The influence of age and breeding technique on a mare's susceptibility to persistent breeding-induced endometritis

Summary

This retrospective study examined factors affecting the susceptibility of mares to persistent breeding-induced endometritis (PBIE). In particular, the differences in susceptibility between young and teenage mares, and between broodmares and embryo donors were investigated, and the efficacy of conventional treatments was assessed. Records were available from mares inseminated at Utrecht University's Faculty of Veterinary Medicine during 2018-2021, this amounted to 1745 inseminated estrous cycles from 769 different mares. PBIE was defined as the presence of more than 2cm of uterine fluid ≥24 h after insemination. Mares were divided into groups based on age (≤ 6 years; 7-13 years; ≥14 years), breeding system (broodmare/ET donor), reproductive status (maiden, foaling and barren) and semen type (frozen-thawed/fresh-cooled). Additionally, the number of inseminations per cycle, straws per (frozen semen) dose, cycle number, use of estrus or ovulation-inducing agents and the month of insemination were assessed. Subsequently, associations between the incidence of PBIE and pregnancy rates were examined. Overall, per cycle incidence of PBIE was 27.6%. PBIE was most frequent in the oldest mare group (42.3%) and least frequent in the youngest mares (11.2%). The incidence of PBIE was higher in donor mares (35.1%) than in broodmares (22.6%; $p<0.05$). PBIE was not significantly associated with a reduced pregnancy rate ($p=0.24$), suggesting that treating susceptible mares with uterine lavage and oxytocin after breeding minimizes the consequences of PBIE on fertility.

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1. Introduction

Inseminating a mare triggers a local immune response known as breeding-induced endometritis (BIE). This is a physiological reaction via which the mare's uterus attempts to remove bacteria and allogenic spermatozoa. The uterine defense mechanism comprises an immunological and a mechanical component. The immunological component is centered around an influx of polymorphonuclear neutrophils (PMNs) and macrophages, which clear the uterus of excess and dead spermatozoa by phagocytosis. The mechanical component involves contractions of the myometrium, which promote drainage of the inflammatory fluids via the cervix.[1] Normally, all spermatozoa and bacteria are cleared from the uterus within 48 hours post-insemination, and the inflammation subsides. If uterine clearance is not completed, the inflammation persists and is referred to as persistent breeding-induced endometritis (PBIE), which is considered pathological.[2] Following ovulation, the corpus luteum starts to produce progesterone which over the subsequent 2-3 days leads to closure of the cervix and a suppression of the mare's local uterine immune mechanisms. This will impair both the immunological and mechanical components of the uterine defense mechanism, such that the mare will be caught in a vicious circle of increasing inflammation if the problem is not resolved in a timely fashion. PBIE can result in premature secretion of PGF2α by the endometrium causing luteolysis and resulting in a shortened cycle and/or early embryonic loss.[3]

Not all mares are equally likely to develop PBIE. Multiple factors determine whether a mare is susceptible or resistant to PBIE, all of which are associated with the competence of the uterine defense mechanisms described above. Conditions that impair the function of these mechanisms will increase the risk of PBIE. The condition of both the mare's internal and external reproductive tract is of significance regarding the mechanical component, and defects are often correlated with age and/or parity of the mare.[4] This poses a dilemma for owners of sport horse mares who want progeny from their top performers. The competitive career of a successful show-jumping or dressage mare may last until her mid-teens, when fertility will start to decline. Breeding before serious training, which starts at around 5-6 years old, is a good alternative because young maiden mares are generally resistant to PBIE; however, carrying a foal to term also comes with risks of complications that could compromise future fertility. Embryo transfer is also an alternative, and can be performed during the course of the mare's sporting career. However, using a mare as an embryo donor and never letting her foal appears to exacerbate the fibrotic changes in the cervix often encountered in old maiden mares, and which impair post-breeding uterine clearance. Moreover, managing mares for insemination and embryo collection can interfere with training and performance. Postponing breeding until a mare has retired from competition is a further possibility. However, when an old maiden mare is presented for breeding, there is a higher risk of impaired uterine defense mechanisms necessitating a more thorough initial examination and closer monitoring to manage the increased susceptibility to PBIE.

With regard to the more intensive monitoring of a susceptible mare, it is important to prevent repeated breeding and, therefore, daily monitoring is required to establish the optimal moment for insemination. In addition, treatments such as flushing the uterus with lactated Ringer's solution prior to insemination and/or from as early as 4-6 hours post-insemination, with the additional use of an ecbolic drug like oxytocin, will help reduce the inflammation and stimulate uterine contractions to facilitate drainage.[1] For some susceptible mares, conventional treatment alone is insufficient, and the use of adjunctive therapies such as systemic corticosteroids may be necessary to attenuate the severity of the post-breeding inflammatory response.[5]

The goal of this study was to retrospectively 1) compare the susceptibility to PBIE of young versus teenage mares, and broodmares versus embryo donors, and 2) assess the efficacy of uterine lavage and ecbolics in preventing the development of PBIE.

2. Material and Methods

2.1. Data recording

For this retrospective study, an existing database of all mares that were inseminated at Utrecht University's Faculty of Veterinary Medicine during 2018-2021 was used. It contained information regarding the mare, the semen and the insemination. The database included patient name, patient number, mare use (ET donor/broodmare), stallion identity, type of semen (frozen-thawed/fresh-cooled), number of straws per dose (for frozen semen), date of AI and, in most cases, the outcome of pregnancy diagnosis (yes/no). Number of straws used per dose was later classified as ≤4, 5-8 or >8 straws.

Subsequently, more information regarding the mare's history, clinical examination pre/post breeding and treatments performed was added to the list by cross-reference to the faculty's veterinary administration program, Provet, to create a more complete data set. The examination and treatment records of every mare that visits the faculty for reproductive management, are filled in by the veterinarians, and subsequently uploaded to Provet. Age, reproductive status (maiden, barren or foaling), number of estrous cycles, estrus induction (yes/no), inseminations per cycle (1/>1) and hormone used to induce ovulation were added to the database. Age was categorized into 3 groups: ≤ 6 years old, 7-13 years old and ≥14 years old. Uterine fluid accumulation was recorded by examining the mare ultrasonographically at least once a day around the time of insemination and classified into 3 categories: no fluid (0 cm), a small amount of fluid (≤2 cm), a significant amount of fluid (2-4 cm) and a lot of fluid (≥4 cm). For this study, fluid accumulation was reclassified into 2 variables: the number of days intrauterine fluid was present post-breeding (fluid days) and the maximal amount of fluid that was found in this period (fluid max.). PBIE was defined as the presence of more than 2 cm of uterine fluid from ≥24 h after insemination. Uterine lavage and oxytocin (ecbolic) administration were the main treatments used to counter fluid accumulation and were recorded as the number of days that they were administered post-breeding; additional treatments, including the use of NSAIDs, corticosteroids and antibiotics were also registered (yes/no). All data was loaded into a single Excel sheet.

The original database consisted of 1925 cycles. During the processing of the data, 180 cycles were excluded due to the absence of information vital for answering the research question. The amended database contained a total of 1745 cycles from 769 different mares. Mares that were inseminated at multiple cycles appear multiple times in the data and, therefore, not every mare contributes equally to the data set, resulting in a bias. Furthermore, for the statistical analysis, the mares were divided into groups based on age, use, reproductive status, and type of semen used. These subsets allow for any given mare to be part of more than one group. For example, the total database consists of information collected over 4 consecutive years, such that it is possible for a mare to appear in 2 different age groups. Similarly, a mare could have been a broodmare one year, and an embryo donor the next, etc. The decision to use the cycle and not the mare as the statistical unit was deliberate, to avoid losing a large amount of potentially interesting data. In the statistical analysis, the potential bias of individual mares was taken into account by including mare ID as a random effect in the model.

2.2. Monitoring and management

Given that the study is retrospective, the conditions in which the data were obtained may not be identical; for example, due to changes in pharmacological treatments available, or to the approach to treatment. Moreover, the clinical examinations were performed by multiple veterinarians, which could also be a potential source of bias; in this respect, the general operating procedures were consistent among the veterinarians concerned.

2.3. Statistical analysis

Data was analyzed using the statistical analysis program RStudio. Firstly, univariable analysis was performed to evaluate individually the effect of each predictor variable on the outcome. Subsequently, multivariable analysis was performed to develop a model. The incidence of PBIE and pregnancy were the two chosen outcome variables, with the estrous cycle being the statistical unit. For the univariable analysis of qualitative data, the Chi-

squared test was used. Variables with an apparent effect at p<0.20 were included in the second step of the analysis. Since both PBIE and pregnancy diagnosis are binary outcomes (yes/no) and we wished to determine which variables were important predictors of the outcome, logistic regression was conducted as the multivariable analysis test. Mare ID was included as a random effect in the model to account for mares with multiple cycles in the data.

3. Results

3.1. Descriptive analysis

3.1.1. Study population

Table 1: The distribution of mares and cycles over different age groups

	Mares		**Cycles**	
Age group	Brood	Donor	Brood	Donor
≤ 6	169	87	274	216
7-13	240	84	421	186
≥14	182	88	347	301
Subtotal	591	259	1042	703
Total	850		1745	

The final database consisted of 569 broodmares and 255 embryo donor mares. However, because 55 mares were used as both donor mare and broodmare over the course of 2018-2021, the actual total number of mares was 769. The total number of mares depicted in Table 1 is 850. The discrepancy arises because 22 broodmares and 4 donor mares appeared in 2 age groups over the course of 2018-2021. Mean age was 10.4 years (min =2, max = 25, σ = 5.5). The mares were bred with semen from 466 different stallions.

3.1.2. Breeding management

Data recorded per cycle are detailed in Supplementary Table 1.

In 772 of the 1745 estrous cycles recorded (55.8%), luteolysis was induced by administration of a prostaglandin F2α-analog: Cloprostenol (Genestranvet®, 75 μg d-Cloprostenol IM; or Estrumate®, 250 μg IM) or Luprostiol (Prosolvin®, 7.5mg IM). Ovulation was induced in 1469 cycles (84.2%), using either hCG (Chorulon®, 1500 IU IM) or a GnRH-analog: buserelin (Suprefact®, 200 μg IM; or Receptal®, 20 μg IM b.i.d.) or deslorelin (Ovuplant®, 2.1mg implant SC).

Mares were inseminated with fresh semen in 999 cycles (57.3%) and with frozen semen in 745 cycles (42.7%). One mare was inseminated with a combination of fresh and frozen semen in the same cycle and, therefore, not included in further analysis. Mean number of straws used per frozen semen dose was 3.2 (min 1, max 15, σ = 2.5). On average mares were inseminated 1.1 times per cycle (min 1, max 3, σ = 0.3).

After breeding, mares developed PBIE (i.e. > 2cm intrauterine fluid ≥24 h post-AI) in 482 cycles (27.6%). In 87 cycles, a maximal fluid score of 3 (>4 cm IU fluid) was noted (18.0%). The mean number of days mares with PBIE presented with intrauterine fluid before the uterus was considered 'clean' again was 1.9 days (min 1, max 5, σ = 0.7). Mares with evidence of PBIE were left untreated in only 6 cycles (1.2%); in 41 cycles treatment consisted of the administration of an ecbolic drug alone (usually oxytocin: 8.5%) and in 23 cycles the uterus was flushed with Ringer's Lactate Solution (4.8%) without additional use of ecbolics. Mostly, however, treatment comprised of a combination of uterine lavage and the administration of an ecbolic drug (85.5%). Mares presented with post-breeding intrauterine fluid that did not reach the threshold for PBIE (i.e. ≤2cm) in 715 cycles (41.0%); the majority (588) of these mares were treated with an ecbolic drug (82.2%) alone, although on 35 occasions the mare was treated with a combination of lavage and an ecbolic (4.9%). NSAIDs were used to ameliorate inflammation in only 4 cycles (0.2%) and corticosteroids in only 20 cycles (1.1%).

Antibiotics were administered in 132 cycles (7.6%), of which 82 were associated with PBIE (62.1%). Pre-breeding swabs were taken for microbiological and/or cytological examination on 131 occasions, with 47 yielding a probable pathogen after culture (35.9%). *Streptococcus equi zooepidemicus* was the most common pathogen cultured (20 times: 42.6%).

Overall pregnancy rate per cycle for mares inseminated with fresh semen was 58.1%, compared to 39.4% for mares inseminated with frozen semen.

3.2. Univariable and multivariable analysis

3.2.1. Factors influencing the likelihood of PBIE

After univariable analysis, variables associated with PBIE at the cutoff value of $p<0.20$ were: age group, reproductive status, type of semen, mare use, the month of AI, number of inseminations per cycle and cycle number. PBIE was not associated with ovulation induction, straws per dose or induction of luteolysis. P-values of the predictor variables obtained from chi-squared analysis are presented in Supplementary Table 2. After multivariable analysis, 5 variables were significantly associated with PBIE ($p<0.05$): age group, type of semen, reproductive status, mare use and month of AI (Table 2).

Table 2: Variables significantly associated with PBIE

Variable	**P-value**
Age group	5.24×10^{-12}
Type of semen	3.03×10^{-7}
Reproductive status	8.18×10^{-5}
Mare use	0.02
Month of AI	0.04

Mare age was strongly associated with the incidence of PBIE. PBIE was most frequent in the oldest group of mares and least frequent in the youngest group of mares. Mares inseminated with cooled-transported semen were more often affected by PBIE than mares inseminated with frozen semen. The incidence of PBIE was highest in barren mares and lowest in foaling mares. PBIE occurred more frequently in donor mares than broodmares. Mares that were bred in February and March developed PBIE more often than mares inseminated later in the year.

3.2.2. Factors influencing the likelihood of pregnancy

For the analysis of the predictor variables for pregnancy, a subset of the database was used because, for some mares/cycles, the pregnancy outcome after breeding was not known. This subset contained 1488 estrous cycles from 654 mares. After univariable analysis, variables associated with pregnancy at the cutoff value of $p<0.20$ were: type of semen, ovulation induction, estrus induction, inseminations per cycle, reproductive status, mare use, age group and cycle number. Pregnancy outcome was not associated with month of AI, PBIE, days of fluid or straws per dose. P-values of the predictor variables obtained from chi-squared analysis are presented in Supplementary Table 3. After multivariable analysis, variables significantly associated ($p<0.05$) with pregnancy were type of semen, age group, and ovulation induction (Table 3).

Table 3: Variables significantly associated with the likelihood of pregnancy

Variable	**P-value**
Type of semen	2.73×10^{-11}
Age group	6.79×10^{-3}
Ovulation induction	0.02

Insemination with fresh semen was more likely to result in pregnancy than insemination with frozen semen. Pregnancy rates were highest in young mares and lowest in the oldest mare group. Mares in which ovulation was induced were more likely to become pregnant than mares in which ovulation occurred spontaneously.

3.3. Incidence of persistent breeding induced endometritis

3.3.1. Effects of age and breeding system

Mare age was strongly associated with the development of PBIE (Table 4). The Odds Ratio (OR) indicated that middle-aged mares were 4.46 times more likely, and old mares, were 7.81 times more likely to develop PBIE than young mares ($p<0.001$).

Table 4: Incidence of PBIE in the different age groups, including OR and 95% confidence interval (CI)

Age group	**PBIE (per cycle)**	**OR (95% CI)**
≤ 6	11.2% (55/490)	1.00
7-13	25.2% (153/607)	4.46 (2.58, 7.72)
≥14	42.3% (274/648)	7.81 (4.46, 13.68)
Overall	27.6% (482/1745)	

Table 5: Incidence of PBIE in broodmares versus embryo donor mares for the different age groups

Age group	**Broodmare**	**Embryo donor**
≤ 6	13.1% (36/274)	8.8% (19/216)
7-13	19.5% (82/421)	38.2% (71/186)
≥14	33.7% (117/347)	52.2% (157/301)
Subtotal	22.6% (235/1042)	35.1% (247/703)
Total	27.6% (482/1745)	

The effect of mare age on the incidence of PBIE was apparent in both broodmares and ET donor mares, with the lowest incidence in the young mares and highest in the old mares (Table 5). However, there was also a difference in the incidence of PBIE between brood and donor mares. With the exception of the young mares, the likelihood of PBIE was higher in embryo donor mares than in broodmares. The OR indicates that the risk of developing PBIE as a donor mare was 1.58 times greater than for a broodmare ($p<0.05$, Supplementary Table 4). Furthermore, age appears to have a more profound effect on the likelihood of developing PBIE in embryo donor mares, with 8.8% of young donor mares developing PBIE compared to 52.2% of old mares (OR = 16.20, $p<0.001$). This difference was less extreme in broodmares: 13.1% in young versus 33.7% in old broodmares (OR=5.28, $p<0.001$).

Table 6: Number of days intrauterine fluid was present after breeding, divided into mare age categories

Days of fluid	**≤6**	**7-13**	**≥14**
0	38.6% (189/490)	33.7% (204/606)	23.9% (155/648)
1	50.6% (248/490)	50.0% (303/606)	43.7% (283/648)
2	9.6% (47/490)	14.0% (85/606)	19.6% (127/648)
3	1.0% (5/490)	2.0% (12/606)	9.4% (61/648)
4	0.2% (1/490)	0% (0/606)	3.1% (20/648)
5	0% (0/490)	0.3% (2/606)	0.3% (2/648)

The number of days that uterine fluid persists is one of the indicators of the severity of the inflammatory reaction after breeding, and the mare's ability to resolve that uterine inflammation. By definition, a mare resistant to PBIE is able to clear the uterus of neutrophils within 48 hours post-breeding, and of significant amounts of fluid within 24 h. Table 6 presents the number of days that mares required to resolve the BIE. Note that the amount of intrauterine fluid is not considered here, such that mares with fluid present 24 hours after breeding did not necessarily develop PBIE (>2cm fluid). Nevertheless, 89.2% of the young mares and 83.7% of the middle-aged

mares cleared the uterus of all fluid within 48 hours, compared to only 67.6% of the old mares. With respect to breeding method, 84.3% of broodmares and 71.6% of ET donor mares resolved the inflammation within 48 hours. Moreover, the mean number of days fluid was present post-breeding in broodmares was 0.86 days compared to 1.13 in ET donor mares.

Table 7: The distribution of cycle number between broodmares and ET donors

Cycle number	Broodmare	Donor
1	63.4% (657/1037)	36.2% (256/707)
2	24.2% (251/1037)	25.7% (182/707)
3	9.1%(94/1037)	16.3% (115/707)
4	2.1% (22/1037)	11.2% (79/707)
5	1.2% (12/1037)	5.9% (42/707)
6	0.1% (1/1037)	3.4% (24/707)
7	0% (0/0)	1.3% (9/707)

The reasons why a mare may be bred multiple times in a season are different for broodmares and ET donors. Broodmares will only be re-bred if they fail to get pregnant or lose their pregnancy. By contrast, a donor mare may be inseminated again, whether an embryo has been recovered or not, if the aim is to produce multiple foals from the mare. This is reflected in Table 7; only 12.5% of broodmares were inseminated more than twice per season, compared to 38.1% of donor mares. Including the embryo flushes, donor mares are therefore more often exposed to cervical manipulation than broodmares.

When the uterus of an ET donor mare was flushed to recover an embryo on day 8 post ovulation, estrus was almost always induced the same day to help prevent the development of endometritis and, in many cases, to enable re-insemination as soon as possible because multiple embryos were desired in the season. In broodmares, pregnancy diagnosis was generally performed on or before day 16, when the embryo becomes fixed at the base of a uterine horn and the reduction of a twin will be harder to perform. At this stage, mares that are not pregnant will be returning to estrus and it should not be necessary to induce luteolysis. This difference was clear in the current dataset. In 65.1% of all donor mare cycles, estrus was induced, compared to just 30.2% of broodmare cycles.

3.3.2. Effect of semen type

Mares inseminated with fresh semen were 2.56 times more likely (OR) to develop PBIE than mares inseminated with frozen semen ($p<0.001$). Furthermore, the OR for developing PBIE as a donor mare was 1.58 times that of a broodmare ($p<0.05$). Accordingly, the highest incidence of PBIE in our population presents in donor mares inseminated with cooled semen, and the lowest incidence was in broodmares inseminated with frozen semen.

Table 8: The incidence of PBIE per cycle in broodmares and donor mares inseminated with fresh or frozen semen.

	Fresh semen	Frozen semen
Broodmares	29.7% (130/438)	17.1% (64/374)
Donor mares	41.0% (188/458)	23.0% (50/217)
Overall	35.5% (318/896)	19.3% (114/591)

The older mares in this study population were more often inseminated with cooled semen (66.8%) than frozen semen (33.2%). Young mares were also bred slightly more frequently with cooled semen (55.3% versus 44.7%: $p=0.02$) whereas for the middle-aged mares there was no difference ($p=0.52$; Supplementary Table 6). With regard to the breeding system, cooled and frozen semen were used equally in broodmares ($p=0.76$), whereas donor mares were more often inseminated with fresh semen (67.4%; $p<0.001$).

The majority of mares that were inseminated more than once per cycle were inseminated with frozen semen (82.4%: 183/222); this was a deliberate breeding management strategy. Mares were inseminated with some of the straws of a frozen semen AI dose before, and the others after ovulation. With cooled semen, mares were normally inseminated only once per cycle; occasionally they were deliberately inseminated twice, e.g. because semen quality or stallion fertility were known to be low. In most mares inseminated with frozen semen, 4 or fewer straws were used per dose (76%), whereas 8 or more straws per dose were used in only 3% of mares. The number of straws per dose was not related to the incidence of PBIE (p=0.35).

3.3.3. Effect of reproductive status

The correlation between the incidence of PBIE and the reproductive status of the mares was examined by Chi-squared analysis of a subset of the data for which the reproductive status was recorded, containing 226 mares and 423 cycles (Supplementary Table 7). In the young group most mares were maiden (69.9%), in the middle-aged group most mares were foaling (70.6%), and in the old group most mares were barren (67.2%). Most broodmares had a foal at foot (53.1%) whereas the majority of donor mares were barren (50.4%).

Table 9: Per cycle incidence of PBIE in maiden, foaling and barren mares in different age groups

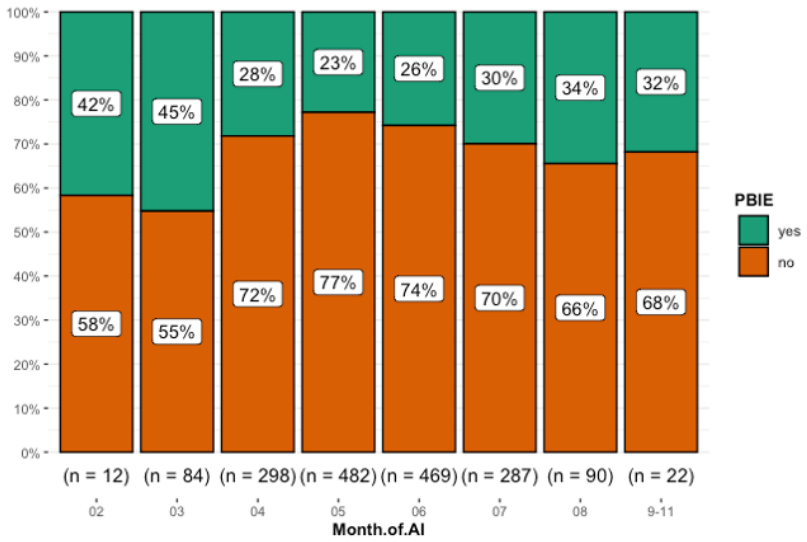
Age group	**Maiden**	**Foaling**	**Barren**
≤ 6	12.8% (11/86)	0% (0/25)	41.7% (5/12)
7-13	20% (1/5)	13.5% (12/89)	42.4% (14/33)
≥14	NA	12.5% (7/56)	49.6% (58/117)
Overall	13.2% (12/91)	11.2% (19/170)	47.5% (77/162)

The incidence of PBIE per cycle in maiden, foaling and barren mares in different age groups is presented in Table 9. Overall, the incidence of PBIE was lowest in foaling mares and highest in barren mares, and only a fraction higher in maiden than foaling mares; this was true for all age categories, although no maiden mares older than 14 years old were present in the data subset. The highest incidence of PBIE was found in barren mares older than 14 years, and the lowest in foaling mares younger than 6 years. However, some groups were too small for meaningful comparison. Nevertheless, reproductive status, age and the incidence of PBIE were clearly inter-related. Compared to foaling mares, barren mares were 6.98 times more likely to develop PBIE ($p<0.001$).

3.3.4. Effect of month of AI

When examining the effect of time of year on the incidence of PBIE, September, October and November were grouped together because of the low number of mares that were inseminated in these months. Although the incidence of PBIE appears to be higher in the earlier and later months of the year, the low numbers of inseminations in February, March and September-November mean that the percentages in those months are less reliable. Taking only the months April to August into account, the incidence of PBIE increased slightly each month from May.

Figure 1: Incidence of PBIE over the breeding season



3.4. Pregnancy rate per cycle

3.4.1. Effect of age

Although univariable analysis suggested little effect of mare age on pregnancy rate per cycle (p= 0.16), multivariable analysis revealed a significant effect (p<0.05: Supplementary Table 5). The difference in pregnancy rates between the young and old mare groups was significant (OR=0.60, p<0.01).

3.4.2. Effect of semen type

Pregnancy rate per cycle for mares inseminated with fresh-cooled semen was 58.1% (521/897) compared to 39.4% (233/591) for mares inseminated with frozen semen. Mares inseminated with cooled semen were 2.81 times more likely to get pregnant than mares inseminated with frozen semen (p<0.001). The number of straws used per frozen AI dose did not affect the likelihood of pregnancy (p=0.70).

3.4.3. Effect of ovulation induction

Ovulation was induced in 84.2% (1469/1745) of all recorded cycles. Of all cycles in which ovulation was induced, 52.4% (658/1255) yielded either an embryo or a pregnant mare; compared to 41.6% (97/233) of cycles in which ovulation was not induced, such that cycles in which ovulation was induced were 1.50 times more likely to result in pregnancy or an embryo. Overall pregnancy rate per cycle in mares treated with Suprefact to induce ovulation was 51.8% compared to 54.3% for mares treated with Chorulon. Ovulation was induced in 87.7% of the mares inseminated with fresh-cooled semen and 79.5% of mares inseminated with frozen semen.

3.5. Effect of treatments to assist uterine clearance

Most mares diagnosed with PBIE received treatment, in the form of either intrauterine lavage or an ecbolic drug but mostly a combination of the two. When uterine fluid was present for 1 day, 87.5% of mares were treated with an ecbolic drug; in mares with >1 day intrauterine fluid, >95% were treated with an ecbolic. Ecbolic administration was maintained in most cases until the uterine fluid was cleared. Lavage was used slightly more conservatively. In 81.3% of all mares with intrauterine fluid for 1 day, uterine lavage was performed whereas for mares with >1 day intrauterine fluid the percentage treated by lavage was >90%. Lavage was generally repeated daily until there was <2cm fluid present, after which ecbolic treatment was continued until the uterus was free of fluid.

Figures 2 and 3 summarize the use of ecbolics and intrauterine lavage, with the number of days that intrauterine fluid was present in mares diagnosed with PBIE on the x-axis and the number of cycles that >2cm of fluid was recorded on the y-axis. The number of days of treatment is indicated by the different colors.

Figure 2: The use of ecbolics in mares with PBIE

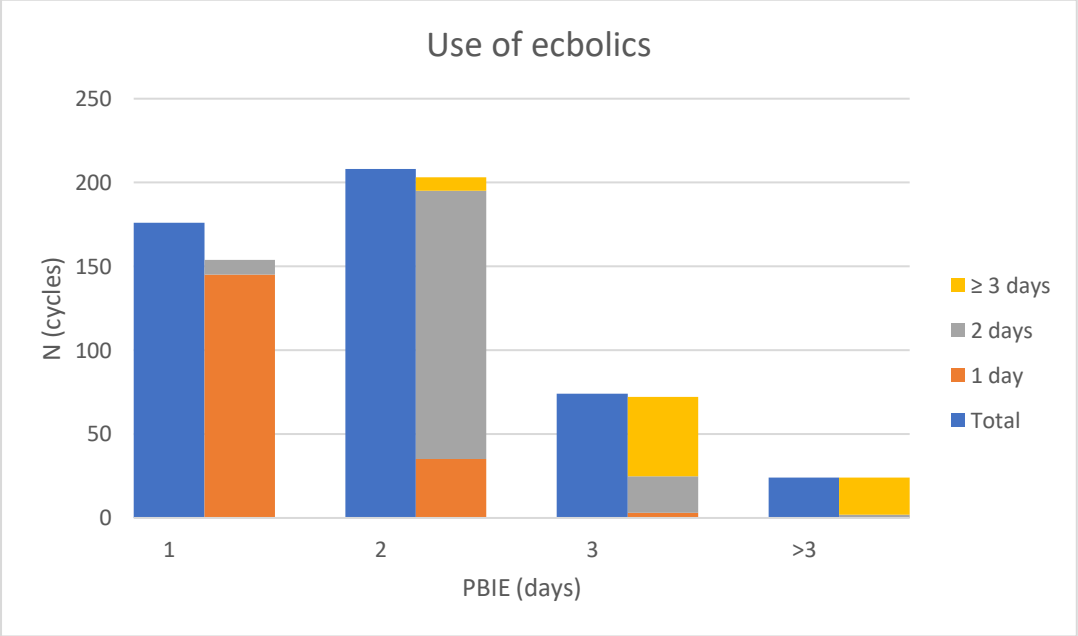
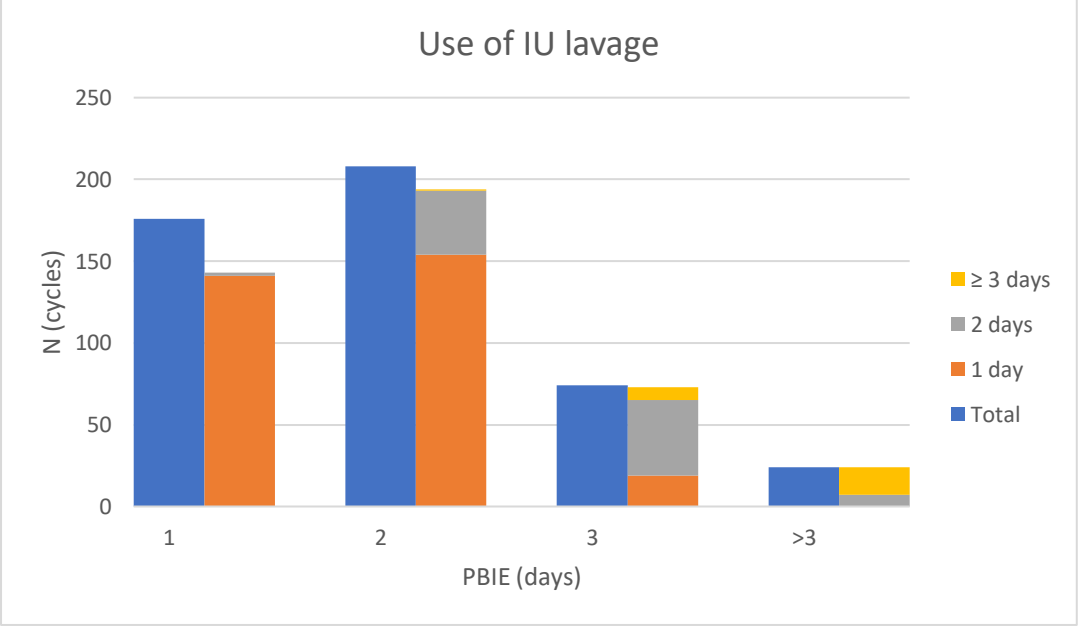


Figure 3: The use of IU lavage in mares with PBIE



The efficacy of the treatment strategy is apparent when the pregnancy rate per cycle in mares diagnosed with PBIE is compared to that in mares without PBIE. It might be expected that the pregnancy rates in mares with PBIE would be much lower. In fact, overall PBIE was not associated with a reduced pregnancy rate (p=0.24), suggesting that the treatments used minimized the impact of PBIE on fertility. However, although PBIE did not influence the pregnancy rate (p=0.29) in mares inseminated with frozen semen, it did have an effect in mares inseminated with fresh semen (p=0.02).

Table 10: Pregnancy rates of mares with and without PBIE, inseminated with fresh or frozen semen; categorized into the different age groups

	≤ 6	**7-13**	**≥14**	**Overall**
Fresh semen				
PBIE	62.1% (18/29)	59.6% (56/94)	48.7% (95/195)	53.1% (169/318)
No PBIE	64.1% (139/217)	63.5% (101/159)	55.4% (112/202)	60.9% (352/578)
Frozen semen				
PBIE	38.1% (8/21)	35.0% (14/40)	34.0% (18/53)	35.1% (40/114)
No PBIE	38.7% (60/155)	43.2% (89/206)	37.9% (44/116)	40.5% (193/477)

4. Discussion

4.1. Persistent breeding-induced endometritis

4.1.1. Incidence

The overall per cycle incidence of PBIE in this study was 27.6%. Barbacini et al. (2003)[6] reported a similar 25.4% incidence of PBIE, although mares in their study were inseminated exclusively with frozen-thawed semen. In the current study, the incidence of PBIE was significantly higher in mares inseminated with fresh-cooled semen, whereas previous studies reported similar incidences of PBIE in mares inseminated with cooled and frozen semen.[7] Recently, Derisoud et al. (2022)[8] investigated factors affecting post-breeding endometritis, pregnancy rate and embryonic/fetal death in sport horse mares in two French commercial stud farms that used fresh, cooled and frozen semen and reported a PBIE incidence of 35.6%.

4.1.2. Effect of age (and parity)

In the studied population, PBIE was most frequent in the oldest mare group and least frequent in the youngest group. The overall incidence of PBIE per cycle was 11.2% in mares ≤6 years old, 25.2% in mares 7-13 years old and 42.3% in mares ≥14 years old. Barbacini et al. (2003) reported an incidence of PBIE of 17.0% in mares aged 3-9 years, 28.2% in mares aged 10-16 years and 67.8% in mares aged >16 years. In our study, the old mare group was significantly smaller than the other groups which could have contributed to the apparently high incidence of PBIE in the old mares. Moreover, mares were examined using ultrasonography as early as 16-18 h post insemination, it may be particularly challenging for the susceptible older mare to clear the uterus of fluid this quickly. Derisoud et al. (2022)[8] reported incidences of PBIE of 8.0% in mares younger than 6 years old, 30.2% in mares 6-15 years old and 31.5% in mares older than 15 years. Although the age categories are not identical, the incidence of PBIE in this study was thus similar to those reported previously.

Age and parity are important factors that can influence the uterine defense mechanisms in various ways, primarily affecting mechanical clearance. Age is associated with degenerative changes in the endometrium collectively known as endometrosis.[9] Indeed, endometrosis and PBIE may be linked. When PBIE recurs in susceptible mares, it can exacerbate endometrosis. As discussed previously, in response to insemination, inflammatory cells such as PMNs enter the uterus and produce pro-inflammatory cytokines. However, profibrotic cytokines are also secreted such that, if inflammation persists, fibroblasts will be activated and differentiate into myofibroblasts, leading to fibrogenesis. Over a prolonged period of time, this process could result in extensive deposition of extracellular matrix proteins and fibrosis within the endometrium. The process triggers a vicious circle because perfusion disorders predispose to further progression of the endometrosis.[4] Insufficient blood supply to the myometrium with impaired uterine contractility as a consequence could explain why older mares become susceptible to PBIE. Fibrosis of the endometrial wall is related to impaired lymph circulation and impaired removal of fluid from the uterus.[10] Moreover, angiopathies causing a decreased perfusion can also affect endometrial edema and uterine clearance.[11]

The foaling histories of the mares in this study were not complete and, therefore, parity was not included as a variable in the data. In any case, it is difficult to evaluate the effect of age and parity separately, because increased parity is generally associated with increased age. Ricketts & Alonso (1991)[12] found that older maiden mares exhibited endometrial changes typical of endometrosis, and concluded that age alone is the primary factor underlying the development of endometrosis. More recently, Esteller-Vico et al. (2012)[11] found that vascular degeneration is present throughout the uterine wall in multiparous mares. Myometrial vessels are affected, as well as larger arteries and veins between the circular and longitudinal myometrial layers. Parity was significantly related with this degeneration, but age was not. In short, the increase in the incidence of PBIE with mare age is probably a result of degenerative changes within the uterine wall that compromise uterine clearance, combined with conformational abnormalities of the caudal reproductive tract, which will be elaborated on later.

4.1.3. Effect of reproductive status

Barbacini et al. (2003)[6] conducted a retrospective study on the incidence of post-insemination uterine fluid in mares inseminated with frozen-thawed semen. Mares were divided into groups based on age and reproductive status. As in the current study, barren mares (38.3%) had a higher incidence of post-breeding fluid accumulation (>2cm) than maiden (19.7%) and foaling mares (17.8%). One of the factors that may affect uterine clearance is the endogenous production and release of oxytocin into the circulation, which can be stimulated in multiple ways including contact with a stallion, nursing, and vaginal stimulation.[13] Nursing a foal should stimulate regular oxytocin release because, during the first month after birth, suckling periods last around 20 min and foals suckle at hourly intervals.[14] Sharma (1974)[15] reported plasma oxytocin concentration peaks of 10 mIU/L, with concentrations of 7-10 mIU/L present 20 minutes after the start of a nursing period. Although Vivrette (1998)[16] reported that nursing was not always related to oxytocin release in postpartum mares, oxytocin would be expected to stimulate uterine contractions and promote uterine drainage and, therefore, nursing could help to prevent the development of PBIE in foaling mares. Maiden mares are mostly young mares with a normal reproductive tract that hasn't been exposed to possible trauma via parturition. So with the exception of age-related changes in the old maiden mare mentioned above or congenital anomalies, maiden mares are generally resistant to PBIE.

In the subpopulation of mares for which the reproductive status was recorded, 72.2% of barren mares were ≥14 years old. Nevertheless, even after accounting for other variables, reproductive status was significantly related ($p<0.001$) to the incidence of PBIE, meaning that being barren was associated with an increased risk of developing PBIE. In the current study, no account was taken of how many years the mares had been barren. When Ricketts & Alonso (1991)[12] studied the effect of age and parity on the development of equine chronic degenerative endometrial disease (CDE), mares with moderate and severe histopathological abnormalities were found to have been barren for significantly more years than the population mean ($P<0.0001$). More recently, de Witt et al. (2022)[17] studied the association between endometrial biopsy grade and reproductive variables. Although their hypothesis was that age and barren status would both be associated with an increased (i.e. worse) endometrial biopsy grade, they found that only age showed this association. Nevertheless, endometrial biopsy score is indicative of susceptibility to PBIE, and mares with severe degenerative endometritis are more likely to suffer uterine fluid retention and reduced ability to clear uterine inflammation.[18]

4.1.4. Effect of mare use

The per cycle incidence of PBIE was significantly higher in ET donor mares (35.1%) than in broodmares (22.6%). Broodmares risk parturition induced-trauma to the perineum, vulva, vestibular-vaginal sphincter, vagina, cervix and uterus. Furthermore, stretching of the broad ligaments in older multiparous mares can result in a pendulous uterus that contributes to the inability to physically clear inflammatory fluid. As mentioned previously, repeated foaling can exacerbate endometrial degenerative changes, such as periglandular fibrosis.[19] In contrast to broodmares, donor mares are exposed to repeated inseminations and embryo collections. Cervical fibrosis is reported to result from these repeated cervical manipulations. The fibrotic cervix is narrow and elongated and fails to relax adequately, predisposing to delayed uterine clearance.[20] In addition, Carnevale et al. (2005)[21]

demonstrated that a higher number of uterine manipulations was associated with increased chronic inflammation within the endometrium, as determined via biopsies. It has also been suggested that the fact that donor mares don't give birth is detrimental to the ability of the cervix to dilate, making them increasingly prone to PBIE.[22] This could explain why, in the current study, the relationship between age and PBIE was stronger in embryo donor mares than in broodmares. It would be interesting to study the difference in the incidence of PBIE between mares that are allowed to carry a foal to term occasionally and mares that aren't. In conclusion, using a mare as a broodmare carries different risks to use as an embryo donor, but both include factors that can increase the chance of developing PBIE, e.g. foaling-induced trauma for broodmares or damage due to repeated cervical and uterine manipulation in ET donors. Clearly, it's not a strict division since a mare could be used as a broodmare one season and an embryo donor the next. In the current study, the incidence of PBIE was significantly higher in ET donor mares, suggesting that repeated transcervical manipulations and absence of parturition-induced cervical dilation are important predisposing factors for PBIE.

4.1.5. Effect of semen type

In this study, the type of semen used was significantly related to the incidence of PBIE ($p<0.0001$), with an overall incidence of PBIE in mares inseminated with fresh semen of 35,5% per cycle compared to 19,2% in mares inseminated with frozen semen. Mares inseminated with fresh semen were 2.56 times more likely to develop PBIE than mares inseminated with frozen semen. In general, it is assumed that the inflammatory reaction in mares bred with frozen semen should be more severe than after breeding with fresh semen, even though there are very few controlled studies on this topic. Kotilainen et al. (1994)[23] found that insemination with frozen semen resulted in higher neutrophil concentrations than insemination with fresh extended semen or after natural breeding. Nevertheless, the second highest neutrophil concentration was found in the mares inseminated with concentrated fresh semen, and the authors concluded that small volumes of highly concentrated semen, whether fresh or frozen, provoke the greatest inflammatory reaction.

The volume of the inseminate does not appear to affect the inflammatory response of the uterus since no significant difference in PMN numbers was observed 4 hours post AI in mares inseminated with 2 mL versus 100 mL of extended frozen semen, containing similar sperm numbers.[24] In the current study, inseminating a mare with frozen semen generally involved introducing a lower volume of semen with a higher concentration of spermatozoa. For fresh semen, doses in the Netherlands are typically 15 mL, whereas the volume of frozen semen depends on the number of straws that are used; in our study, most frozen semen AIs used only one straw, i.e. 0.5mL (32.8%). The World Breeding Federation of Sport Horses (WBFSH) recommends AI doses of 600×10^6 progressively motile sperm (pms) for cooled-transported semen and 250×10^6 pms post-thaw for cryopreserved semen. While these numbers are generally adhered to for cooled semen, yielding a concentration of approximately 40×10^6 pms per ml, frozen semen is increasingly used on a per straw basis. As a result, although the concentration of spermatozoa in frozen semen is generally much higher ($200\text{-}400 \times 10^6$ per ml) than in cooled semen, the sperm number per AI is often much lower ($50\text{-}100 \times 10^6$ pms).

In this respect, although spermatozoa are eliminated rapidly from the uterus and a only relatively small proportion reach the tip of the uterine horn, deep-horn insemination allows a greater percentage of the motile spermatozoa to reach the oviduct, such that fewer spermatozoa are necessary to achieve acceptable pregnancy rates than with conventional insemination.[25] Although very dependent on the fertility of the stallion, acceptable pregnancy rates can be achieved using deep horn insemination of 1 straw of frozen-thawed semen in doses $<60 \times 10^6$ pms.[26,27] On the other hand, it has been suggested that introducing the AI pipette into the tip of the horn could irritate the uterus and therefore increase inflammation. Güvenc et al. (2005)[28] studied the effect of insemination dose (20×10^6 versus 200×10^6 spermatozoa/straw) and site (tip of the uterine horn or uterine body) on the uterine inflammatory response in mares. They reported no significant difference in intrauterine fluid accumulation between mares inseminated with the high dose in the uterine horn versus the body, suggesting that advancing the pipette deeper into the uterus does not lead to more intense inflammation. In fact, mares inseminated with the low dose in the uterine horn had significantly less intrauterine fluid than the

other groups, suggesting that low sperm numbers inseminated into the tip of the horn results in less fluid accumulation. That deep horn AI of low spermatozoa doses was the routine for frozen semen AI in the current study, presumably explains the lower incidence of PBIE than for cooled semen.

Finally, seminal plasma has an immunomodulatory effect on the endometrial reaction to spermatozoa, which is elicited by specific seminal plasma proteins. However, seminal plasma is removed prior to cryopreservation because of the negative effects on post-thaw sperm viability.[29,30] *In vitro*, seminal plasma increases the mRNA expression of pro-inflammatory cytokines IL-8 and IL-1β in the endometrium whilst downregulating TNF, hereby suppressing complement activation, PMN chemotaxis and phagocytosis.[31,32] *In vivo* the presence of seminal plasma was found to shorten the duration of breeding-induced inflammation, although the initial influx of PMNs (2-12 h post AI) was stronger.[33] Although it would therefore be anticipated that frozen-thawed semen would have less seminal plasma to dampen the inflammatory response, it is standard practice in the Netherlands to remove the bulk of the seminal plasma from cooled semen such that the possible advantage would be minimal.

Overall, while insemination with frozen semen is often assumed to provoke a more severe inflammatory reaction in the mare's uterus than insemination with fresh semen, multiple studies have reported similar post-breeding inflammation with the two types of semen.[7,8] In the current study, the incidence of PBIE was higher in mares inseminated with fresh semen, presumably because of the greater spermatozoa numbers present in an AI dose.

4.1.6. Effect of time of year

Derisoud et al. (2022)[8] reported that month of insemination was significantly related to post-breeding endometritis ($p<0.01$). They recorded the lowest incidence of PBIE in April with the incidence subsequently increasing monthly to reach a peak in July-August. The results of the current study are comparable in the sense that the incidence increased gradually after relatively low levels early in the breeding season. Derisoud et al. explained the peak incidence of PBIE in July-August in 2 ways. Firstly, resistant broodmares would have become pregnant in the first or second cycle, leaving a higher proportion of susceptible mares among those inseminated later in the season. However, mares bred later in the season will also include a higher proportion of foaling mares, which may explain why the incidence of PBIE in broodmares in the months June, July and August was consistent (23%, 24% and 22%) in the current study. This is supported by a marked increase in the incidence of PBIE in embryo donor mares in the months June, July and August (30%, 38% and 49%). The seasonal increase in the incidence of PBIE was therefore primarily within the donor mares. Secondly, increased environmental temperatures in late spring and summer have been hypothesized to predispose to an increased incidence of PBIE.[8] Although there is no evidence for this hypothesis in mares, heat stress in dairy cows that caused the rectal temperature to rise by almost one degree has been shown to reduce uterine blood flow, which could reduce resistance to inflammation.[34,35] Neither hypothesis explains why the highest incidence of PBIE in our study was observed at the beginning of breeding season, although this could relate to a population of predominantly barren and ET donor mares early in the season.

4.2 Pregnancy rate per cycle

4.2.1. Effect of semen type

Jasko et al. (1992)[36] reported that the fertility of cooled semen is typically greater than that of frozen semen. In general, pregnancy rate per cycle is 5-10% lower for frozen-thawed compared to cooled semen.[37] However, Squires et al. (2006)[38], Loomis (2001)[39] and Lewis et al. (2015)[40] reported similar pregnancy rates for cooled and frozen semen. In the current study, pregnancy rate per cycle for cooled-transported semen was 58,1% compared to 39,4% for frozen semen. Sieme et al. (2003)[41] reported very similar pregnancy rates, namely 55.7% in mares inseminated once with cooled semen between 24 h before and 12 h after ovulation, and 41.3% in mares inseminated with frozen semen between 12 h before and 12 h after ovulation. The success of breeding with frozen semen is dependent on multiple factors including stallion fertility, semen quality, mare status, management, and appropriate semen handling. The recorded pregnancy rate per cycle for frozen semen in this

study is perhaps on the low side compared to other studies in a commercial setting that reported 45.0% and 48.6%.[40,42] However, the fertility of 2 individual stallions was known to be variable or poor but was used in 132 cycles, i.e. 22,5% of all frozen semen cycles, eventually yielding a pregnancy rate of 34,9%.

4.2.2. Effect of age

In the study population for which pregnancy diagnosis was known, pregnancy rate per cycle was similar for mares ≤ 6 years old (53.3%) and mares 7-13 years old (52.1%) whereas for mares ≥14 years old it was lower (47.6%). The effect of age on mare fertility has been studied extensively. Sharma et al. (2010)[43] divided commercial mares into 4 age groups (3-7, 8-12, 13-17 and ≥18 years old) and reported that the day 16 pregnancy rate was negatively correlated with mare age (54.08%, 51.93%, 45.21%, 35.90%) with the difference reaching significance for mares of 3-7 and 8-12 years compared to mares ≥18 years old (P<0.008). Similarly, Morris & Allen (2002)[44] found the day 15 pregnancy rate of naturally mated mares of 14-18 (51.4%) and >18 years old (50.0%) to be significantly lower than for mares of 3-8 (62.7%) and 9-13 years (61.2%). In Morris & Allen's study, post-AI treatments were conducted in 12.24% of all mated cycles, which is significantly less than in the current study. However, their study was performed on naturally mated broodmares, where mares ≥14 years accounted for only 20.1% of the total cycles. The age difference and absence of ET donor mares presumably explains the reduced need for post-mating treatment.

4.2.3. Effect of ovulation induction

The follicular phase in mares is relatively long with a reported mean of 7.6 days, and a range of 2-14 days.[45] The preovulatory follicle the day before ovulation is also variable in size, ranging from 31 to 59 mm.[46] This makes it difficult to determine the best moment to inseminate. Frozen semen remains viable in the mare's reproductive tract for approximately 12 hours and is ideally inseminated between 12 h before and up to around 8 hours post ovulation. Cooled semen is ideally inseminated between 24h before and up to 6 h after ovulation.[47] In order to overcome the unpredictability of when to inseminate and achieve optimal pregnancy rates, ovulation-inducing drugs are commonly used. In the current study, pregnancy rate per cycle was improved by the induction of ovulation. By contrast, neither Sharma et al. (2010)[43] nor Morris & Allen (2002)[44] found an effect of induction of ovulation on the pregnancy rate.

Suprefact (1153 cycles) was the most frequently used induction agent in the current study, followed by Chorulon (299 cycles). Ovulation typically occurs 36 to 42 hours after IM administration of 1500 IU hCG in estrous mares.[48] However, mares are thought to develop antibodies after repeated hCG administration, which reduces the efficacy of hCG in some.[49] Buserelin is a GnRH agonist that induces the release of luteinizing hormone from the adenohypophysis and has the advantage over hCG of not losing efficacy after repeated administration. Accordingly, Newcombe & Cuervo-Arango (2017)[50] found that in barren and older mares the ovulation response to hCG was significantly lower than that to buserelin. Indeed, multiple studies have reported the efficacy of hCG to be reduced in older mares.[51,52,53] In the Netherlands, hCG is registered for use in horses as Chorulon® while buserelin is registered for horses as Receptal®, Veterelin®, Busol® and Fertigest®. Suprefact (1 mg/mL buserelin) is registered only for human medicine, and its use in horses is off-label under the cascade. However, the low concentration of buserelin (4.2 μg/mL) in the registered products means that they are only effective if administered repeatedly (12 h intervals), which is also off-label. Moreover, studies have shown that at least 200 μg of buserelin is required to effectively induce ovulation in mares with a single treatment, which would mean an impractical 50 mL of the registered buserelin products would be required.[54]

In this study, pregnancy rates per cycle were similar for mares treated with Suprefact and Chorulon. However, the mean age of mares in which ovulation was induced with hCG was 7.2 years and the mean age of mares in which ovulation was induced using buserelin was 12.1. To justify the off-label use of buserelin instead of using hCG, mares were selected for use of Suprefact on the basis of age (>12 years old), previous failure to react to hCG, or use at repeated cycles.

4.3 Effect of treatment

Overall, the pregnancy rate per cycle in mares with PBIE differed little to that in mares with no PBIE (p=0.24). However, in mares bred with cooled semen, PBIE was associated with a reduced pregnancy rate (p=0.02). In this respect, the incidence of PBIE and the mean number of days that intrauterine fluid was present after breeding, were both higher in mares inseminated with cooled semen. Similarly, Barbacini et al. (2003)[6] found that the pregnancy rate after breeding with frozen-thawed semen was lower in mares with uterine fluid (45.2%) than in mares without uterine fluid (50.8%). Their treatment strategy was similar in that mares with less than 20 mm of intrauterine fluid were treated with oxytocin, and mares with more than 20 mm were treated with oxytocin and uterine lavage.

Karam et al. (2021)[55] compared the effects of 6 different post-breeding treatment regimes in Arabian mares divided into 3 age groups (5-10, 11-15, and ≥16 years old); the treatments compared were; intrauterine antibiotics (1 g gentamicin), uterine lavage (normal saline 500 mL), IM oxytocin (10 IU), intrauterine antibiotics and IM oxytocin, uterine lavage and IM oxytocin, and uterine lavage with IU antibiotics and IM oxytocin. Foaling rates were higher in mares treated with oxytocin in the age categories 5-10 and 11-15 years, but not in the ≥16 year old group. The authors suggested that multiparous older mares respond less well to oxytocin than younger mares. However, the highest pregnancy rates in all age groups were found in mares treated with oxytocin combined with uterine lavage, which was concluded to be the best treatment strategy.

Overall, it appears that the negative effect of PBIE on fertility can be minimized by treating susceptible mares with oxytocin and/or uterine lavage after breeding. Although recorded, corticosteroids and NSAIDs were used too infrequently in the current population to evaluate their effects on PBIE. With regard to the use of antibiotics, it would be more useful to analyze the antibiotic-treated cycles as a separate data set to examine the rationale and effect of the treatment.

5. Conclusion

Older mares are more susceptible to developing PBIE than young mares. This is probably a result of a combination of caudal reproductive tract conformational abnormalities and degenerative changes within the uterine wall that compromise uterine clearance. Using a mare as a broodmare as opposed to as an embryo donor also reduces the likelihood of developing PBIE. This presumably relates to differences in predispositions to PBIE. Although some predispositions apply to both groups, for example age, other risk factors specifically affect one group, e.g. foaling-induced trauma for broodmares or incremental damage due to repeated cervical or uterine manipulation in ET donors. In this study, the incidence of PBIE was significantly higher in ET donor mares, suggesting that repeated cervical and uterine manipulations and the absence of parturition-induced cervical dilation are the most important factors predisposing to PBIE. Fortunately, the consequences of PBIE on fertility can be minimized by treating affected mares with oxytocin and/or uterine lavage after breeding. Other factors significantly affecting the likelihood of PBIE are semen type, reproductive status and month of AI. PBIE was more common in mares inseminated with fresh-cooled rather than with frozen-thawed semen, presumably because of the greater sperm numbers present in an AI dose. Barren mares were more likely to develop PBIE than foaling mares, and in some cases this may explain why they failed to get pregnant the previous year.

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Supplementary material

Supplementary Table 1: Characteristics of the 1745 recorded cycles

Variable	Cycles	Percentage
Cycle number (per year)		
1	914	52.4
2	433	24.8
3	209	12.0
4	101	5.8
5	54	3.1
6	25	1.4
7	9	0.5
Induction of luteolysis		
No	973	55.8
Yes	772	44.2
Induction of ovulation		
No	276	15.8
Yes	1469	84.2
Type of semen (n=1744)		
Fresh	999	57.3
Frozen	745	42.7
Number of straws		
≤4	566	76.0
5-8	157	21.1
>8	22	2.9
Inseminations per cycle		
1	1522	87.2
>1	223	12.8
Month of AI		
February	12	0.7
March	84	4.8
April	299	17.1
May	482	27.6
June	469	26.9
July	287	16.4
August	90	5.2
September-November	22	1.3
PBIE		
No	1263	72.4
Yes	482	27.6
Treatment (n=482)		
None	6	1.2
Ecbolic drug	41	8.5
Intrauterine lavage	23	4.8
Ecbolic drug + lavage	412	85.5
Additional treatments		
NSAID	4	0.2
Corticosteroid	20	1.1
Antibiotic	132	7.6

Swabs, bacteriological		
Taken	131	7.5
Negative	46	35.1
Positive	47	35.9
Mixed culture	28	21.4
Unknown	10	7.6
Pregnancy diagnosis = 1		
Fresh (n=897)	521	58.1
Frozen (n=591)	233	39.4

Supplementary Table 2: P-values of the predictor variables for the incidence of PBIE, obtained by chi-squared analysis

Variable	**P-value**
Age group	$1{,}58 \times 10^{-30}$
Reproductive status	$6{,}59 \times 10^{-15}$
Type of semen	$4{,}43 \times 10^{-13}$
Mare use	$7{,}24 \times 10^{-9}$
Month of AI	$1{,}30 \times 10^{-3}$
Inseminations per cycle	0,10
Cycle number	0,11
Ovulation induction	0,20
Estrus induction	0,25
Straws per dose	0,35

Supplementary Table 3: P-values of the predictor variables for the likelihood of pregnancy, obtained from chi-squared analysis

Variable	**P-value**
Type of semen	$1{,}38 \times 10^{-12}$
Ovulation induction	$2{,}46 \times 10^{-3}$
Estrus induction	$5{,}61 \times 10^{-3}$
Inseminations per cycle	0,01
Reproductive status	0,10
Mare use	0,13
Age group	0,16
Cycle number	0,18
Month of AI	0,20
PBIE	0,24
Fluid days	0,25
Straws per dose	0,85

Supplementary Table 4: Odds ratio including 95% confidence interval for factors influencing the likelihood of developing PBIE

Variable	OR	95% CI	P
Age group, ≤ 6 yo vs;			<0.001
7-13 yo	4.46	2.58, 7.72	
≥14 yo	7.81	4.46, 13.68	
Mare use, broodmare vs donor	1.58	1.07, 2.34	<0.05
Type of semen, fresh vs frozen	0.39	0.27, 0.56	<0.001
Inseminations, <1 vs >1	1.42	0.87, 2.31	0.16
Cycle number, 1 vs;			0.83
2	1.03	0.73, 1.47	
3	0.82	0.51, 1.34	
4	1.25	0.66, 2.36	
5	0.80	0.34, 1.90	
6	1.31	0.39, 4.34	
7	0.51	0.08, 3.36	
Reproductive status, barren vs;			<0.001
Foaling	0.14	0.06, 0.34	
Maiden	0.70	0.24, 2.06	
Month of AI, February vs;			<0.05
March	1.28	0.24, 6.70	
April	0.46	0.09, 2.36	
May	0.34	0.07, 1.75	
June	0.40	0.08, 2.07	
July	0.45	0.08, 2.40	
August	0.53	0.09, 3.09	
September-November	0.48	0.06, 3.90	

Supplementary Table 5: Odds ratio including 95% confidence interval for factors influencing the likelihood of pregnancy

Variable	OR	IC 95%	P
Age group, ≤ 6 yo vs;			<0.01
7-13 yo	0.97	0.68, 1.38	
≥14 yo	0.60	0.42, 0.86	
Mare use, broodmare vs donor	0.89	0.65, 1.20	0.43
Type of semen, fresh vs frozen	0.36	0.26, 0.48	<0.001
Inseminations, <1 vs >1	0.97	0.65, 1.45	0.88
Cycle number, 1 vs;			0.21
2	0.95	0.71, 1.29	
3	1.21	0.80. 1.82	
4	1.30	0.75, 2.23	
5	1.33	0.66, 2.68	
6	3.54	1.16, 10.76	
7	4.45	0.77, 25.76	
Ovulation induction, no vs yes	1.50	1.07, 2.10	<0.05
Luteolysis induction, no vs yes	1.17	0.89, 1.52	0.25
Month of AI, February vs;			0.12

March	0.83	0.20, 3.40	
April	0.51	0.13, 2.00	
May	0.83	0.21, 3.40	
June	0.61	0.16, 2.41	
July	0.55	0.14, 2.22	
August	0.82	0.19, 3.50	
September-November	0.37	0.06, 2.26	

Supplementary Table 6: Type of semen used for insemination, divided into mare age categories

Age group	***Fresh***	***Frozen***
≤ 6	*55,3% (271/490)*	*44,7% (219/490)*
7-13	*48,7% (295/606)*	*51,3% (311/606)*
≥14	*66,8% (433/648)*	*33,2% (215/648)*

Supplementary Table 7: Distribution of mares and cycles over the different mare status groups

Reproductive	**Mares**	**Cycles**
Maiden	50	91
Foaling	101	170
Barren	75	162