ORIGINAL ARTICLE

Expression of interleukins 6 and 10 and population of inflammatory cells in the equine endometrium: diagnostic implications

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Abstract

Endometritis consists of an acute or chronic inflammatory process involving the endometrium and together with endometrosis constitute the main causes of infertility in mares. The aim of this study was to associate the histopathological findings with the immunohistochemical markers interleukins 6 (IL-6) and 10 (IL-10) to evaluate the inflammatory changes and progression of uterine tissue lesions of mares in the diestrus phase and their diagnostic implications. Twelve crossbred cyclic mares were used for endometrial biopsy collection. Samples were collected in the diestrus period (6 ± 1 day after ovulation) without previous artificial insemination. In the histopathological analysis the samples were classified according to the type and intensity of inflammation, alterations regarding endometrial fibrosis and biopsy categories (I, IIA, IIB and III). In the immunohistochemical analysis, the markers of IL-6 and IL-10 were evaluated by scores (0, 2, 4, 6) according to the intensity of the immunostaining and inflammatory cells (CD-3, CD-20, CD-68 and MPO antibodies) and were counted according to the number of cells immunostained in brown, in ten random fields. An association ($p \leq 0.05$) occurred between low score (2) for IL-6 in the endometrial glandular area and moderate fibrotic nets; and between high scores (4 and 6) for IL-10 in sub-epithelial connective tissue and moderate periglandular fibrosis. In conclusion, immunohistochemical analysis demonstrated an association between interleukins and inflammatory cells with endometrial lesions. In addition, this research may be useful in the future to evaluate the progress of the inflammatory process, contributing to the adequate optimization of the reproductive management of the mares.

Keywords IHC · Uterine biopsy · Mares · Interleukins

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Introduction

Endometritis consists of an acute or chronic inflammatory process involving the endometrium [1]. Uterine infections and inflammation generate large losses in the breeding industry of horses leading to subfertility and infertility of mares [2, 3].

The causes of endometritis are multifactorial and were subdivided into four classes according to etiology and physiopathology: sexually transmitted diseases, endometrosis, persistent post-breeding endometritis and chronic infectious endometritis [4].

Endometrosis is considered a degenerative disease characterized by periglandular and endometrial fibrosis, lymphatic lacunae, endometria atrophy, and reduction of uterine glands, usually irreversible [5]. This degenerative process may be due to delayed uterine clearance [6], resulting from repeated conditions of uterine inflammation or even aging [5]. Similarly, several studies have been developed regarding the mechanisms involved in the susceptibility to endometritis and the physical clearing of the uterus is one of the most important factors described [7].

In terms of the physiological drainage of the uterus, during the estrus period, under the effect of estradiol myometrial contractions are greater in intensity, duration and coordination, and the uterine folds become more prominent and the cervix relaxed, while on the other hand, under the predominance of progesterone (P4) these contractions are smaller, the folds become reduced and the cervix is closed [8].

Thus, during diestrus period, under the effect of P4, the ability to clear fluids and particles is reduced, increasing the occurrence of infections [9]. In addition, there are important differences in endometrial cells due to estrous cycle phase [10]. Minor variations occur in the diestrus phase, although the samples can be collected at any stage of the estrous cycle [11]. Then, the monitoring of the uterine health of mares in diestrus becomes an important tool for the management and evaluation of fertility before the season breeding.

Endometrial biopsy has been the main procedure for collecting uterine samples in the last 40 years and the histopathological evaluation remains the main support for the evaluation of uterine health [10, 12]. This technique allows the detection of endometrial degenerative changes, based on the classification system established by Kenney and Doig [11] and also to assess the distribution and severity of the inflammatory process [13].

Immunohistochemistry (IHC) is an advantageous technique compared to conventional histopathological analysis, since this method can be performed using specific antibodies for the identification of defense cells produced in inflammatory processes, such as neutrophils, T lymphocytes and macrophages [14].

Interleukin 6 (IL-6) is one of the most important proinflammatory cytokines of the innate immune system, being extremely relevant in acute inflammatory responses. It is synthesized by mononuclear phagocytic cells, vascular endothelial cells, fibroblasts and other cells in response to molecular patterns associated with pathogens (PAMPs), IL-1 and TNF. In contrast, interleukin 10 (IL-10) participates in the control of acquired and cell-mediated immunity reactions, acting in the inhibition of activated macrophages and dendritic cells [15].

The interleukin (IL) markers used in immunohistochemical analysis have been shown to be good predictors for the evaluation of the progression of the inflammatory process in the animal endometrium [16, 17]. However, few studies were carried out in mares. Thus, the aim of this study was to associate the histopathological findings with the immunohistochemical markers interleukins 6 (IL-6) and 10 (IL-10) to evaluate the inflammatory changes and progression of uterine tissue lesions of mares in the diestrus phase and their diagnostic implications.

Materials and methods

Animals

The study was conducted between November 2014 to March 2015 at the farm of the Instituto Vital Brazil, located at Cachoeiras de Macacú, Rio de Janeiro, Brazil and was approved by the Ethics Committee on Animal Use of the Universidade Federal Fluminense (protocol number 586).

Twelve crossbred cyclic mares with a mean age of 12.6 years (range 8–18 years) and without breeds in this breeding season were selected for the study. All the mares received the same feed and water ad libitum. The collection of the samples was carried out at diestrus (6 ± 1 days after ovulation), without prior artificial insemination.

Histopathology

The uterine samples were obtained by endometrial biopsy, using a Yeoman forceps, and fixed in 10% buffered formalin. After fixation, the sections were stained with hematoxylin-eosin and observed under optical microscope in ×40.

Samples were classified into categories (I, IIA, IIB and III) according to the characteristics of glands, lymphatic vessels, fibrotic alterations and infiltrate of inflammatory cells, as established by Kenney and Doig [11]. The inflammatory infiltrate was classified as mild, moderate and severe, according to the intensity and type of inflammatory cells.

The presence and intensity of different types of endometrial fibrosis were also considered in the evaluation.

Immunohistochemistry

The sections of uterine samples were deparaffinized, hydrated and the endogenous peroxidase was blocked with 3% hydrogen peroxide solution. The sections were then incubated in a solution with Target Retrieval, ph 6 (DAKO Corporation, Carpinteria, CA, USA) at 96 °C in a water bath during 30 min for antigen recovery.

Samples were incubated overnight with the primary antibodies (Table 1). The sections were treated with LSAB System-HRP (DAKO Corporation, Carpinteria, CA, USA) for IL-6 and IL-10 and with Advance HRP (DAKO Corporation, Carpinteria, CA, USA) for CD3, CD20, CD68 and MPO antibodies, as recommended by the manufacturer. Diaminobenzidine chromogen (Chromogen System, DAKO Corporation, Carpinteria, CA, USA) was used to evidence the immunostaining and all samples were counterstained with Harris' hematoxylin. For all antibodies, the negative control was performed, omitting the primary antibody.

The evaluation of interleukins was performed using scores (0, 2, 4, 6), according to the intensity of the immunostaining in the surface epithelium, the sub-epithelial connective tissue and the glandular epithelium of the endometrium, adapted from Jiwakanon et al. (2011). All fields of the fragment were counted in a ×40 objective, and the most frequent score was adopted as a result of the evaluation.

The evaluation of immunostaining intensity for the antibodies was performed by counting the immunostained cells in brown, in ten random fields, with a ×40 objective. The mean number of cells per field was obtained for comparison with the other parameters.

Statistical analysis

A descriptive statistical analysis was performed. Subsequently, association between qualitative variables (histopathology and immunohistochemistry for IL-6 and IL-10) were assessed by Fisher's exact test. Qualitative variables (histopathology or immunohistochemistry of IL-6 and IL-10) and quantitative variables (immunohistochemistry for CD3, CD20, CD68 and MPO) were associated by the Mann–Whitney test. Pearson's linear correlation coefficient used was 0.97 between the number of T lymphocytes and mononuclear total. For all data, $p \leq 0.05$ was considered significant. The software Statistical Package for Social Sciences (SPSS) version 17.0® was used.

Results

Histopathology

The mares evaluated in this study were classified in IIB (8/12) and III (4/12) categories according to Kenney and Doig [11]. Most of the mares showed mild PMN infiltrate, both in the compact and spongy stratum (66.7% and 75%, respectively). Similarly, mild PMNs were found in gland lumen and in venules in the SC in most mares (100% and 66.7%, respectively). Few eosinophils were found in general, and one mare (8.3%) presented moderate infiltrate and another one (8.3%), severe eosinophil infiltrat. On the other hand, all mares presented moderate or severe mononuclear infiltrates (8.3% and 91.7%, respectively) (Fig. 1a). Moderate periglandular fibrosis and moderate fibrotic nests were observed in more than half of the mares (58.3% and 66.7%, respectively), whereas glandular dilatation (Fig. 1b) and lymphatic lacunae were predominantly mild (66.7% and 100%, respectively) (Table 2).

In addition, all mares (100%) presented extensive and multifocal fibrosis, fibrosis in lamina propria, abundant collagen and hypertrophied arteries, and absence of non-glandular endometrial cysts. Only one mare (8.3%) presented a decrease in the glandular population.

Immunohistochemistry

In the cytoplasm of the surface epithelium, most mares (91%) presented score 2 or 4 for the immunostaining for IL-6 (Fig. 2c). In the sub-epithelial connective tissue, approximately half (58.3%) of the mares presented the score 4 and in the endometrial glandular area, and 75% presented score 2 (Table 3). For IL-10, a more homogeneous distribution was observed between the scores 2, 4

Table 1 Panel of primary antibodies used on immunohistochemistry of the uterine tissue lesions of mares, with their respective dilutions and manufacturer

| Target cell	Antibody	Pre-treatment	Type of antibody	Dilution	Manufacturer
IL-6	IL-6	Target retrieval ph 6	Polyclonal goat	1/100	R&D Systems
IL-10	IL-10	Target retrieval ph 6	Polyclonal goat	1/150	R&D Systems
T lymphocyte	CD3	Target retrieval ph 6	Polyclonal rabbit	1/150	Dako
B lymphocyte	CD20	Target retrieval ph 6	Monoclonal mouse	1/300	Dako
Macrophages	CD68	Target retrieval ph 6	Monoclonal mouse	1/100	Dako
Neutrophils	MPO	Target retrieval ph 6	Polyclonal rabbit	1/3000	Dako
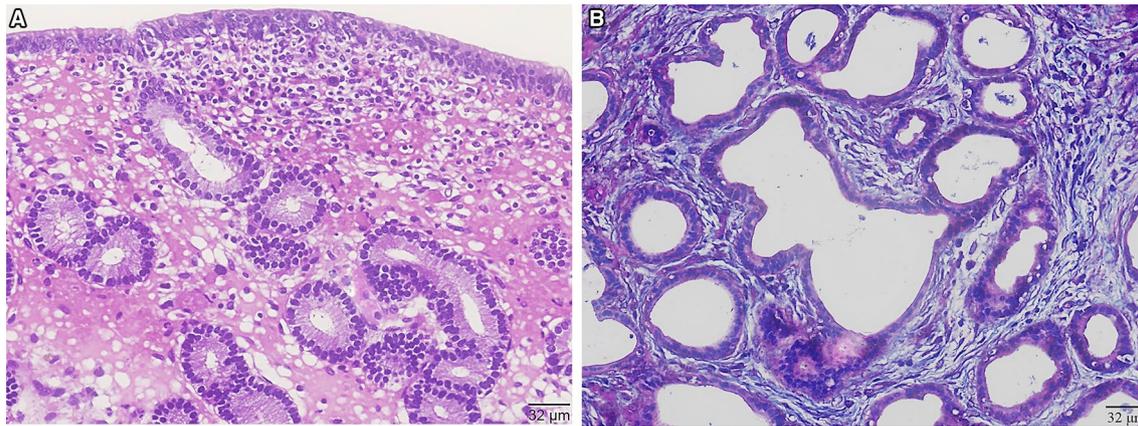
Fig. 1 Photomicrographs of equine endometrial biopsy: **a** predominantly mononuclear inflammatory infiltrate, HE; **b** glandular dilatation and collagen fibers, Masson's trichrome

Table 2 Percentage of acute and chronic inflammation of mares according to the intensity in different biopsy categories

Intensity	Evaluated parameters in the categories of biopsy				
	PMN ISC	PMN ISS	PMN G	PMN V	E
Parameter of acute inflammation					
Mild	66.7% (8/12)	75% (9/12)	100% (12/12)	66.7% (8/12)	83.3% (10/12)
Moderate	33.3% (4/12)	21% (3/12)	0% (0/12)	33.3% (4/12)	8.3% (1/12)
Severe	0% (0/12)	0% (0/12)	0% (0/12)	0% (0/12)	8.3% (1/12)
Intensity	Evaluated parameters in the categories of biopsy				
	MI	PF	FN	GD	LL
Parameter of chronic inflammation					
Mild	0% (0/12)	25% (3/12)	25% (3/12)	66.7% (8/12)	100% (12/12)
Moderate	8.3% (1/12)	58.3% (7/12)	66.7% (8/12)	33.3% (4/12)	0% (0/12)
Severe	91.7% (11/12)	16.7% (2/12)	8.3% (1/12)	0% (0/12)	0% (0/12)

PMN ISC Polimorfonuclear infiltrate in stratum compactum, *PMN ISS* polimorfonuclear infiltrate in stratum spongiosum, *PMN G* polimorfonuclear in the lumen of the glands, *PMN V* polimorfonuclear in venules of the stratum compactum, *E* eosinophils, *MI* mononuclear infiltrate, *PF* periglandular fibrosis, *FN* fibrotic nests, *GD* glandular dilatation, *LL* lymphatic lacunae

and 6 (25%, 33.3% and 41.7%, respectively) especially in the cytoplasm of the surface epithelium (Fig. 2b). In the sub-epithelial connective tissue and endometrial glandular area, 50% of the mares presented score 4 and 2, respectively (Table 3). The count of inflammatory cells showed a predominance of mononuclear cells (51.94) compared to the number of polymorphonuclear cells (5.72) (Table 4).

In this study, an association ($p \leq 0.05$) occurred between low score (2) for IL-6 in the endometrial glandular area and moderate fibrotic nets (Fig. 3a). Additionally, an association ($p \leq 0.05$) was observed between high scores (4 and 6) for IL-10 in sub-epithelial connective tissue and moderate periglandular fibrosis (Fig. 3b). Positive correlation (Pearson's coefficient = 0.97) was found between the number of T lymphocytes (Fig. 2a) and total mononuclear cells.

Discussion

The cytokines are one of the main variables that influence the immune response in a tissue [18]. The present study showed cytokines in the mare endometrium by IHC. This positive labelling of IL-6 and IL-10 was found in cells of the cytoplasm of the surface epithelium, sub-epithelial connective tissue and endometrial glandular area. Similarly results were found in porcine endometrium on which an immunohistochemical labelling of IL-6, IL-10 and TGF-1 was evident, especially in the surface and glandular epithelium [16].

IL-6 is described in early inflammatory response [16]. In this study, there was an association between low score (2) for IL-6 in the endometrial glandular area and
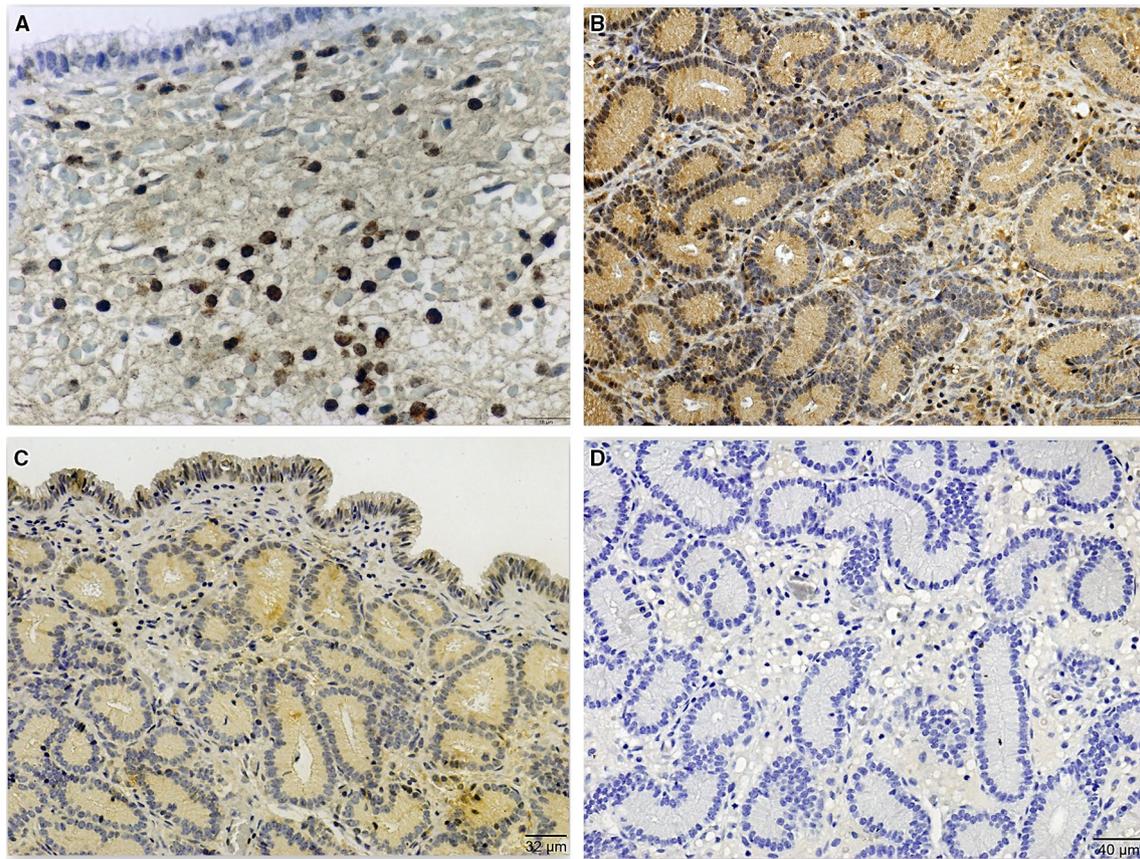
Fig. 2 Photomicrographs of equine endometrial samples. **a** Immunohistochemistry for CD3 antibody showing T lymphocyte labelling; Observe also immunohistochemical labelling in the surface epithelium, sub-epithelial connective tissue and the epithelial glandular: **b** IL-10; **c** IL-6; **d** negative control

Table 3 Percentage of immunohistochemical labelling of IL-6 and IL-10 in the equine, according to the score system used for evaluation

Score	Compartment		
	CSE	SECT	EGA
Interleukin IL-6			
0	0% (0/12)	0% (0/12)	0% (0/12)
2	50.0% (6/12)	16.7% (2/12)	75.0% (9/12)
4	41.7% (5/12)	58.3% (7/12)	25.0% (3/12)
6	8.3% (1/12)	25.0% (3/12)	0.0% (0/12)
Interleukin IL-10			
0	0% (0/12)	0% (0/12)	0% (0/12)
2	25.0% (3/12)	16.7% (2/12)	50.0% (6/12)
4	33.3% (4/12)	50.0% (6/12)	41.7% (5/12)
6	41.7% (5/12)	33.3% (4/12)	8.3% (1/12)

CSE Cytoplasm of the surface epithelium, *SECT* sub-epithelial connective tissue, *EGA* endometrial glandular area

Table 4 Inflammatory cell count of immunohistochemical labelling (mean/field) in the equine endometrium

Antibody	Mean/field	SE
MPO	5.72	0.8
CD3	47.19	6.36
CD20	1.87	0.47
CD68	2.87	1.16
Total mononuclear cells	51.94	5.91

moderate fibrotic nests of mares, which reaffirms the acute character of this cytokine. However, Szóstek et al. [17] also identified immunostaining to IL-6 in all three Kenney and Doig [11] categories in mare endometrium.

IL-10 immunostaining in sub-epithelial connective tissue was predominant in high scores (4 and 6) in the presence of moderate periglandular fibrosis, suggesting the role of IL-10 in chronic inflammation. This cytokine is synthesized relatively late in the inflammatory response and acts as a generalized anti-inflammatory effector by reducing the transcription of pro-inflammatory cytokines
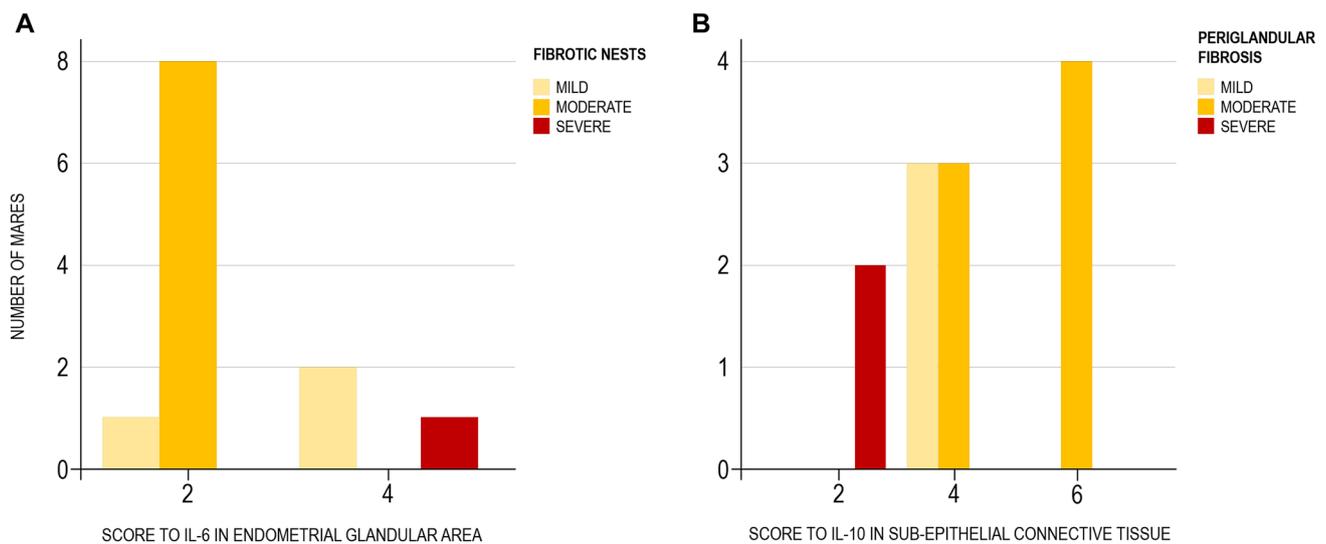
Fig. 3 Number of mares by immunohistochemical score for IL-6 in endometrial glandular area (**a**) and for IL-10 in sub-epithelial connective tissue (**b**)

by monocytes and macrophages [19]. In the same sense, studies in mice and humans have already demonstrated such an anti-inflammatory role of IL-10 in other tissues more clearly in chronic inflammatory conditions [20–23].

Antigens from pathogens are presented and exposed to T cells by antigen-presenting cells so an effective immune response to pathogens was required [3]. Positive correlation was identified between the number of T lymphocytes and total mononuclear cells, since T lymphocytes were the predominant cell type, and their increase consequently leads to an increase in total mononuclear cells, corroborating with the severe intensity of mononuclear inflammatory infiltrate observed in most mares. Similarly, a greater number of T lymphocytes were found by IHC in the luminal epithelium, stratum compactum and stratum spongiosum in the different groups of mares (pregnant, non-pregnant, prolonged luteal phase with an intrauterine device, and normal luteal phase with an intrauterine device) [24]. The predominant presence of mononuclear cells is characteristic of chronic inflammation. In addition, these mares presented degenerative lesions characteristic of endometrosis and therefore classified in categories IIB (8/12) and III (4/12).

The main tissue alteration with predominance of moderate or severe intensity, observed on histopathology analysis, was mononuclear infiltrate, periglandular fibrosis and fibrotic nests and all mares presented extensive and multifocal fibrosis, fibrosis in lamina propria, abundant collagen and hypertrophied arteries. These lesions are characteristic of endometrosis, which constitutes a collective disease comprising progressive and degenerative conditions of the equine endometrium

and the severity and extent of the pathologic changes aggravates with aging [5]. It has been found that the fertility outcome correlates with the presence of endometrium changes characteristic of endometrosis [25].

Degenerative changes of the endometrium (biopsy score IIB and III) can also predict susceptibility to persistent endometritis [26]. Increased age has been related to susceptibility to persistent endometritis and it is related with anatomical defects [27]. The mean age of mares in this study was 12.6 years old (range 8–18 years) and it may have contributed to the predominance of the results obtained. Others factors associated with susceptibility increase included internal and external conformation of the female genital tract [28], intrinsic contractile dysfunction of the myometrium affecting uterine clearance [29] and position of the uterus in the abdomen [30].

The immunohistochemical analysis allows an association between endometrial lesion and the degree of staining for both interleukins and inflammatory cells (specific cell type). In addition, this research may be useful in the future to evaluate the progress of the inflammatory process, contributing to the adequate optimization of the reproductive management of the mares.

Compliance with ethical standards

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