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Donkey endometrium: Characterization of resident immune cells

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ABSTRACT

The *Burro de Miranda* is an endangered donkey breed. The dynamics of the immune system of the donkey's reproductive tract are essential to manage the fertility of these animals for the improvement and survival of the species. In mares, high numbers of immune cells infiltrating the endometrium promote endometrosis, which is still to be confirmed in jennies. Uterine biopsies of 32 jennies were evaluated based on the Kenney and Doig grading system used in mares. Hematoxylin and eosin stain was used to assess the infiltration and distribution of neutrophils and eosinophils. Macrophage and B and T lymphocytes endometrial distribution was conducted through immunohistochemistry.

T lymphocytes were the most predominant cells in jenny endometrium, macrophages being the second. T lymphocytes were also found in the superficial and glandular epithelium. Eosinophils, neutrophils and B lymphocytes were the least common cells. No differences were found in the inflammatory infiltrate compared to the different endometrosis grades (IIA, IIB and III).

This study mapped the immune cells in jenny's endometrium, providing core valuable information for additional immunological and reproductive studies in this species. It also highlighted significant differences in endometrial immune cell distribution between the jenny and the mare during estrus and diestrus, supporting the need to develop of a more suitable scoring system than the current Kenney and Doig categorization to assess the morphology and clinical feature of jenny's endometrium.

1. Introduction

Burro de Miranda is an endangered donkey breed (Quaresma et al., 2014). In Europe, most donkey populations consist of an elderly demographic, with low reproductive rates and decreasing population sizes due to agricultural industrialization (Carluccio et al., 2008). The reported average number of females foaling yearly in *Burro de Miranda* donkeys' population is between 50 and 70 (Quaresma et al., 2014). Today, donkeys are playing an increasingly role in nature conservation and tourism, as well as in the cosmetic industry through milk production (Camillo et al., 2018). This has led to significant rise in new studies focused on this species (Catalán et al., 2022; Miró et al., 2020; Miró et al., 2021; Quaresma et al., 2019).

The equid endometrium can be divided into two strata (Kenney, 1978), which undergo morphological changes due to hormonal influence during the estrous cycle. The stratum compactum is the most

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superficial endometrial layer. It is composed of the luminal epithelium (a single layer of epithelial cells), the surface endometrial stroma, and the first layer of the endometrial glands. The stratum spongiosum is comprised of the stratum compactum (deep glands and stoma) and the myometrium (Morel, 2021; Silva, 2024).

Among other functions already reported in other animal species, the endometrium acts as a barrier against pathogenic agents. As such, the immune cells in the uterine stroma contribute to the tissue regulation and remodeling that physiologically occurs during the estrous cycle and post-partum (Pires and Payan-Carreira, 2015). Immune cells present in the uterus can be part of the innate immune system [macrophages, dendritic cells, neutrophils, eosinophils, and Natural Killer (NK) cells] or associated with adaptive immune responses, such as lymphocytes (B cells and T cells) (Akbalik et al., 2018; Horne et al., 2008; Wira et al., 2005). The interactions between the immune cells and their mediators (cytokines and immunoglobulins) with the endometrial cells contribute to a controlled microenvironment, maintaining adequate uterine homeostasis, whose primary function is to allow successful reproduction (Lee et al., 2015). Besides the resident immune cells depicted in the endometrium, a physiological influx of neutrophils after mating or artificial insemination was described as part of the normal immune response of the uterus in mares (LeBlanc and Causey, 2009) and jennies (Miró et al., 2013). Also, resident immune cells have been depicted in the woman's endometrium (Horne et al., 2008).

In contrast, immunity failure and imbalance will predispose the uterus to inflammation and subsequent infertility (Turner et al., 2012). In mares, malfunction of uterine clearance mechanisms may promote persistent endometritis (Fantini et al., 2021). Recent studies reported that endometritis induced by the seminal plasma is greater in jennies than in mares (Mateo-Otero et al., 2022).

Endometrosis is an endometrial degenerative change, with fibrous tissue deposition on the endometrial stroma, and is an inevitable consequence of aging in mare, reflecting of gynecological senility and chronic inflammation (Lehmann et al., 2011). These advanced-stage conditions lead to endometrial fibrosis, progressive and irreversible damage of the uterine fitness, resulting in endometrial incompetence and progressive reduction of the potential for gestation while increasing infertility, due to early pregnancy failure and abortion (Aresu et al., 2012; Mambelli et al., 2013; Ricketts and Barrelet, 1997).

In mammals, irrespective of the species, maintaining adequate uterine health is necessary for reproductive efficiency. However, its deregulation promotes inflammation of the uterus and infertility (Pires and Payan-Carreira, 2015; Turner et al., 2012). As an equid, the jenny is frequently compared to mare, whose endometrial disorders are responsible for lower fertility rates (LeBlanc and Causey, 2009). In the mare uterus, several diseases are described, such as inflammatory (endometritis), degenerative (that includes angiosclerosis and endometrosis), as well as glandular differentiation disorders (Kenney and Doig, 1986; Rudolph et al., 2017).

The Kenney and Doig scale has been used to assess mare endometrial health (Kenney and Doig, 1986), particularly in evaluating the presence and distribution of immune cell infiltrates and degenerative lesions, which can indicate underlying infertility. However, it has still to be proven adequate to score the donkey endometrium.

Due to the still scarce knowledge of the aging process of donkey's endometrium, this study aimed (i) to characterize the main resident immune cell population of the jenny's endometrium in a broad and representative sample population; and (ii) to correlate the immune cell population with endometrium histopathological grade.

2. Materials and methods

2.1. Animal recruitment and endometrial biopsy retrieval

Thirty-two endometrial biopsies from *Burro de Miranda* jennies were sampled as part of routine gynecological evaluation. Each female was submitted to a thorough reproductive exam, which included the collection of an endometrial biopsy, with the owner's consent. Animal handling complied with the national regulations and the European Council Guidelines (Directive 2010/63/EU) for protecting animals and respecting Animal Care and Welfare protocols. The study was approved by the Ethics Commission of the University of Trás-os-Montes e Alto Douro, Vila Real, Portugal (reference Doc37-CE-UTAD-2021).

The uterus and ovaries were evaluated by transrectal palpation and ultrasound (Shenzhen Veterinary US Scanner™, Shenzhen, China) coupled to a 5mHz linear probe, to confirm the estrous cycle phase and to detect signs of uterine disease.

Estrus was defined by the presence of a large follicle ($\geq$30 mm) in the ovaries and uterine edema, coexisting with heat signs exhibition. Behavioral signals of estrus included mouth clapping and at least one of the following signs during the teasing period: winking (rhythmic eversion of the vulvar labia with exposure of the clitoris), urinating, raising the tail, and posturing. Diestrus phase was assigned upon the presence of an active corpus luteum, absence of uterine edema, and non-receptive behaviors, namely 1) tail down (holding tail down between hind legs when mounted), (2) lack of interest (no positive or negative responses to the presence or teasing of the jack), and (3) refusing the jack by moving away or kicking.

Following transrectal evaluation, a Kruuse™ uterine biopsy forceps, 62 cm long (Kruuse, Langeskov, Denmark), was introduced inside into vagina, and guided across the cervix to the uterus for biopsy collection. When in place, controlled by a transrectal palpation, a portion of the endometrium was pushed between the forceps jaws to retrieve a 3 $\times$ 6 mm sample of endometrial tissue. The same practitioner obtained all the endometrial biopsies from the right uterine horn, 2 cm above the uterine body-horn transition.

2.2. Histological evaluation

The biopsies were fixed in 10 % buffered formaldehyde solution, routinely processed for paraffin embedding, cut into consecutive 3 μm thick slices, and stained with hematoxylin-eosin (HE). Three independent Veterinary evaluators conducted the histological analyses of HE slides by optical microscopy and categorized them according to Kenney and Doig (Kenney and Doig, 1986). Briefly, the biopsies were classified into different categories considering the amount of collagen and the severity of endometrial inflammatory changes, as follows: Category I - normal glands, without signs of fibrosis or inflammation and normal cellular morphology of epithelial cells; Category IIA - scarce to mild inflammatory cells infiltrate, scant peri-glandular fibrosis, or less than two glandular nests by visual microscopic field, lymphatic ectasia or partial endometrial atrophy could be present; Category IIB: severe inflammatory cells infiltrate and presence of two or more lesions described above for the IIA category. Besides, it may also be observed moderate to severe peri-glandular fibrosis with two or four glandular nests; Category III - extensive fibrosis of the endometrial glands and lamina propria, with nests of the dilated gland, moderate to severe infiltration of inflammatory cells and widespread changes in cellular morphology of gland epithelial cells (Fig. 1). As this classification has never been adapted to jenny's endometrium, the same parameters were applied to this species in the absence of other alternative classifications as previous done by other authors (Miró et al., 2020). Biopsy samples were blindly scored, as the identification of each animal was replaced by a code number unknown by the three independent evaluators.

Sequential sections of endometrial tissue were submitted to indirect immunohistochemistry. The cross-reactivity of the antibodies has not been commercially tested for horses and donkeys; however, previous publications have confirmed they work in horses (Silva et al., 2024). Nevertheless, in order to certify and validate the technique, the antibodies were tested on donkey lymph nodes (data not shown), which serve as a positive control for immune cells, to ascertain the appropriate dilution and incubation time.

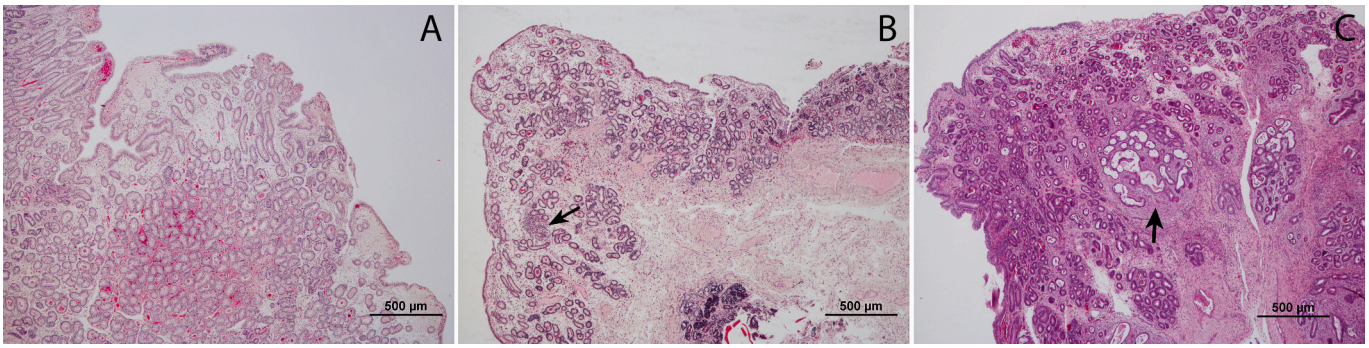

Fig. 1. Morphological aspects of endometrial biopsies classified according to Kenney and Doig categories: A. category IIA; B. category IIB; C. category III. Hematoxylin and eosin staining. A – Endometrial category IIA in an animal in diestrus. Note the scant inflammatory cells infiltrate, edema in the stratum compactum and absence or slight peri-glandular fibrosis. B – Endometrial category IIB in an animal in estrus. The inflammatory cells infiltrate can be observed, sometimes in disposed in periglandular nests (arrow). Glandular disorganization and moderate peri-glandular fibrosis is also present. C – Endometrial category III in an animal in estrus. Observe the extensive peri-glandular fibrosis, most notorious in the stratum spongiosum. Glandular nests isolated by fibrous tissue (arrow) are also present, along with glandular ectasia and moderate inflammatory cell infiltrate.

The 3 µm sections were deparaffinized and hydrated, and antigen retrieval was performed in a microwave in a target retrieval solution (Dako™; Agilent, Santa Clara, CA, USA; pH 6.0) for CD3 and CD20 slides, or in EDTA at pH 9.0 for Iba-1 slides. The Novolink™ Max-Polymer detection system (Leica Biosystems, Newcastle, UK), developed for visualizing mouse IgG, mouse IgM, and rabbit IgG primary antibodies, was used according to the manufacturer's instructions. Slides were incubated overnight at 4 °C with polyclonal anti-CD3 antibody (A0452, Dako, Glostrup, Denmark; 1:100) to identify T lymphocytes; anti-CD20 antibody (L26, Dako, Glostrup, Denmark; 1:100) to identify B lymphocytes; and anti-Iba-1 antibody (ab178846, Abcam, Cambridge, UK; 1:2000) to identify total macrophage population. The slides were then sequentially incubated with the Novolink™ post-primary and Novolink™ Polymer reagents (30 min each). The reaction was visualized with Novolink™ DAB Polymer, for 5 min and counterstained with Gill's Hematoxylin. Neutrophils and eosinophils were evaluated based on their histological morphology with hematoxylin and eosin stain in sequential sections to those of IHC. The slides were dehydrated and mounted in Entellan™ New (Merck, Darmstadt, Germany,).

All slides were evaluated using a Nikon E-600 optical microscope, and images were acquired with the Nikon Imaging Software (NIS) elements D program from Nikon® (Tokyo, Japan) at 400× magnification. Five areas in each endometrial compartment (stratum compactum, spongiosum, and the glandular and luminal epithelia) were counted, using ImageJ® software (https://imajej.nih.gov/ij/index.html). The positive T and B cells and macrophages were identified by positive brown staining of the cell membrane and cytoplasm.

2.3. Statistical analysis

The statistical package R (V 4.0.3, R Core Team; Vienna, Austria) was used to analyze the data, and the GraphPad Prism software (V 8.4.0, GraphPad Software LLC; San Diego, CA, USA) was used to elaborate the figures. The Kolmogorov-Smirnov and Levene tests were used to verify the data's normality and the variances' homoscedasticity, respectively. When necessary, the data were transformed with the square root of the arcsine (arcsin $\sqrt{x}$) to perform the analyses. A two-way ANOVA was applied to analyze the differences in the different inflammatory cell (T lymphocytes, B lymphocytes, macrophages, neutrophils, and eosinophils) distribution in the stroma of the stratum (compactum and spongiosum) and the glandular and luminal epithelium per each endometrial category according to Kenney and Doig's classification (IIA, IIB, and III), and the estrous cycle phase (estrus and diestrus). The Bonferroni post-hoc test was applied to all the analyses, considering $p \leq 0.05$. Results are expressed as mean ± standard error of the mean (SEM).

3. Results

The present study included 32 females aged 8.74 + 5.07 years (range = 3–20; one female of undetermined age). Nineteen jennies were in the estrus phase, and 13 were in diestrus. Fifteen jennies were nulliparous, ten were primiparous, and seven recorded two or more pregnancies (Table 1).

Regarding Kenny and Doig's category, ten donkeys were classified as IIA, 15 as IIB, and 7 in category III (Table 2). None of the females enrolled in this study was in category I. Animals scored in category III were significantly older than those in categories IIA and IIB ($p < 0.011$), but age differences were negligible between the latter. The parity was unevenly distributed among the three categories of Kenny and Doig

Table 1
Information retrieved on the 32 donkeys used: parity, estrous cycle phase and endometrial Kenney and Doig grade.

Jenny identification	Number of foalings	Age	Estrus phase	Kenney and Doig Grade
1	0	20	Estrus	III
2	0	7	Estrus	IIB
3	0	5	Estrus	IIB
4	1	8	Estrus	IIA
5	1	13	Estrus	IIA
6	1	7	Estrus	IIA
7	1	16	Estrus	IIB
8	0	6	Estrus	IIB
9	1	6	Estrus	IIA
10	1	3	Diestrus	IIA
11	1	4	Diestrus	IIB
12	0	5	Estrus	IIB
13	2	7	Diestrus	IIB
14	0	4	Diestrus	IIB
15	0	3	Estrus	IIB
16	3	13	Estrus	IIB
17	0	8	Estrus	IIB
18	3	14	Diestrus	IIB
19	2	8	Diestrus	IIA
20	0	5	Diestrus	IIA
21	1	6	Diestrus	IIA
22	0	4	Diestrus	IIB
23	2	12	Diestrus	IIB
24	4	14	Diestrus	III
25	1	7	Diestrus	IIA
26	1	6	Diestrus	IIA
27	0	20	Estrus	III
28	0	20	Estrus	III
29	0	7	Estrus	III
30	0	5	Estrus	III
31	2	8	Estrus	IIB
32	0	Unknown	Estrus	III

Table 2
Age distribution, parity and phase of the cycle in the jennies according to their endometrial Kenney and Doig grade.

Kenney and Doig grade	Mean age ± SD (years)	Parity/ number of foaling			Phase of the estrous cycle	
		Nulliparous	Primiparous	Pluriparous	Estrus	Diestrus
IIA	6.9 ± 2.6	1	8	1	4	6
IIB	7.73 ± 4.11	8	2	5	9	6
III	14.33 ± 6.89	6	0	1	6	1

classification (Table 2). Nonetheless, parity showed no effects on the endometrial sample categorization in the present population.

Four animals classified as Kenny and Doig IIA were in estrus, and six were in diestrus. Of the fifteen animals classified as IIB, nine were in estrus and six were in diestrus. Six females categorized as III were in estrus, and only one was in diestrus (Table 2).

3.1. Inflammatory cells in the different strata and epithelia

The T lymphocytes were the predominant cells in the stratum compactum and spongiosum (306.3 ± 39.4 and 288.1 ± 45.3, respectively, Fig. 2). They were at a significantly higher number than the other evaluated cell' types ($p < 0.001$). T cells were also present in the glandular and luminal epithelia (32.0 ± 8.47 and 44.8 ± 5.99, respectively), although not in significantly different numbers compared to the other inflammatory cells. All samples present T cell clusters in the stroma and around the glands (Fig. 3). The macrophage population (Fig. 4) was more abundant in the stratum spongiosum than in the luminal epithelium (94.26 ± 6.91 vs. 14.91 ± 1.60; $p = 0.0006$). While T cells predominated in the luminal epithelium, macrophages were in higher numbers in the glandular epithelium. Eosinophils were also observed (Fig. 5), predominantly located in the stroma of the compactum and spongiosum strata (47.6 ± 8.7 and 45.4 ± 8.6, respectively), and B lymphocytes (Fig. 4) and neutrophils (Fig. 5), rarely observed inside the different evaluated epithelia.

B lymphocytes and neutrophils the rarest inflammatory cells present in the endometrium (1.3 ± 0.3 and 0.9 ± 0.4, respectively, in glandular epithelium; and 1.3 ± 0.4 and 3.5 ± 2.4 in the luminal epithelium, respectively). B lymphocytes were the scarcest immune cell type in jenny endometrium. No significant statistical differences were observed in B lymphocyte, neutrophil, and eosinophil cell counts in the stratum and all epithelia.

3.2. Inflammatory cell count and estrous phase

Although no significant differences were found between diestrus and estrus for most cells studied, a slightly larger number of immune cells was observed during diestrus, except for neutrophils, which were the most common in estrus (Fig. 6). Only T lymphocytes showed significant differences between these two phases, with predominance in diestrus compared to estrus (808.5 ± 132.0 vs. 575.0 ± 94.0; $p = 0.002$).

3.3. Inflammatory cells and endometritis grade

A significantly higher number of T lymphocytes were recorded in all the categories of the Kenney and Doig scoring system (Fig. 7) compared to the remaining inflammatory cells ($p < 0.001$). A significant reduction ($p < 0.05$) in T cell counts was observed in category IIB (545.8 ± 116.4) compared to category III (722.6 ± 139.6). In contrast, no significant differences were observed in the cell count of B lymphocytes, neutrophils, eosinophils and macrophages according to the categories of Kenney and Doig.

4. Discussion

Studies on the presence of immune cells in donkey endometrium are scarce. A significant influx of neutrophils and eosinophils into the uterine lumen, along with augmented cytokine expression, has been reported to occur in jennies with endometritis (Fantini et al., 2021). Like in mares, neutrophil influx in the endometrium occurs in association with artificial insemination with frozen semen (Vilés et al., 2013; Miró and Papas, 2018), which might explain the infertility reported for artificial insemination in the species (Vidament et al., 2009). Another study focused on neutrophil and eosinophil counts and their association with fibrosis in the jennies' endometrium, reported a fall in eosinophil and neutrophil count as endometrial fibrosis progressed (Miró et al., 2020). It was also noted that the presence of seminal plasma, but not sperm cells, the neutrophils rearrange morphologically and form neutrophils extracellular traps (Mateo-Otero et al., 2022). In our study, only a small population of neutrophils was detected, in contrast to the high number of T lymphocytes. However, we only focused on animals in the estrous cycle that had not been inseminated.

The endometrial resident immune cells differ between species in their distribution and proportion, as it has been shown in cows (Cobb and Watson, 1995), sows (Kaeoket et al., 2001), mares (Watson and Dixon, 1993; Rudolph et al., 2017) and dogs (Pires and Payan-Carreira,

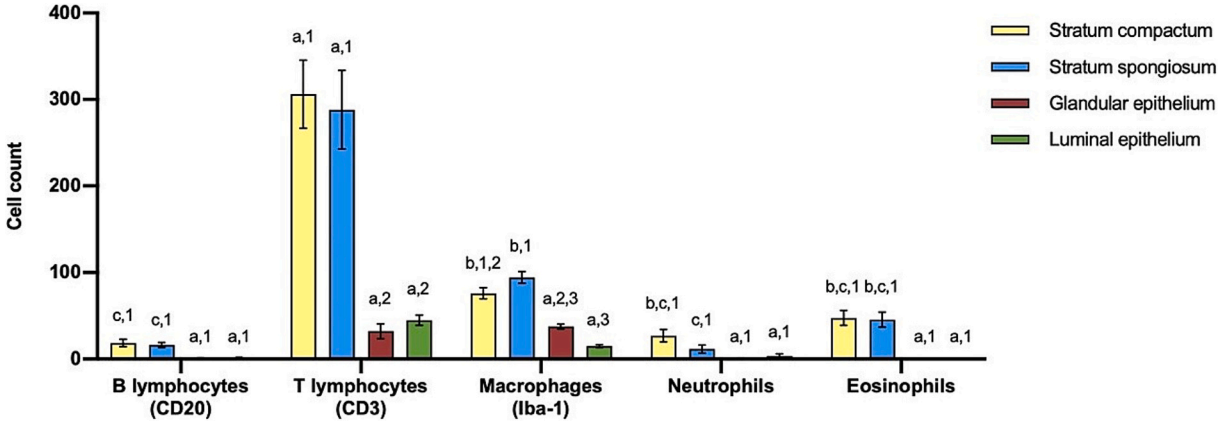

Fig. 2. Distribution of cell types in the endometrium (Mean ± SEM). (a-c) Different letters indicate significant differences ($p \leq 0.05$) between inflammatory cells within each stratum and epithelium. (1–3) Different numbers indicate significant differences ($p \leq 0.05$) between stratum and epithelium, for each inflammatory cell.

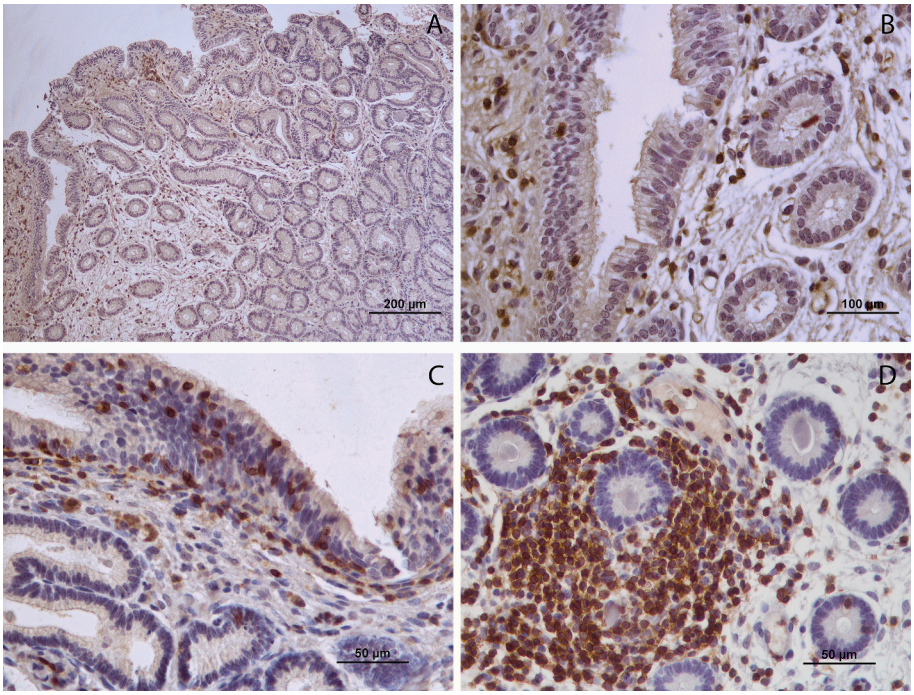

Fig. 3. Immunopositivity for T lymphocytes (antibody anti-CD3). A) and B) – Animal in estrus, with Kenney and Doig stage IIA. A – T lymphocytes are scattered throughout the different strata. B – detail of the image in A, showing T lymphocytes scattered in the stroma of the stratum compactum and in between the surface epithelium of the endometrium. C) and D) – Animal in diestrus with Kenney and Doig stage IIB. C – presence of T cells among the superficial and glandular epithelium in the stratum compactum. D – T cells surrounding the endometrial glands in the stratum spongiosum, organized in nodules; some can locate between the glandular epithelial cells, revealing their epitheliotropic properties. Gill's hematoxylin counterstaining.

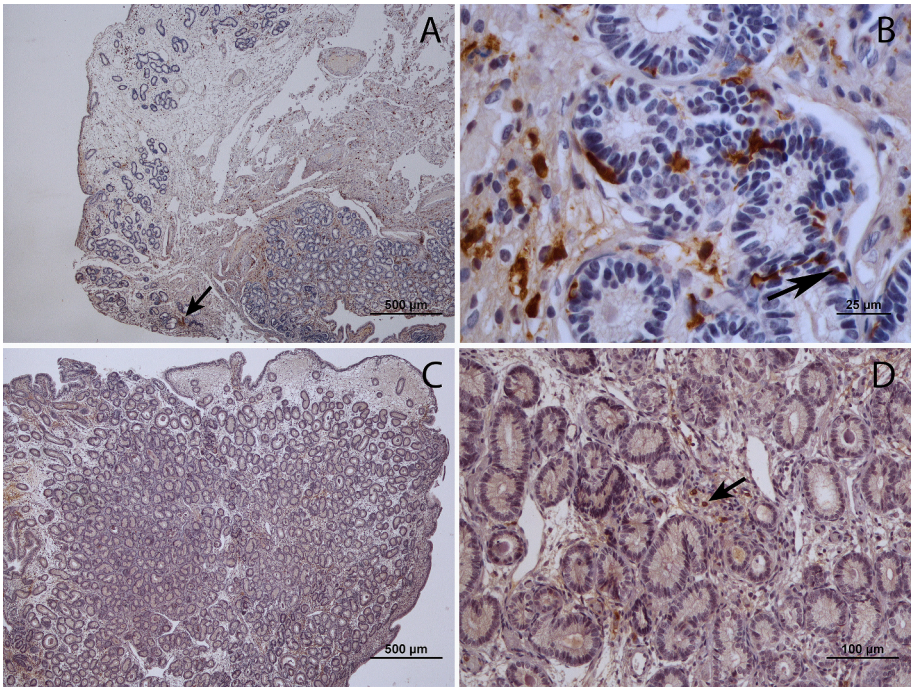

Fig. 4. Immunostaining for macrophages (antibody anti-Iba1) and B lymphocytes (antibody anti-CD20) in the jenny's endometrium. A and B (antibody anti-Iba1) – Animal in estrus, with Kenney and Doig stage IIB. A – diffuse infiltration of macrophages in both strata, sometimes being displayed in small clusters (arrow). B – higher magnification of image A. The macrophages are scattered in the spongious strata and between the glandular epithelial cells (arrow). C and D (antibody anti-CD20) – Animal in diestrus, with Kenney and Doig stage IIA. C – B lymphocytes are sparsely distributed in the endometrium, without particular preponderance in the different strata of the endometrium. D – detail of image C), showing a scant population of B lymphocytes (arrow). Gill's hematoxylin counterstaining.

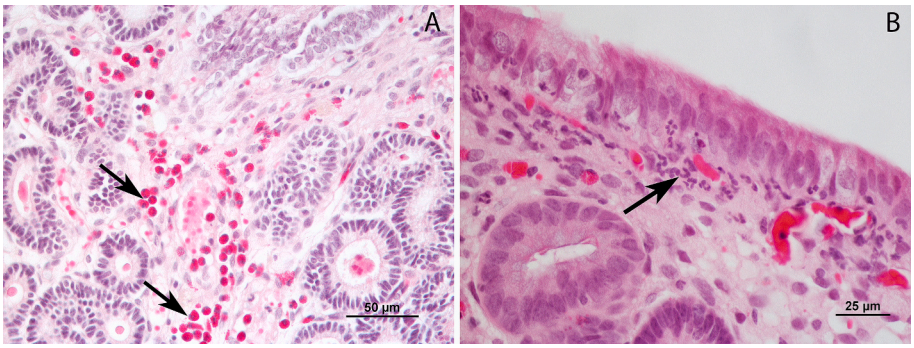

Fig. 5. Eosinophils (A) and Neutrophils (C) infiltrate in jenny's endometrium. A – Intense infiltrate of eosinophils (arrow), recognized by their typical eosinophilic granules, in the stratum spongiosum. Animal in diestrus, with Kenney and Doig stage IIB. B – Neutrophils (arrow) in the stratum compactum. Animal in estrus, with Kenney and Doig stage IIA. Hematoxylin and eosin staining.

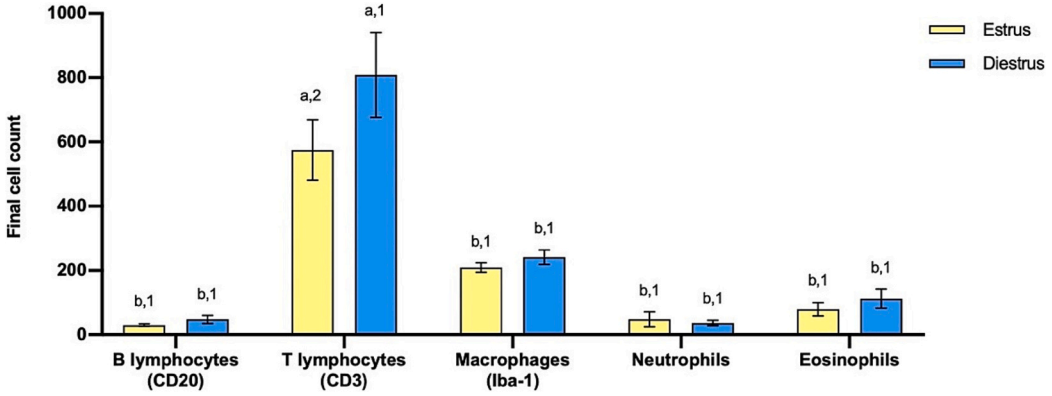

Fig. 6. Cell count of the different inflammatory cells in the estrus and diestrus (Mean ± SEM). (a, b) Different letters indicate significant differences ($p \leq 0.05$) between inflammatory cells within each estrous cycle phase. (1,2) Different numbers indicate significant differences ($p \leq 0.05$) between estrous cycle phase, for each inflammatory cell.

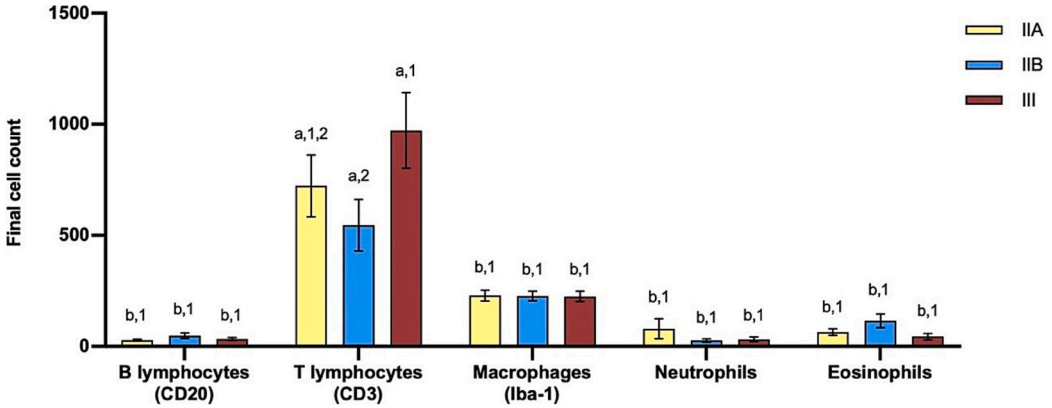

Fig. 7. Cell count (Mean ± SEM) of the different inflammatory cells in the different endometrial grades. (a, b) Different letters indicate significant differences ($p \leq 0.05$) between inflammatory cells within each grade of endometritis. (1,2) Different numbers indicate significant differences ($p \leq 0.05$) between grades of endometria for each inflammatory cell.

2015). Similarly, the present study showed that the reproductive tract of the jenny is rich in immune cells, particularly T lymphocytes, which prevail in the luminal and glandular epithelium, similar to what is reported in the mare (Watson and Dixon, 1993; Rudolph et al., 2017), cows (Cobb and Watson, 1995), and humans (Kamat and Isaacson, 1987). Moreover, T cell count appears slightly higher in diestrus, suggesting a possible association of sex hormones, particularly progesterone, in the T lymphocytes infiltrating jenny endometrium. Similar findings have been described in cows (Oliveira et al., 2013) and mares (Frayne and Stokes, 1994).

In the jenny endometrium, we have shown that macrophages represent the second most portrayed immune cell population studied. Neutrophils, and B lymphocytes were found in lower numbers than T lymphocytes or macrophages. Similar findings have been reported earlier in mares (Watson et al.,1996; Rudolph et al., 2017), dogs (Pires and Payan-Carreira, 2015), and cats (Butterworth et al., 2001).

Peri-glandular T lymphocytes were sometimes arranged to form clusters around the glands and inside the glandular and surface

epithelia. As described for the mare, these immune cells could influence the endometrial microenvironment by producing pro-fibrotic cytokines and promoting collagen fibers deposition, but without correlation with Kenney and Doig's morphological classification. Some authors hypothesize that the T cells increase in the equine endometrium during estrus is part of the antigen immune response associated with the deposition of semen and other antigens at this stage (Frayne and Stokes, 1994).

The presence of lymphocytes in the jennies' endometrium, while not necessarily indicating an existing tissue lesion or antigenic stimulation, can relate to the physiological microenvironmental characteristic of this specific tissue and species. Immune cells in jennies may integrate a species-specific immune surveillance mechanism to the natural endometrial remodeling through the estrous cycle.

Even though it has been suggested that the presence of a high number of T lymphocytes in the mare endometrium could be related to the existence of an organized lymphoid tissue associated with the endometrium (Klose and Schoon, 2016), like the mucosa-associated lymphoid tissue (MALT) described for the human endometrium (Morris et al., 1985; Yeaman et al., 2001), our observations do not allow us to infer the existence of such lymphoid aggregates.

The presence of T lymphocytes has been linked to fibrosis progression in mare endometrosis and is associated with up-regulation of TGFβ-1 (Rebordão et al., 2014). These observations may explain the increase in T lymphocyte count in severe endometrosis, where the amount of collagen is in the highest quantity. Inflammatory cytokines, such as IL33, are involved in innate and adaptive immunity and are a crucial regulator of chronic inflammation and eosinophil activation (Miller et al., 2017) and may also influence the promotion of grade III in jennies through microenvironment cytokines up-regulation (Miró et al., 2020). In jenny's endometrium, a notorious increase in IL33 transcripts was reported as fibrosis increased, which might be associated with eosinophil infiltration (Miró et al., 2020).

In the present study, a high number of T lymphocytes was found across the estrous cycle phases and in all the categorized endometrial grades. This finding was observed even in cases where the endometrial score was IIA.

Scattered neutrophils could be observed within the stratum compactum and spongiosum of the jenny endometrium and the luminal epithelium, during all estrus and diestrus. The neutrophil count tended to be higher in IIA grade compared with other endometrosis categories. These findings do not entirely agree with Miró et al. (2020), who reported a fall in neutrophil count with increased endometrial classification. It is worth noting that in mares, neutrophils are usually observed during estrus, located in the endometrium stratum compactum and luminal epithelium (Brunckhorst et al., 1991; Schöniger and Schoon, 2020). It has been demonstrated that neutrophils rearrange their morphology in the endometrium of mares to form extracellular traps (NETs) and may also be involved in the jenny reproductive tool as an instrument to select spermatozoa (Rebordão et al., 2014). This may also play a long-term role, in association with PMN granules, contributing to the endometrosis process by inducing myofibroblast differentiation and catalyzing collagen deposition (Chrysanthopoulou et al., 2014). Neutrophils in jenny's endometrium may be involved in fibrogenesis since they correlate to collagen I deposition (Miró et al., 2020).

In the present study, the eosinophil infiltrate is noted through both endometrial strata, contrary to the reported to mares, where the stratum compactum contains slightly higher numbers than the stratum spongiosum (Grimm et al., 2017; Schöniger and Schoon, 2020). Fewer eosinophils were found in the estrus phase, which agrees with what was reported in the mare (Grimm et al., 2017). A higher number of eosinophils was observed in categories IIB, compared to IIA and III, similar to the study in Catalan jennies, which showed an increase in endometrial fibrosis (i.e., the Kenney and Doig degree III), in parallel to a decrease in eosinophil count (Miró et al., 2020). Since one feature of endometrosis is endometrial fibrosis, eosinophils could be linked to collagen remodeling and be actively requested to promote establishing fibrosis in grade IIB endometrium.

An increase in endometrial fibrosis determines the abnormal endometrium architecture and function, which could also be associated with eosinophils, and particular cytokines (Miró et al., 2020). With the advancing of endometrosis, mares chronically progress to irreversible grade III, where most of the endometrium is replaced by thin collagen fibers (Snider et al., 2011). At this stage, a drastic morphological change occurs in endometrial cells, including the glandular epithelia and stroma cells, which are replaced by collagen fibers (Lehmann et al., 2011; Snider et al., 2011). In jennies, grade III samples may no longer present an active inflammatory process, while evidencing destructive inactive endometrosis (following the classification by Hoffmann et al., 2009).

5. Conclusions

To the best of our knowledge, this is the first study to evaluate several immune cells infiltrating in the donkey endometrium and relate it to the Kenney and Doig scale and the estrous cycle. An innovative view of the dynamics of the immune system of the donkey's reproductive tract is essential to managing the fertility of these animals for the improvement and survival of the species.

This study describes the endometrial distribution pattern of major immune cell populations. It highlights the differences between mares in the estrus and diestrus phases and at Kenney and Doig's classification. Based solely on the inflammatory cell infiltrate, this study suggests that Kenney and Doig's scale might not be the most adequate grading scale to assess jenny's endometrium as it does not present significant differences between grades IIA and IIB, not even in the case of T lymphocytes. Therefore, these findings suggest developing an adapted scale for donkeys' endometrosis morphology classification.

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CRediT authorship contribution statement

Ariana Radar-Chafirovitch: Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Miguel Quaresma:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Conceptualization. **Ivan Yánez-Ortiz:** Writing – review & editing, Software, Formal analysis, Data curation. **Belén Leiva:** Methodology. **Graça Ferreira-Dias:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Rita Payan-Carreira:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Jordi Miro:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maria dos Anjos Pires:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

None.

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