average endometrial lesions (from I to IIa, IIa-b) we found only a few myofibroblasts. As expected, in severe endometrosis (IIb-III) we found many myofibroblasts around

the affected glands. However myofibroblasts were present in tissues with mild or even without signs of endometrosis (I). Meanwhile, there was no reaction to antibodies in a few of the formatted gland nests.

Probably these results may be explained due to the pathogenesis of fibrosis. For fibroblast activation, inflammatory cells and their mediators are necessary (4, 8, 19). During long-term inflammation, matrix fibroblasts are activated by these factors and also are responsible for tissue fibrosis. Further development of fibroblasts to myofibroblasts is regulated by cytokines and inflammatory mediators (1, 16). Due to these factors during chronic or recurrent endometritis (with stable and high concentrations of inflammatory mediators), activation of fibroblasts may occur causing their transformation to myofibroblasts. Another opinion is that appearance of myofibroblasts around dilated glands may result from mechanical or other stress factors (2, 19). Probably the incidence of myofibroblasts in the mildly injured endometrium (IIa-IIb) indicates long-term inflammation, leading towards increased occurrence of endometrosis.

In the present study it was demonstrated that myofibroblasts can be found in every endometrium tissue, despite to the level of fibrotic changes. We think, that this indicates long-term lasting former endometritis and activation of fibroblasts. Also the appearance of myofibroblasts can be due to the uterus adaptation. However further studies are needed to investigate the role of myofibroblastic cells and further pathological changes in the equine endometrium.

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SINGH B. R., ALAM J., HANSDA D.: Wyłysienie na tle salmonellozy u świnek morskich. (Alopecia induced by salmonellosis in guinea pigs). Vet. Rec. 156, 516-518, 2005 (16)

Chroniczną postać salmonellozy wywołano u 120 świnek morskich w wieku 60-75 dni podzielonych na 12 grup (A-L). Do zakażenia użyto szczepów *Salmonella enterica serovar Abortusequi* opornych na kwas naliksydowy: typ dziki S-787, szczep referencyjny E156, aroA mutant S-28, htr A mutant S-29, aroA – htrA mutant S-30 mutant. Zwierzęta z grup A-F zakażono domięśniowo, z grup G-J *per os* 24 godz. hodowlą salmonelli. Dawka zakaźna przy zakażeniu domięśniowym wynosiła w zależności od grupy $7{,}22\text{-}7{,}65\times10^3$, *per os* $7{,}22\text{-}7{,}65\times10^4$. Nasilenie utraty włosa określono wg skali 0-3. Wyłysienia pojawiły się po 27 dniach po zakażeniu domięśniowym szczepem S-787, po 24 dniach po zakażeniu szczepem referencyjnym E-156. Pod koniec 3. miesiąca większość zwierząt utraciła prawie 50% owłosienia, podczas gdy zwierzęta zakażone mutantem albo nie straciły owłosienia, albo ubytek włosów był niewielki. Po 91 dniach po zakażeniu u świnek z utratą włosa zastosowano w iniekcji domięśniowej ampicylinę w dawce 100 mg/dzień przez okres 7 dni oraz przez 45 dni dietę wzbogaconą w witaminę C. U zwierząt leczonych, za wyjątkiem kilku sztuk zakażonych szczepem referencyjnym E 156, okrywa włosowa odrosła po 21 dniach. Iniekcje ampicyliny uwolniły zwierzęta od nosicielstwa salmonelli.

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