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Intrauterine infusion of killed semen adversely affects uterine blood flow and endometrial gene expression of inflammatory cytokines in mares susceptible to persistent breeding-induced endometritis

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ABSTRACT

Persistent breeding-induced endometritis (PBIE) is a leading cause of infertility in mares. The objective of the study was to assess genital perfusion and endometrial gene expression of inflammatory cytokines in mares classified as susceptible (n = 5) or resistant (n = 5) to PBIE. Ten mares were examined daily during estrus until 6 d after hCG-induced ovulation for two estrous cycles. Twenty-four hours after application of 1500 IU hCG, 4 mL of killed (by repeated freezing in liquid nitrogen and thawing at 50 °C) deep-frozen semen or sterile saline was instilled into the uterine body and examinations were carried out immediately before and 3, 6, and 12 h after intrauterine infusion. Examinations included blood sampling to determine plasma progesterone (P_4) concentrations, and transrectal ultrasonography in B- and color Doppler mode to determine follicular and luteal size and blood flow, the extent of intrauterine fluid, as well as time-averaged maximum velocity (TAMV), blood flow volume (BFV), and blood flow resistance (expressed as pulsatility index, PI) of the uterine arteries. Additionally, endometrial biopsies were obtained at 24 h before, and 2 and 7 d after infusion, and mRNA expressions of *IL1B*, *IL6*, *IL8*, *IL10*, *TNF*, *CASP3*, and *COX2* were determined by qRT-PCR. Statistical analyses were performed with mixed models. Intrauterine fluid retention (diameter >20 mm for at least 3 d) was found after infusion of killed semen in five susceptible mares. There was no treatment effect (semen vs saline; P > 0.05) on genital blood flow, plasma P_4 concentration, and endometrial gene expression. In comparison to resistant mares, susceptible mares had an increased (P = 0.04) BFV of the uterine arteries at 24 h before intrauterine infusion of killed semen, and an increased (P = 0.03) PI at 2 d after infusion. The TAMV, plasma P_4 concentrations, and follicular and luteal size and blood flow did not differ (P > 0.05) between resistant and susceptible mares. Endometrial mRNA expression of *IL1B* increased (P = 0.05) at 2 d after the infusion of killed semen in the susceptible mares, and the expression of *IL10* increased (P = 0.003) at 7 d after the infusion within the resistant mares. Interleukin 6 mRNA was increased (P = 0.05) in susceptible compared to resistant mares at 2 d after infusion. In summary, an intrauterine infusion of killed semen increases uterine blood flow resistance and alters endometrial gene expression of inflammatory cytokines for at least 7 d but does not affect ovarian blood supply and luteal function in mares susceptible to PBIE.

1. Introduction

An inflammatory response of the equine endometrium to breeding is regarded as a physiological event that is necessary to remove excess spermatozoa and contaminating bacteria from the uterus [1]. A reproductively healthy (resistant) mare is able to clear this inflammation within 48 h [2,3], whereas a subset of mares

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(susceptible mares) remain inflamed beyond 72 h [4]. The delayed uterine clearance of inflammation in these mares leads to a persistent breeding-induced endometritis (PBIE) and is associated with intrauterine fluid retention [5,6]. Consequently, the uterus of mares with PBIE is not well-prepared for the admittance of the conceptus at 5–6 d after breeding [7], leading to reduced pregnancy rates [8,9]. Occurring in about 15% of normal Thoroughbred mares [10], PBIE is considered by equine practitioners to be the leading cause of equine infertility [11].

The mechanisms by which PBIE can impair fertility are multifarious and recent reviews have summarized the current level of knowledge [3,12]. However, little is known about the role of the genital blood flow in the context of PBIE. Adequate blood supply to the reproductive tract is essential for hormonal signaling, uterine contractility, feto-endometrial interactions, and placentation [13]. In particular, sufficient uterine blood flow has been associated with enhanced fertility in mares, while reduced perfusion due to elastosis and degeneration of uterine blood vessels compromised uterine contractility in aged, pluriparous, and subfertile mares [14,15]. Color Doppler ultrasonography has been proven to be a useful and noninvasive method for evaluating uterine blood flow in mares [16–18].

There is increasing evidence that uterine inflammation additionally impairs the function of the ovaries. For instance, blood progesterone (P_4) concentration, reflecting corpus luteum (CL) function, was reduced in mares with uterine inflammation [19,20]. The function of CL can also be assessed by measuring luteal blood flow, as there is a high correlation between luteal blood flow and plasma P_4 concentrations in horses [21] and cows [22]. Consistently, the luteolysis induced by repeated intrauterine infusions of LPS was accompanied by a reduction of plasma P_4 concentrations as well as luteal blood flow in heifers [23]. This effect is probably based on the local utero-ovarian pathway, since $PGF_{2\alpha}$ release from the bovine endometrium induces luteolysis *via* the countercurrent between the uterine vein and the ovarian artery [24,25]. In mares, lacking a local utero-ovarian pathway, the same outcome is by no means certain, but the effect of a uterine inflammation on the blood flow of the CL has not been studied yet. Admittedly, the blood flow in the ovarian artery ipsilateral to the dominant follicle increased after intrauterine application of raw semen [26]. This effect is possibly attributable to the interaction between sperm and oviduct, since the ovarian artery supplies both the ovary and the oviduct. However, newer facilities like Power Doppler Imaging (PDI) mode ultrasonography allow a distinct evaluation of follicular and luteal blood flow in mares.

The innate immune response after breeding influences the susceptibility to PBIE in mares [3]. Inflammatory cytokines are cell-signaling molecules that are expressed in the equine endometrium and control the inflammatory response [27]. Pro-inflammatory cytokines interleukin 1β (*IL1B*), interleukin 6 (*IL6*), tumor necrosis factor α (*TNF*), and the chemokine interleukin 8 (*IL8*) initiate and maintain inflammation [28–30], whereas the anti-inflammatory cytokine interleukin 10 (*IL10*) modulates the pro-inflammatory cytokines [31]. Consistently, the endometrial mRNA expression of *IL1B*, *IL6*, *IL8*, and *TNF* was increased and gene expression of *IL10* was reduced 24 h after AI with killed semen [28,32]. Woodward et al. [30] reported that susceptible mares had a reduced mRNA expression of *IL6* and *IL10* at 6 h after intrauterine infusion of killed spermatozoa compared with resistant mares, indicating that these mares were less able to respond to and initiate the modulation of inflammation. However, a tight balance between multiple pro- and anti-inflammatory factors is required for resolving the breeding-induced endometritis within 24–36 h in the reproductively healthy mare [12]. Although endometritis is considered persistent only after 48 (–72) h post breeding, it appears that the inflammatory changes differentiating resistant and susceptible mares already occur within the first hours [3]; therefore, previous research on endometrial gene expression of inflammatory cytokines has focused on the first 72 h after breeding. Since the endometrial expression of inflammatory cytokines is likely to influence the survival of the approaching embryo, this study expanded the investigation up to 6 d after ovulation.

The present study aimed to investigate the hypothesis that in mares susceptible to PBIE, an intrauterine infusion of killed semen has a detrimental, fertility-compromising effect on uterine, and possibly ovarian, perfusion and on endometrial gene expression of inflammatory cytokines.

2. Materials and methods

2.1. Animals, housing and feeding

Ten clinically healthy, non-lactating, non-pregnant and normally cycling Warmblood (n = 5) and Franches Montagnes (n = 5) mares with a mean age of 10 years (range, 3–21 years) were used. Six mares were maiden and four mares had at least one foal. In the latter, the last foaling was five years or more ago. The mares had no severe anatomical defects of the genitalia, such as poor vulva conformation, incompetent vestibule-vaginal sphincter, or adversely increased vulva angle of declination. Due to their breeding history, five mares were expected to be susceptible and five mares were potentially resistant to PBIE. The study was conducted during three consecutive breeding seasons in the Northern hemisphere. The examinations of each mare were performed within one breeding season. All mares were housed in an open stable day and night, were fed with hay or haylage, and had *ad libitum* access to water.

The experimental procedures complied with the Swiss Federal Law on Animal Protection and were approved by the Committee of Animal Experiments of the Cantonal Veterinary Office Zurich, Switzerland (application 2012153).

2.2. Experimental design

The mares were examined every two to three days by transrectal ultrasonography (B-mode) to detect the onset of estrus. Examinations were performed daily once the dominant follicle had a diameter of approximately 30 mm combined with the presence of uterine edema (Fig. 1), and included blood sampling from the right or left jugular vein and transrectal ultrasonography (B- and color Doppler mode) of the pre-ovulatory follicle, the developing corpus luteum (CL), the uterus, and both uterine arteries. Ovulation (Day 1) was induced by intravenous application of 1500 IU human chorionic gonadotropin (hCG, Chorulon® 1500 ad us. vet.; Veterinaria AG, Pfäffikon, Switzerland) when a follicular diameter of at least 35 mm was observed. Twenty-four hours after application of hCG, either 4 mL of killed deep-frozen semen or sterile saline (NaCL 0.9%) were instilled into the uterine corpus of randomly chosen mares, and additional examinations were carried out immediately before and 3, 6, and 12 h after the infusion. Furthermore, 24 h before, and 2 and 7 d after infusion, endometrial cells were collected for cytology (including a bacteriological examination of the first sample), and endometrial tissue was obtained by biopsy for RNA extraction and histological examinations. Daily examinations (blood sampling and ultrasonography) were continued until 6 d after ovulation. All mares were short cycled on Day 9 by intramuscular injection of 7.5 mg luprostiol ($PGF_{2\alpha}$ analogon, Prosolvin® ad us. vet.; Virbac, Glattbrugg, Switzerland) and one estrous cycle without treatment was awaited before the trial was repeated using the alternative treatment (mares that were formerly treated with saline were

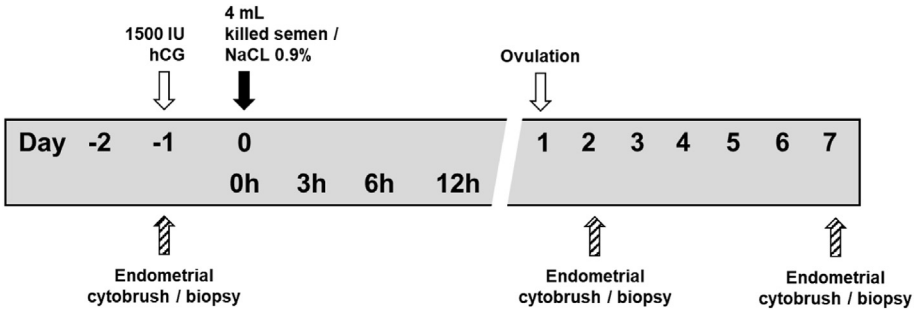

Fig. 1. Treatment schedule of cycling mares treated with 1500 IU human chorionic gonadotropin (hCG) during estrus of two different, non-consecutive estrous cycles and assigned randomly to receive either an intrauterine infusion with 4 mL killed semen or sterile saline. Blood samples were collected and ultrasonographic measurements were performed at all times shown. Endometrial cytobrush and biopsy samples were collected 24 h before and 2 and 7 d after intrauterine infusions.

infused with killed semen and *vice versa*).

Classification of mares as resistant (n = 5) or susceptible (n = 5) to PBIE was based on their uterine inflammatory response to the intrauterine infusion of killed semen or saline. With reference to Brinsko et al. [33], the accumulation of intrauterine fluid (IUF) with a diameter >20 mm for at least 3 d was regarded as IUF retention and confirmed mares as susceptible to PBIE. In resistant mares without fluid retention, some intrauterine fluid was observed but was limited to the first 12 h after intrauterine applications.

2.3. Preparation of killed semen and intrauterine application

Eight insemination straws, each with 0.5 mL semen (containing approx. 10^8 spermatozoa) of the same stallion, were used for the treatments in all mares. Spermatozoa were killed by repeated (three times) freezing in liquid nitrogen and thawing at 50 °C as reported by Brinsko et al. [34]. Lack of viability was confirmed in aliquots by motility determination and by flow cytometric analysis after propidium iodide staining [34].

Treatments with killed semen and saline were performed transcervically in an aseptic manner, using a single use uterine catheter (IUI pipette with stylet for horses 57 cm; Minitüb, Tiefenbach, Germany) guided by transvaginal palpation, and reagents were infused into the body of the uterus.

2.4. Plasma concentrations of progesterone

Immediately prior to each ultrasonographic examination, a blood sample was collected from the right or left jugular vein into an evacuated tube containing EDTA as anticoagulant (Vacuette 9 mL K3EDTA; Greiner bio-one, Kremsmünster, Austria). Tubes were immediately placed on ice and plasma was separated by centrifugation (3000×*g*, 15 min), and frozen at −20 °C until P_4 concentrations were measured with a commercial RIA (RIA kit IM1188; Immunotech s. r.o., Prag, Czech Republic).

2.5. Ultrasonography

Transrectal ultrasonographic examinations of the ovaries, the uterus, and the uterine arteries were performed using a portable ultrasound device (GE LOGIQ e Premium BT11; General Electric Medical System, Solingen, Germany), equipped with a 4.0–12.0 MHz, linear-array transducer.

Three cross-sectional B-mode images with maximal areas were frozen and recorded for each ovarian follicle with a diameter of ≥30 mm and for every detectable CL. The total areas of the follicle and CL were measured using computer-assisted image analysis software (PixelFlux Version 1.0; Chameleon Software, Leipzig, Germany) and were defined as follicular size (FS) and luteal size (LS), respectively. For each follicle and CL, mean values of measurements of the three recorded images were used for data analysis.

Uterine edema was evaluated visually using cross-sectional B-mode images of both uterine horns, and the degree of edema was scored semiquantitatively from 0 to 3, with 0 indicating no edema, and 1 to 3 indicating edema of low, moderate and high degree. If IUF was detectable, three cross-sectional B-mode images of the uterus at the location with the maximum accumulation of fluid were frozen and recorded. The area of IUF was measured using PixelFlux software and the average of the calculations of three images was used for data analysis.

For a subsequent determination of uterine blood flow, the diameters of both uterine arteries were measured in three B-mode images depicting a maximal cross-sectional area of the vessel. All images were recorded in the region where the uterine artery crosses the deep circumflex iliac artery. For each uterine artery, the mean value of the three measurements was used for further calculations.

The second part of the ultrasonographic examination used color Doppler mode. More specifically, PDI-mode was applied to assess the absolute follicular (aFBF) and luteal blood flow (aLBF). Therefore, the transducer was positioned at the maximal diameter of the follicle or CL, and small adjustments in location (relative to the ovary) were made to achieve a maximal number of color pixels in the follicular wall or luteal parenchyma, prior to freezing and recording the image. The number of pixels with color was determined (PixelFlux software), as a semiquantitative assessment of aFBF and aLBF. For each structure in each mare, three images were recorded, and the average was used for data analysis. To calculate the relative follicular (FBF) and luteal blood flow (LBF), aFBF and aLBF were divided by FS and LS, respectively.

In case of an ovulation of two dominant follicles (double ovulation), and a subsequent development of two CL, the mean and sum were applied for follicular and luteal measurements, respectively, as suggested by Bollwein et al. [21].

To determine the blood supply to the uterus, Color Flow (CF)- and Pulsed Wave (PW)-mode ultrasonography were used as previously described [17]. Color Flow-mode ultrasonography is a method of visually detecting blood flow using a color map that is incorporated into a standard B-mode image. Detected blood flow is assigned a color, typically blue or red, depending on whether the flow is moving toward or away from the transducer, whereas all stationary objects are represented on a gray scale (as in B-mode imaging). By using PW-mode ultrasonography, which allows to sample at a specific depth along the Doppler line, it becomes possible to accurately measure the velocity of blood in a precise

location and in real time. In the present study, the blood flow of the left and right uterine artery was examined in the region where the artery crosses the deep circumflex iliac artery (localized using CF-mode). Typical blood flow waveforms of both uterine arteries were obtained using PW-mode and were recorded for analysis in the laboratory. Doppler calculations were performed with PixelFlux software using two similar consecutive blood flow waveforms. The analysis was based on the envelope of the Doppler shift spectrum and blood flow was characterized by the time-averaged maximum velocity (TAMV), the blood flow volume (BFV), and the pulsatility index (PI).

The TAMV was calculated from the time-averaged maximum frequency shift (TAMF) over the cardiac cycle in the equation

$$\text{TAMV [cm/s]} = (\text{TAMF [Hz]} \times \text{c [cm/s]}) / (2 \times \text{F [Hz]} \times \cos(\alpha)),$$

where c is the ultrasound propagation speed, F the transmitted wave frequency and α the angle between the ultrasound beam and the direction of blood flow. Additionally, the BFV as quantitative measure of uterine blood supply was calculated as previously described [35], using the formula

$$\text{BFV [mL/min]} = \text{TAMV [cm/s]} \times 60 \times (\text{D [cm]} / 2)^2 \times \pi,$$

where D is the diameter of the uterine artery.

Uterine blood flow resistance was calculated as PI, the ratio of the difference between peak systolic frequency shift (PSF) and minimum diastolic frequency shift (MDF) to TAMF:

$$\text{PI} = (\text{PSF-MDF})/\text{TAMF}.$$

The dimensions of PSF, MDF and TAMF were derived from maximum frequency shift envelopes. The PI increases when the vascular conditions proximal to the site of measurement remain constant and the vascular bed distal to the site of measurement constricts. Conversely, a low PI value indicates decreased impedance to blood flow in the vascular bed distal to the site of measurement [36].

The TAMV, BFV and PI values of the two uniform consecutive pulse waves were averaged and the mean was used for statistical analyses.

2.6. Endometrial bacteriology and cytology

After a thorough cleaning of the vulva as described previously for intrauterine application, endometrial cells were collected using a double guarded uterine cytobrush for horses (Minitüb). Guided by transvaginal manual assistance, the cytobrush passed through the cervix and a cell sample was collected from the uterine corpus.

Immediately after cell collection, a swab sample of the cell-bearing end of the cytobrush was taken, placed into a transport medium, and sent to the laboratory (Institute of Veterinary Bacteriology, University of Zurich) for further processing on the same day. The swab was streaked on blood agars and incubated aerobically for 24 h at 37 °C. Bacterial growth was identified according to the morphology of the colonies and standard tests for bacterial identification. If after enrichment a small amount of *E. coli* or more than three different isolates were identified, contamination was suspected.

After the swab sample was taken, the cytobrush was immediately streaked on an object slide, air-dried, and stained with Diff-Quick (Henry Schein, Melville, NY, USA). According to a modified schedule of Prieto et al. [37], the number of polymorphonuclear neutrophils (PMNs) was counted within ten different, randomly chosen fields of view using light microscopy at 400 × magnification. A total of >5 PMNs was considered an acute endometritis.

2.7. Endometrial biopsy, histology, RNA extraction, and RT-qPCR

Endometrial tissue was collected by biopsy immediately after the cytobrush was taken. Therefore, a sterile Kevorkian uterine biopsy punch (Eikemeyer, Tuttlingen, Germany) was passed through the cervix guided by transvaginal manual palpation, and a piece of the endometrium (approximately 10 × 5 × 5 mm) was obtained from the anterior part of the uterine body under transrectal manual assistance. Repeated endometrial biopsy collection can be performed within the same estrous cycle without causing significant trauma to the endometrium and without provoking clinical signs or altering endometrial gene expression [27].

Tissue samples were divided into two pieces with a sterile scalpel. One half was immediately placed in a sterile DNase- and RNase-free cryo tube (Brand, Wertheim, Germany), frozen in liquid nitrogen and stored at −80 °C until expression analysis, whereas the other half was stored in Lillie's neutral buffered formaldehyde (3.7% formaldehyde solution) at 5 °C until histological analysis was performed at the Institute of Veterinary Pathology, University of Zurich.

For histological evaluation of endometrial tissue, fixed tissue samples were routinely embedded in paraffin and sectioned at 2.5 μm. Histologic sections stained with hematoxylin-eosin (HE) were examined using light microscopy at 40 × to 400 × magnification for the presence of inflammatory cells in the luminal epithelium, the *stratum compactum,* and the *stratum spongiosum*. Infiltration of one or more PMNs per five fields of high magnification (400 ×) was considered as evidence of acute endometritis [38]. Furthermore, HE-stained sections were examined for lymphatic lacunae and angiopathies, and were used to categorize the endometrium of mares as I, IIA, IIB or III, according to Kenney and Doig [39]. Additionally, trichrome staining (Gomori's blue Trichrome; Agilent, Santa Clara, CA, USA) was performed to evaluate periglandular and stromal fibrosis, as suggested by Blanchard et al. [40].

Endometrial mRNA expression was determined for *IL1B*, *IL6*, *IL8*, *IL10*, *TNF*, caspase 3 (*CASP3*), and cyclooxygenase 2 (*COX2*) at the Institute of Veterinary Physiology, University of Bern.

Total RNA was extracted from endometrial tissues with peqGOLD TriFast™ (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Quantity and purity of RNA was measured by using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, Germany). Complementary DNA (cDNA) was made by reverse transcription of 500 ng of total mRNA with Moleney Murine Leukemia Virus Reverse Transcriptase RNase H Minus, Point Mutant (MMLV-RT; Promega Corp., Madison, WI) and random hexamer primers (Invitrogen, Leek, The Netherlands).

Quantitative real-time PCR analysis was performed with the Sensimix DNA Kit (Quantace, Biolabo, Châtel St. Denis, Switzerland) on a Rotor-Gene 6000 (Corbett Research, Sydney, Australia). One reaction mixture contained 2 μl of cDNA equivalent to 25 ng of total RNA, 0.8 μl RNase-free water (Qiagen, Hilden, Germany), 1 μl (5 pmol) of forward primer, 1 μl (5 pmol) of reverse primer, and 5.2 μl of 2× SensiMix plus SYBR-Green (1 mM $MgCl_2$).

The following 3-step PCR program was used: initial denaturation for 10 min at 95 °C, followed by 40 cycles with denaturation for 15 s at 95 °C, 30 s at primer-specific annealing temperature, and elongation for 20 s at 72 °C. Fluorescence was acquired at 72 °C after each cycle, and a dissociation melt curve of the PCR product was determined at the end of each run to verify the specificity of the PCR reactions. The primers used to amplify specific fragments referring to selected regulated genes are shown in Table 1. As

Table 1
Sequences and accession numbers of PCR primers for assayed genes from equine endometrial cells, and annealing temperature (AT) of PCR products.

Gene	Gene symbol	Reference		Forward primer	Reverse primer	AT
		[acc. no.]	[Source]	[5'- … -3']	[5'- … -3']	[°C]
Interleukin 1β	*IL1B*	NM_001082526.1	[41]	CAGTCTTCAGTGCTCAGGTTTCTG	CATTGCCGCTGCAGTAAGT	60
Interleukin 6	*IL6*	NM_001082496.2	[42]	GGATGCTTCCAATCTGGGTTCAAT	TCCGAAAGACCAGTGGTGATTTT	60
Interleukin 8	*IL8*	AY184956.1	[41]	CTTTCTGCAGCTCTGTGTGAAG	GCAGACCTCAGCTCCGTTGAC	60
Interleukin 10	*IL10*	NM_001082490.1	[41]	GCTGGAGGACTTTAAGGGTTAC	CATCACCTCCTCCAGGTAAAA	60
Tumor necrosis factor α	*TNF*	NM_001081819.2	[41]	GGCCCAGACACTCAGATCAT	TTGGGGGTTTGCTACAACAT	60
Caspase 3	*CASP3*	NM_00116396.1	[43]	GTGATTCTAAGCCATGGGGATGAAGG	CCCCGGCAGGCCTGAATGATGAAGAG	58
Cyclooxygenase 2	*COX2*	AB041771	[43]	CGCAAACGCTTTCGGCTGAC	CCACCAGAAGGGCAGGATACAG	58
Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	NM_001163856.1	[44]	GGGTGGAGCCAAAAGGGTCATCAT	AGCTTTCTCCAGGCGGCAGGTCAG	60

housekeeping gene *GAPDH* was used.

Cycle threshold (Ct) values were determined by the Rotor-Gene software version 1.7.75, and the relative mRNA expression was calculated by means of the comparative Ct method using the following equation [45]:

$$\Delta Ct = Ct\ target\ gene - Ct\ endogenous\ control\ (housekeeping\ gene)$$

2.8. Statistical analysis

Intrauterine fluid retention was observed after treatment with killed semen but not with saline in three mares, after treatment with killed semen and with saline in two mares, and not at all in five mares. The former five mares were regarded as susceptible to PBIE and the latter five mares as resistant. The effect of an intrauterine infusion of killed semen (*versus* saline) on repeated measurements of P_4, ultrasonographic parameters of the ovaries and uterus, and mRNA expression in endometrial biopsies was statistically analyzed using a repeated measures analysis of variance procedure in SAS (PROC MIXED), as recommended by Wang and Goonewardene [46]. This procedure revealed no significant treatment effect. Using the same procedure in SAS, there was no significant effect of the mares' breed on all examined parameters.

The MIXED model (PROC MIXED) was also used to analyze the effect of susceptibility to PBIE on plasma P_4 concentrations, ovarian and uterine ultrasonographic parameters, and endometrial mRNA expression. Significant results were further evaluated on a per-time basis using a two-sample *t*-test (PROC TTEST) and a Student's *t*-test (PROC UNIVARIATE) for independent or dependent pairwise comparisons, respectively. Normal distribution of the data was assessed visually and confirmed using the Shapiro-Wilk test (PROC UNIVARIATE).

For non-normal data, Wilcoxon's two-sample test (PROC NPAR1WAY) was applied for independent pairwise comparisons (between groups), and Wilcoxon's signed rank test (PROC UNIVARIATE) for dependent pairwise comparisons (within group). Differences in categorical variables were evaluated using Fisher's exact test (PROC FREQ).

The relationship between the blood flow in the uterine artery ipsilateral to the dominant follicle or CL (dominant artery) and the uterine artery contralateral to the dominant follicle or CL (non-dominant artery) was assessed using Pearson's correlation (PROC CORR). The measurements of TAMV, BFV, and PI in dominant and non-dominant uterine arteries were highly correlated ($r = 0.56$, $r = 0.64$ and $r = 0.83$, respectively; each $P < 0.0001$); therefore, the mean of measurements of both uterine arteries was calculated and used for further evaluations. The similarity of correlations was examined using Fisher's r-to-z transformation and revealed a higher correlation for PI compared to TAMV and BFV values ($P \leq 0.0005$).

Statistical analyses were conducted using the Statistical Analysis System V9.3 (SAS Institute Inc., Cary, NC, USA). Data were presented as mean ± standard error of the mean (SEM) or median ± interquartile range (IQR), depending on the distribution of the data, and differences were considered significant at $P \leq 0.05$.

3. Results

3.1. Age and parity

The average age of mares susceptible to PBIE was higher ($P = 0.003$) compared to the resistant mares (15.8 ± 2.4 vs 4.8 ± 1.1 years; mean ± SEM). There was a lower percentage of nulliparous mares within the group of susceptible compared to the group of resistant mares (20 vs 100%; $P = 0.05$).

3.2. Endometrial bacteriology, cytology, and histology

The endometrial tissue of the susceptible mares was histologically categorized as Kenney grade IIA ($n = 1$) or IIB ($n = 4$). All of these mares had periglandular and/or stromal fibrosis, but none had lymphatic lacunae (Table 2). Angiopathies were observed in three out of five susceptible mares (Table 2). Exemplary histological findings of susceptible mares are depicted in Fig. 2(D–F). The resistant mares had a grade I ($n = 4$) or IIA ($n = 1$) endometrium. Periglandular fibrosis was found in one out of five resistant mares, whereas none of these mares had lymphatic lacunae or angiopathies (Table 2; Fig. 2A–C).

Before intrauterine infusion, bacteriological and cytological examinations of the endometrium in all mares did not reveal any pathogenic bacteria or neutrophils. Additionally, physiological non-inflammatory edema due to estrus was verified by histological examination. Two days after intrauterine infusion with killed semen, acute endometritis was detected in one resistant and one susceptible mare by cytology (>5 PMNs per ten fields of 400 × magnification), and in two resistant and four susceptible mares by histological examinations (≥1 PMN per five fields of

Table 2
Results of semiquantitative histological examinations of endometrial biopsies in ten mares (A-J) susceptible and resistant to persistent breeding-induced endometritis.

Histological findings	Susceptible mares						Resistant mares					
	A	B	C	D	E	Mean	F	G	H	I	J	Mean
Fibrosis[a]	1	2	2	1	1	1.75*	0	1	0	0	0	0.20*
Lymphatic lacunae[b]	0	0	0	0	0	0	0	0	0	0	0	0
Angiopathies[c]	0	1	0	1	1	0.6*	0	0	0	0	0	0.0*

Within rows values with asterisks (*) are different ($P < 0.05$).
[a] 0 = perivascular fibrosis; 1 = periglandular or stromal fibrosis; 2 = periglandular and stromal fibrosis.
[b] 0 = no lymphatic lacunae; 1 = lymphatic lacunae.
[c] 0 = no angiopathies; 1 = angiopathies.

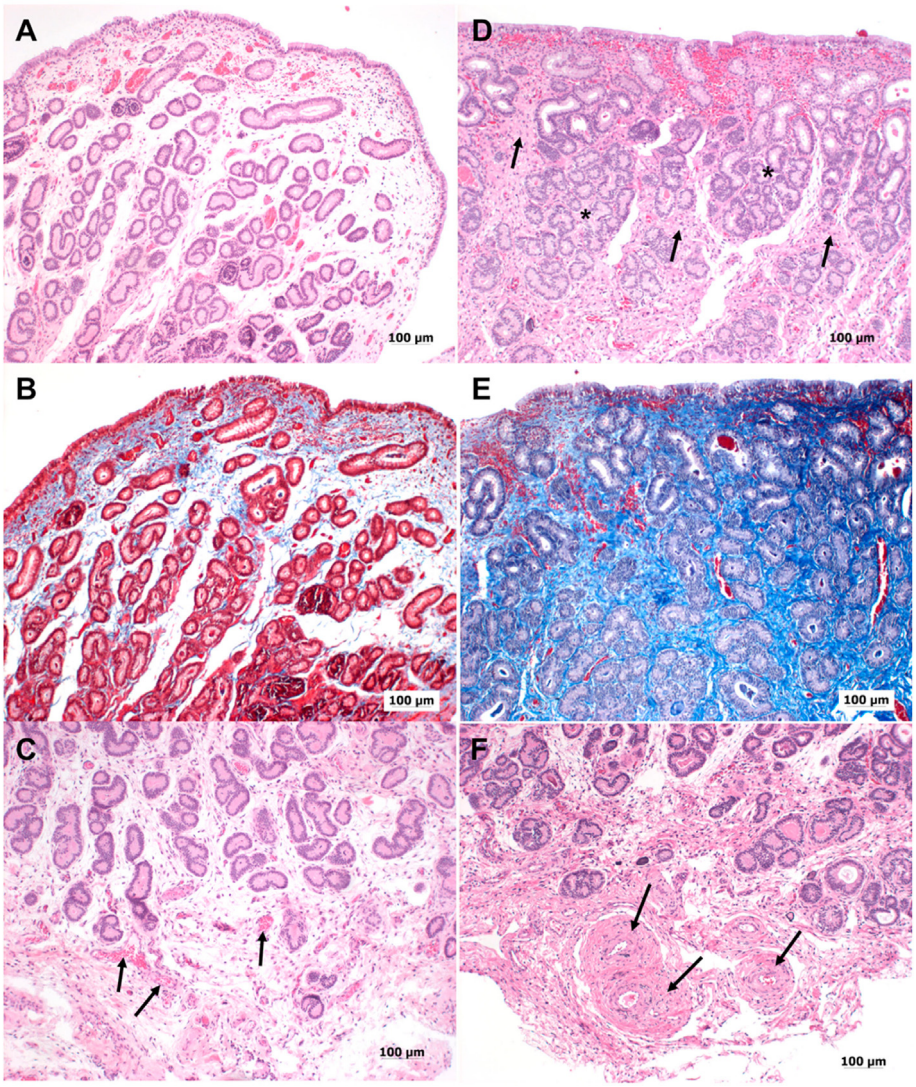

Fig. 2. Endometrial biopsies of a resistant, biopsy category I mare (A–C) and a susceptible, biopsy category IIB mare (D–F) stained with hematoxylin and eosin (HE; A, C, D, F) or Gomori's blue Trichrome (B, E). Magnification 100 ×. A. Unaltered *Stratum compactum* and *Stratum spongiosum*, and a moderate edema in the *Stratum compactum*. B. Normal amount of collagen (blue) in the *Stratum compactum* and *Stratum spongiosum*. C. Unaltered vessels (arrows) in the *Stratum spongiosum*. D. Glandular nest formation (asterisks) and periglandular and stromal fibrosis (arrows). E. A high amount of collagen (blue) in the *Stratum compactum* and *Stratum spongiosum* (stromal and periglandular fibrosis). F. Severe angiopathy (arrows) of vessels in the *Stratum spongiosum*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

400 × magnification). Seven days after intrauterine infusions with killed semen, cytological examinations revealed no acute endometritis in any mare.

3.3. Uterine edema and intrauterine fluid accumulation

Three days before ovulation, uterine edema of moderate degree (score 2) was observed in all susceptible and resistant mares. The average edema score (median ± IQR) during the three days before intrauterine infusion of killed semen amounted to 3 ± 1 in susceptible and to 2 ± 1 in resistant mares, whereas uterine edema during the seven days after infusion scored 1 ± 1 in susceptible and 0 ± 1 in resistant mares. At 1 d before and 1–6 d after infusion, uterine edema was one score higher in susceptible compared to resistant mares. Seven days after infusion, no edema (score 0) was observed in all mares.

The amount of IUF revealed a group effect (difference between groups; $P = 0.003$) but neither a time effect (within group difference) nor group*time interactions ($P > 0.05$). Using per-time analysis, IUF accumulation was higher in susceptible mares compared to resistant mares at 12 h ($P = 0.02$) and 1, 2, and 3 d (each $P = 0.03$) after intrauterine infusion of killed semen (Fig. 3).

3.4. Diameter and blood flow of uterine arteries

The diameters of the dominant and non-dominant uterine arteries did not differ ($P > 0.05$) between susceptible (3.14 ± 0.29 and 3.10 ± 0.30 mm, respectively; mean ± SEM) and resistant mares (2.39 ± 0.26 and 2.34 ± 0.45 mm, respectively). There was a time effect ($P = 0.003$) for TAMV, and group and time effects for BFV ($P = 0.02$ and $P = 0.004$), and PI ($P = 0.03$ and $P < 0.0001$). None of these parameters revealed any group*time interaction ($P > 0.05$). On a per-time basis, susceptible mares had higher ($P = 0.04$) BFV compared to resistant mares at 1 d before infusion of killed semen, and higher PI at 2 d after infusion ($P = 0.03$; Fig. 4).

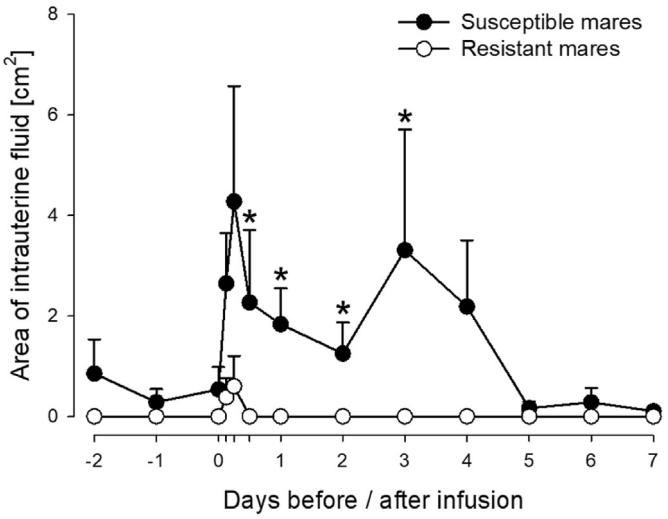

Fig. 3. Changes (means ± SEM) in the area of intrauterine fluid accumulation of ten mares susceptible (n = 5) and resistant (n = 5) to persistent breeding-induced endometritis from 2 d before to 7 d after intrauterine infusion with killed semen (Day 0). * Difference (P ≤ 0.05) between susceptible and resistant mares at times indicated.

3.5. Endometrial mRNA expression

Group effects were observed for the mRNA expressions of *IL1B* (P = 0.003), *IL8* (P = 0.04), *IL10* (P = 0.03), *TNF* (P = 0.0001), and *CASP3* (P = 0.007). Furthermore, there was a time effect for the mRNA expression of *TNF* (P = 0.05) and group*time interactions for the mRNA expressions of *IL6* (P = 0.04), *IL8* (P = 0.0008), and *COX2* (P = 0.02).

On a per-day basis, a higher mRNA expression in susceptible compared to resistant mares was observed for *IL6* (P = 0.05) at 2 d after intrauterine infusion of killed semen, as depicted in Fig. 5. Lower mRNA expressions in susceptible mares were revealed for *IL1B* and *CASP3* (each P = 0.02) at 1 d before infusion, for *TNF* (P = 0.004) and *CASP3* (P = 0.05) at 2 d after infusion, and for *IL1B* (P = 0.008), *IL8* (P < 0.0001), *IL10* (P = 0.01), *TNF* (P = 0.0002), *CASP3* (P = 0.03), and *COX2* (P = 0.01) at 7 d after infusion.

Endometrial mRNA expression of *IL1B* increased (P = 0.05) between 1 d before and 2 d after intrauterine infusion in susceptible mares but remained constant (P > 0.05) in resistant mares. In susceptible mares, mRNA expressions of *IL6* (P = 0.04) and *IL8* (P = 0.03) decreased between 1 d before and 2 d after infusion and between 2 and 7 d after infusion, respectively, whereas expression of *IL10* remained unchanged (P > 0.05). In resistant mares, mRNA expression of *IL6* remained constant (P > 0.05), whereas expressions of *IL8* (P = 0.02) and *IL10* (P = 0.003) increased between 2 and 7 d after infusion and between 1 d before and 7 d after infusion, respectively. The mRNA expression of *TNF* did not change (P > 0.05) in susceptible mares, but increased (P = 0.03) between 1 d before and 7 d after infusion in resistant mares. An increase (P = 0.04) in mRNA expression of *CASP3* was observed in susceptible mares between 2 and 7 d after infusion, whereas mRNA abundance of *CASP3* did not change (P > 0.05) in resistant mares. Expressions of *COX2* mRNA did not differ (P > 0.05) over time, neither within susceptible nor within resistant mares.

3.6. Plasma P_4 concentrations and follicular and luteal size and blood flow

Time effects (P < 0.0001) but no group effects (P > 0.05) or group*time interactions (P > 0.05) were observed for plasma P_4 concentrations, FS and LS, and FBF and LBF. Using per-time analysis, none of these parameters differed (P > 0.05) between susceptible and resistant mares (Fig. 6). In one resistant mare, a double ovulation with subsequent development of two CL occurred.

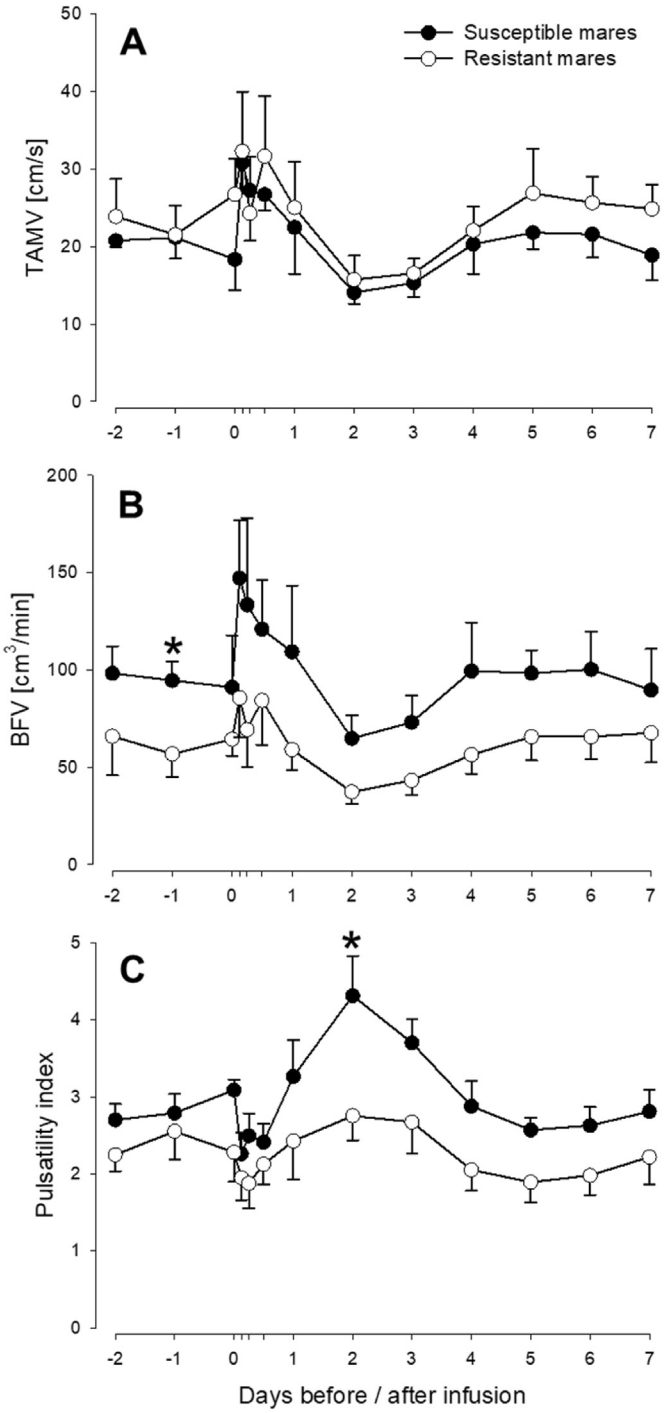

Fig. 4. Changes (means ± SEM) in A) time-averaged maximum velocity (TAMV), B) blood flow volume (BFV), and C) the pulsatility index of uterine arteries in ten mares susceptible (n = 5) and resistant (n = 5) to persistent breeding-induced endometritis from 2 d before to 7 d after intrauterine infusion with killed semen (Day 0). * Difference (P ≤ 0.05) between susceptible and resistant mares at times indicated.

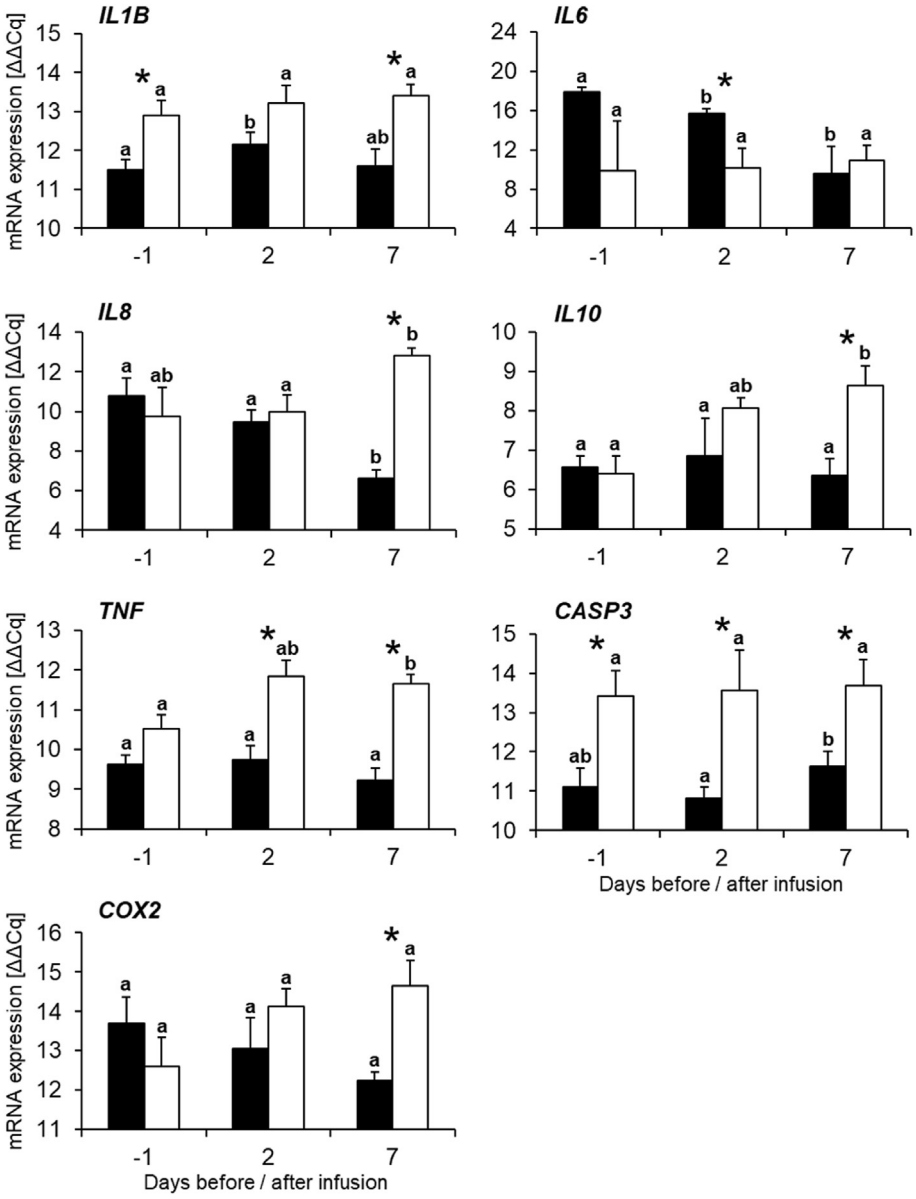

Fig. 5. Endometrial mRNA expressions (means ± SEM) of interleukin 1β (*IL1B*), interleukin 6 (*IL6*), interleukin 8 (*IL8*), interleukin 10 (*IL10*), tumor necrosis factor α (*TNF*), caspase 3 (*CASP3*), and cyclooxygenase 2 (*COX2*) in ten mares susceptible (black columns; n = 5) and resistant (white columns; n = 5) to persistent breeding-induced endometritis at 1 d before and 2 and 7 d after intrauterine infusion with killed semen (equivalent to 2 d before and 1 and 6 d after ovulation). An asterisk (*) represents a difference between groups ($P \leq 0.05$) at times indicated; different letters (a,b) represent a difference between times ($P \leq 0.05$) within groups indicated.

4. Discussion

The susceptibility to PBIE in mares in the present study was associated with advanced age and previous maternity. This is consistent with long standing observations regarding the lower ability of old mares to resolve uterine inflammation compared to young ones [47,48]. Age- and parity-related factors, such as impaired lymphatic drainage, poor perineal conformation, an incompetent vestibule-vaginal sphincter, an increased declination of vulva angle, and periglandular fibrosis as well as degenerative angiopathies of the endometrium make mares susceptible to persistent inflammation [27,49]. Although severe anatomical defects were not found in susceptible mares of the present study, adverse histological findings of the endometrium were present in all of them. Additionally, repeated foaling causes a ventrally dropped uterus within the abdomen (due to a loss of structural support of the caudal reproductive tract and stretching of the broad ligaments) that predisposes to uterine fluid retention after breeding [50]. Since all but one susceptible mare had at least one foal before, a relaxed suspension of the uterus might be jointly responsible for PBIE in these mares.

The histopathologic evaluation of endometrial biopsies revealed a higher Kenney grade and a higher proportion of biopsies with angiopathies and periglandular and stromal fibrosis in susceptible compared to resistant mares. These observations are in agreement with the advanced age and previous maternity of susceptible mares in the present study, since age and parity represent independent predisposing factors for the incidence of angiopathies [51]. Moreover, the severity and frequency of endometrosis, which is characterized by periglandular fibrosis, rises with increasing age of the mare [52] and is an important factor for the categorization as grade IIB or higher, according to Kenney and Doig [39].

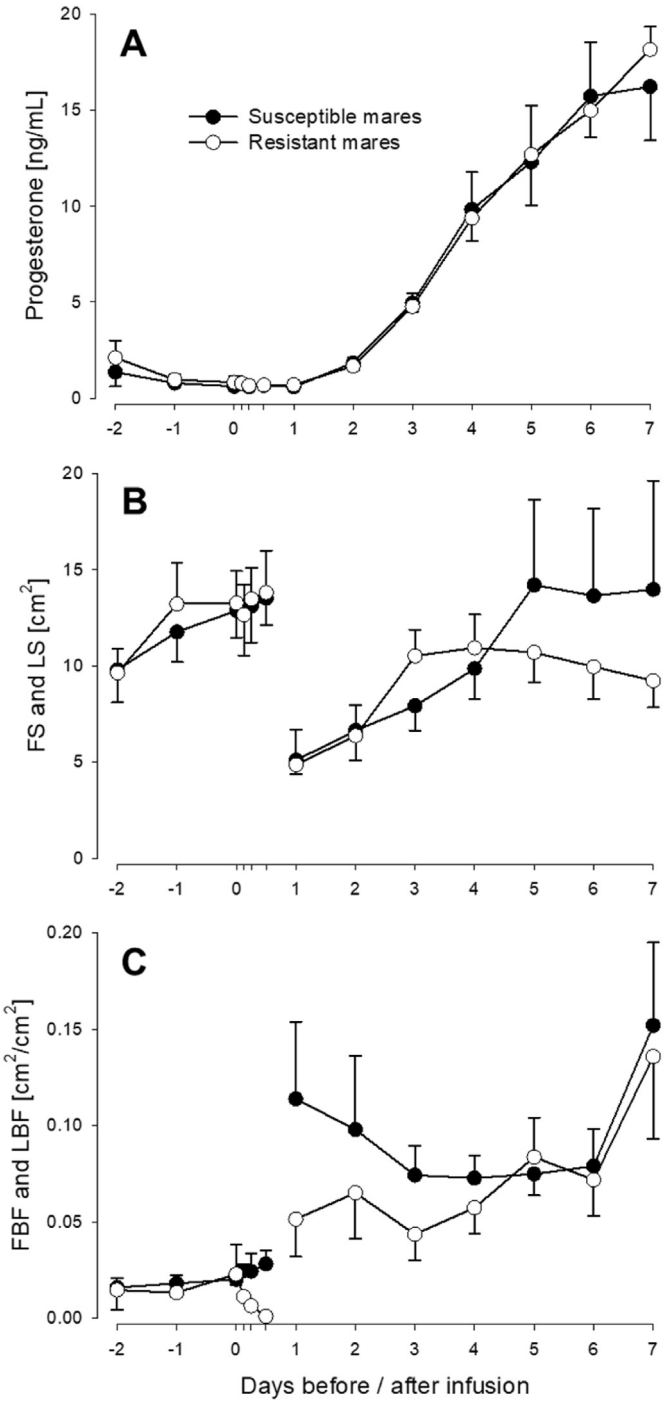

Fig. 6. Changes (means ± SEM) in A) plasma progesterone concentrations, B) the total areas of the follicle and corpus luteum (FS during 3 d before ovulation (Day 1), and LS during 6 d after ovulation), and C) the areas of colored pixels relative to FS and LS expressing relative follicular and luteal blood flow (FBF during 3 d before ovulation, and LBF during 6 d after ovulation) of ten mares susceptible (n = 5) and resistant (n = 5) to persistent breeding-induced endometritis from 2 d before to 7 d after intrauterine infusion with killed semen (Day 0).

Bacteriology and cytology did not reveal any infection or acute inflammation before the intrauterine infusion, neither in susceptible nor in resistant mares in the present study. Similarly, Christoffersen and Troedsson [12] reported that susceptible mares can be difficult to identify prior to breeding as most of them do not have a positive uterine culture at this time. Interestingly, two days after intrauterine infusion of killed semen in the present study, acute endometritis was recognized in one susceptible mare using cytology, and in four susceptible mares using histology. In fact, the examination of an endometrial biopsy was found to be the most sensitive method for determining the presence of endometritis [38]. The histological threshold for positive inflammation diagnosis was 1 PMN per five fields of 400 × magnification, which might be too low, since scattered neutrophils are found in the normal estrous uterus [53]. Furthermore, the time of examination (2 d after infusion) might be important since neutrophil numbers were increased after treatment with frozen/thawed semen at 8, 16, and 24 h in cytological examinations, and at 24 and 72 h in histological examinations [54]. However, PMNs play a crucial role in clearing uterine inflammation by stimulating the synthesis and release of $PGF_{2\alpha}$ from the endometrium activating uterine contractility [55], and by exerting phagocytosis and bactericidal activity [56]. Therefore, the increase in endometrial PMNs in susceptible mares may reflect longer persistence of PMN accumulation rather than differences in cellular responses and may be an outcome rather than the cause of prolonged inflammation [30].

Apart from endometrial PMN counts, the measurement of IUF accumulation that is assessed by ultrasonography represents a common method of evaluating inflammation of the equine endometrium [33,57]. In the present study, the accumulation of IUF was significantly higher in susceptible compared to resistant mares at 12, 24, 48, and 72 h after the intrauterine infusion of killed semen. Also, other studies have identified an association between the sustained inflammatory response in susceptible mares and the accumulation of fluid, pathogens and inflammatory products within the lumen of the uterus [1,5,6]. The amount of IUF increased significantly in the first hour after intrauterine infusion of semen extender, seminal plasma, and raw semen in mares [26]. The presence of 2 or more centimeters of fluid is considered a good indicator for the susceptibility to PBIE [33]. In the present study, neither intrauterine infusion of killed semen nor infusion of saline induced IUF accumulation in resistant mares. The same was found in another study [27] when resistant mares were inoculated with PBS or *E. coli*. In contrast, all five susceptible mares in the present study had IUF accumulation after infusion with killed semen but, interestingly, two of these mares also produced IUF after infusion with saline. This observation is in accordance with the study of Christoffersen et al. [27] that reported IUF accumulation after *E. coli* infusion in all seven and after PBS infusion in two out of seven susceptible mares.

The present study aimed to evaluate - for the first time - the uterine blood flow in mares with and without PBIE because inflammation is typically characterized by hyperemia of the affected organ [58]. In the susceptible mares, BFV was higher even before the intrauterine infusion of killed semen. The formula to calculate BFV includes both TAMV and the diameter of the uterine arteries. Since TAMV did not differ between susceptible and resistant mares, the higher diameter of the uterine arteries found in susceptible compared to resistant mares might have contributed to this result. Consistently, BFV was significantly higher in aged, primi- or pluriparous and mares with endometrial fibrosis compared to young, nulliparous and mares without endometrial fibrosis [59]. Increased BFV was due to increased diameters of the uterine arteries, which were in turn mainly the result of parity [59]. In contrast to the present study, Bollwein et al. [26] found that uterine TAMV values significantly increased within 1 h after infusion of both seminal plasma and raw semen. These changes in uterine perfusion were thought to be associated with endometrial inflammation and vasodilatory components in the seminal plasma. However, in the same study [26], infusion of skim milk semen extender had no effect on uterine blood flow. Consistently, the

inflammatory reaction of the endometrium, quantified by the neutrophil concentrations, was most severe after insemination with frozen or concentrated fresh semen, and only mild after infusion of semen extenders [60]. The intrauterine infusion of thawed deep-frozen semen with killed spermatozoa in the present study is not directly comparable with the treatments used in the study of Bollwein et al. [26]. Marked differences in the present study include the absence of live sperm and seminal plasma, the use of egg yolk extender instead of skim milk extender, and the infusion of a remarkably lower volume (4 mL vs 20 mL). Probably, the volume is the crucial reason for the different outcome between the two studies, since the intensity of uterine inflammation depends on the volume of the inseminate that changes sperm concentration [60]. Interestingly, the PI was significantly higher 2 d after intrauterine infusion of killed semen in the susceptible compared to the resistant mares, indicating an association between susceptibility to PBIE and increased uterine blood flow resistance. The PI is defined as the ratio of the difference between PSF and MDF to TAMF. The observed increase in the PI was due to a higher PSF and to a lower MDF in the non-dominant uterine arteries, and indicates a constriction of the distal vascular bed, whereas proximal vascular conditions remain constant [17]. The observation is in agreement with angiopathies detected in the endometrium in the susceptible mares. These angiopathies result in endometrial malperfusion and drainage disturbances caused by a reduced function of veins and lymphatic vessels [61].

In contrast to the uterine blood flow, there were no differences in the ovarian follicular and luteal perfusion. Previously, an increased TAMV in the dominant ovarian artery was observed at 1–12 h after the intrauterine infusion of raw semen, whereas no changes in the ovarian TAMV were determined after infusion of seminal plasma or skim milk semen extender [26]. However, it was assumed that the increase in the ovarian blood flow after infusion of raw semen was attributable to changes in the oviduct, as the equine ovarian artery supplies the ovary as well as the oviduct [62]. Consequently, an interaction between killed semen and the oviduct is not probable in the present study. The lack of differences in the FBF and LBF between susceptible and resistant mares is accompanied by a similar course of the follicular and luteal size as well as plasma P_4 concentrations. Positive correlations between LS, LBF, and P_4 have already been published [21].

The production of IL1B in the equine endometrium initiates the inflammatory cascade [63]. In the present study, the mRNA expression of *IL1B* remained constant within the resistant mares at all examination times, whereas *IL1B* mRNA increased between 24 h before and 48 h after infusion of killed semen in the susceptible mares. Consistently, the endometrial mRNA expression of *IL1B* was increased at 24 h and 72 h after intrauterine infusion of *E. coli* in susceptible compared to resistant mares [27]. Presumably, the increased mRNA expression of *IL1B* at 48 h after infusion of killed semen in the susceptible mares can be explained by a sustained or delayed abundance of *IL1B* mRNA, whereas the increase in the expression of *IL1B* mRNA in the resistant mares was short-termed and already over at 48 h after infusion. Since the events initiated by IL1B lead to the transcription of iNOS [64] and NO is known as a smooth muscle relaxer [65], IL1B is thought to contribute to the retention of IUF in susceptible mares. Interestingly, the mRNA expression of *IL1B* in the present study was higher in resistant compared to susceptible mares at 24 h before and 7 d after infusion of killed semen. In contrast, Fumuso et al. [28] found higher mRNA expression of *IL1B* in susceptible compared to resistant mares during estrus and diestrus (7 ± 1 d after ovulation) but values ranged from the 2.26- to 133-fold difference relative to *IL1B* mRNA expression in the sample with the lowest expression. Due to the wide range, the absolute baseline levels do not seem to be as important for the development of PBIE as the duration of mRNA abundance after challenge. Probably, the higher mRNA expressions of *IL1B* in resistant compared to susceptible mares at 24 h before and 7 d after challenge in the present study indicate that the resistant mares are continuously on alert defending detrimental stimuli on the endometrium and are therefore less predisposed to a delayed inflammatory response. The lack of difference in *IL1B* mRNA between resistant and susceptible mares at 48 h after infusion of killed semen in the present study is in agreement with the observations at 2, 6, 12, and 24 h after insemination with killed spermatozoa by Woodward et al. [30], and might be explained by the increased *IL1B* mRNA in the susceptible mares that partly adjust the difference to the higher baseline expression in the resistant mares.

Interleukin 6 has both pro- and anti-inflammatory properties. While IL6 is initially pro-inflammatory in response, it has the ability to activate varying receptors and pathways to function as anti-inflammatories later in the inflammatory process [63]. The present study found a constant mRNA expression of *IL6* within the resistant mares. This observation might be explained by a rapid, short-time release of IL6 after the intrauterine infusion of killed semen that is followed by an immediate expression of modulatory cytokines in the resistant mares. Consequently, the mRNA expression of *IL6* was back to normal already at 48 h after the infusion. The assumption is confirmed by recent studies that found an increased mRNA expression of *IL6* as early as 3 h after intrauterine infusion of *E. coli* [27] and 6 h after insemination with killed spermatozoa [30] in resistant mares, whereas at 12 h after challenge, *IL6* mRNA had already returned to normal in both studies. However, in susceptible mares of the present study the expression of *IL6* was high at 24 h before infusion of killed semen and decreased at 48 h and 7 d after infusion compared with the initial value. By Day 7, *IL6* mRNA expression had returned to levels observed in resistant mares. This result was in agreement with the initially pro-inflammatory, clinical response to breeding observed in susceptible mares. Moreover, the decreased mRNA expression of *IL6* at one week after infusion might suggest a reduced capability to modulate inflammation. At 48 h after infusion of killed semen, the mRNA expression of initially pro-inflammatory *IL6* was higher in susceptible compared to resistant mares. This is notable since the persistent inflammation in susceptible mares stands in contrast to the ability of resistant mares to clear the breeding-induced inflammation within 48 h [2].

Interleukin 8 is released by macrophages and endothelial cells and acts as a potent chemoattractant for PMNs [66]. Interleukin 8 mRNA expression did not differ between 24 h before and after infusion of killed semen in both resistant and susceptible mares, with the exception of a decreased expression of *IL8* at 7 d after infusion in the susceptible mares. Moreover, *IL8* mRNA increased between 2 and 7 d after infusion within the resistant mares. In a previous study [30], the mRNA expression of *IL8* increased at 2 h after insemination with killed spermatozoa in susceptible mares, and at 2 and 6 h after insemination in resistant mares. However, it did not differ between before and 12 and 24 h after insemination within each group of mares. Nash et al. [54] also found that the mRNA expression of *IL8* did not change at 24 h after challenge with live sperm in mares that were free from acute or persistent inflammation. However, differences in *IL8* mRNA were not examined until 7 d after challenge in these studies. The comparison of *IL8* mRNA between susceptible and resistant mares in the present study revealed no differences at 24 h before and 48 h after infusion of killed semen, but a higher expression at 7 d after infusion in the resistant compared to the susceptible mares was evident. Recently, it has already been shown that the initial *IL8* mRNA expression and PMN numbers are the same in susceptible and resistant mares [30]. In contrast with the present study and that of Woodward et al. [30],

an increased endometrial mRNA expression of *IL8* in susceptible compared to resistant mares has been observed at 24 h after insemination with killed sperm [32]. Since the peak concentration of PMNs in the uterine lumen is expected after 4–8 h [2,30,67] and PMNs are mostly removed due to resolution of inflammation within 24–36 h [4], the present study might have missed the peak of *IL8* mRNA expression. However, the decreased mRNA expression of *IL8* at 7 d after infusion in the susceptible compared to the resistant mares was in contrast to a previous study [32] that found increased *IL8* mRNA at 7 ± 1 d after ovulation in susceptible compared to resistant mares irrespective of whether mares were inseminated during estrus or not. Although IL8 is mainly known as neutrophil chemotactic factor, it is moreover a potent promoter of angiogenesis [68]. Therefore, increased *IL8* mRNA in resistant mares at 7 d after infusion might promote regeneration of the endometrium by increased angiogenic activity. However, it is debatable if the mRNA expression of *IL8* at 7 d after infusion has any importance in the pathophysiology of PBIE.

The inflammatory response is modulated by anti-inflammatory cytokines like IL10 [69] that reduce the transcription of pro-inflammatory cytokines by PMNs [70] and macrophages [71]. In the present study, the mRNA expression of *IL10* remained unchanged in the susceptible mares, whereas it increased at 7 d after infusion of killed semen in the resistant mares, thus leading to a significant higher mRNA expression of *IL10* in the resistant compared to the susceptible mares. Similarly, the gene expression of *IL10* in circulating leukocytes was reduced at 7 d after inoculation with *E. coli* in susceptible compared to resistant mares [27]. Resistant mares had a higher mRNA expression of *IL10* than susceptible mares already at 6 h after insemination with killed spermatozoa [30]. Therefore, the 6 h after breeding was regarded as a critical time for the development of PBIE and susceptible mares were considered to experience a defect in the inflammatory modulating mechanism [3].

The release of the pro-inflammatory cytokine TNF is part of the pro-inflammatory response [3]. In the present study, the mRNA expression of *TNF* remained constant in the susceptible mares, whereas it was increased in resistant mares at 7 d after intrauterine infusion of killed semen compared with its expression at 24 h before infusion. In another recent study [30], *TNF* mRNA in resistant but not in susceptible mares was increased at 2 h compared to the pre-insemination level. This early increase in *TNF* mRNA might have been missed in the present study, since the mRNA expression after infusion was not examined until after 48 h. In the present study, there was no difference in the mRNA expression of *TNF* at 24 h before infusion between resistant and susceptible mares, whereas a significantly higher expression of *TNF* mRNA was observed at 2 and 7 d after infusion in resistant compared to susceptible mares. In previous studies [28,30], there was no difference in *TNF* mRNA expression between susceptible and resistant mares up to 24 h after insemination with killed spermatozoa. However, 3 h after *E. coli* infusion, resistant mares had a higher endometrial mRNA expression of *TNF* compared to susceptible mares [27]. The early up-regulation has been suggested as a first line of defense to avoid uterine bacterial invasion. Possibly, the higher mRNA expression of *TNF* in the resistant compared to susceptible mares at 2 and 7 d after infusion in the present study indicates a sustained disposition to defend against bacteria in resistant mares. However, it is questionable if the long-term effect at 7 d after infusion is relevant to the pathophysiology of PBIE. In untreated mares, the variability of *TNF* mRNA expression at 7 ± 1 d after ovulation ranged from a 2.4- to 288-fold difference relative to the mRNA expression in the sample with the lowest expression [28]. Most notably, this high variability of *TNF* mRNA expression during diestrus and the small number of mares in the present study create doubt about the relevance of increased *TNF* mRNA in resistant mares at 7 d after infusion.

In the present study, the increased mRNA expression of *TNF* in resistant compared to susceptible mares was accompanied by an increased expression of *CASP3* mRNA. This observation is consistent with TNF inducing apoptosis in infected cells [3] with CASP3 being the key enzyme of induced apoptosis [72].

During inflammation, $PGF_{2\alpha}$ synthesis occurs due to the action of its synthesizing enzyme COX2 [73]. Both the increase in the mRNA expression of *COX2* in the resistant mares and the decrease in *COX2* mRNA in the susceptible mares were not significant, but resulted in a significantly higher expression of *COX2* mRNA in resistant compared to susceptible mares at 7 d after infusion. In a previous study [29], an increase in the mRNA expression of *COX2* has been noted in the endometrium of mares at 12 h after exposure to semen extender. Moreover, Nash et al. [54] found a local increase in $PGF_{2\alpha}$ concentration in the uterus of mares at 16 h after insemination with frozen/thawed semen. Apparently, this early increase in *COX2* mRNA leading to enhanced synthesis of $PGF_{2\alpha}$ could not be detected and compared because the first sampling at 48 h after infusion in the present study was too late. It is again a debatable point whether the higher expression of *COX2* mRNA in the resistant compared to susceptible mares at 7 d after infusion is considered of importance regarding the prevention of PBIE. The increased endometrial *COX2* mRNA is associated with increased uterine release of $PGF_{2\alpha}$ and enhanced tone of the myometrium [1], which might be beneficial with respect to regenerative processes during early diestrus. However, the difference at 7 d after infusion should not be overrated, since the mRNA expression of *COX2* did not differ significantly within each group of mares over time. A re-evaluation of cytokine mRNA expression during diestrus after infusion of killed semen in a higher number of resistant and susceptible mares might enlighten this uncleared issue.

In conclusion, an intrauterine infusion of killed semen does not affect the ovarian perfusion and luteal function but is accompanied by an increased uterine blood flow resistance in mares susceptible to PBIE. This finding indicates a constriction of the distal vascular bed that is associated with hypoperfusion of uterine tissue in susceptible mares. Furthermore, the infusion of killed semen in susceptible and resistant mares provokes changes in the endometrial gene expression of inflammatory cytokines. These changes are most notably the increased mRNA expression of the pro-inflammatory cytokine *IL1B* after the infusion of killed semen in the group of susceptible mares, the increased expression of the anti-inflammatory cytokine *IL10* after infusion within the resistant mares, and the higher expression of pro-inflammatory *IL6* at 48 h after infusion in susceptible compared to resistant mares. At 7 d after infusion, i.e. the time of embryo arrival in the uterus, the mRNA abundance of all cytokines, except for *IL6*, was decreased in susceptible compared to resistant mares. This result might indicate that resistant mares are on higher alert regarding uterine insult during diestrus. However, due to the high variability of cytokine mRNA expression in the diestrus of untreated and artificially inseminated mares [28], this result should be validated in a higher number of mares. Nevertheless, the overall pattern of expressions suggests increased anti-inflammatory properties of the endometrium in resistant compared to susceptible mares that are lasting until the time of embryo arrival in the uterus. Taken together, these changes in endometrial gene expression and in perfusion of uterine tissue may contribute to the retention of IUF and the reduced capability to modulate local inflammation, both of which are known to compromise fertility in susceptible mares.

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CRediT authorship contribution statement

J. Lüttgenau: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **I. Imboden:** Conceptualization, Methodology, Investigation, Writing - review & editing, Project administration. **O. Wellnitz:** Formal analysis, Investigation, Writing - review & editing. **R. Romer:** Investigation, Writing - review & editing. **I. Scaravaggi:** Investigation, Writing - review & editing. **A.P. Neves:** Investigation, Writing - review & editing. **N. Borel:** Investigation, Resources, Writing - review & editing, Visualization. **R.M. Bruckmaier:** Methodology, Resources, Writing - review & editing. **F. Janett:** Conceptualization, Resources, Writing - review & editing, Supervision. **H. Bollwein:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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