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Effects of repeated embryo flushing without $PGF_{2\alpha}$ administration on luteal function, percentage of unwanted pregnancy and subsequent fertility in mares

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Abstract

Background: $PGF_{2\alpha}$ is commonly given at the end of embryo flushing (EF) to shorten the interval to the next oestrus and ovulation.

Objectives: To determine the effect of repeated EF on plasma progesterone concentration, percentage of mares with endometritis, unwanted pregnancy and subsequent fertility in mares flushed without the use of $PGF_{2\alpha}$.

Study design: Controlled experiments.

Methods: Nine mares were inseminated in seven consecutive cycles ($n = 63$), to either perform an EF ($n = 54$) 7–9 days after ovulation or left pregnant ($n = 9$). $PGF_{2\alpha}$ was not used to induce oestrus. Ultrasound examination and blood sampling were performed just before the EF and 72 h later to determine changes in progesterone concentration and signs of endometritis.

Results: The overall percentage of positive EF/pregnancy was 55.5% (30/54) and 66.7% (6/9), respectively. The likelihood of pregnancy/positive EF in the first three cycles was 55.5% (15/29). This was not different ($p > 0.1$) from the fertility of the last four cycles (69.4%, 25/36). In five EF cycles (9.3%), mares had signs of endometritis and early luteolysis (progesterone <2 ng/mL) 72 h after EF. The reduction in progesterone concentration by 72 h after EF was greater ($p < 0.05$) for Day 9 (−2.3 ± 0.7 ng/mL) than Day 7 (−1.0 ± 0.8 ng/mL) or Day 8 (−1.3 ± 1.1 ng/mL) cycles. The progesterone concentration in non-flushed mares did not vary significantly during the sampled period (Day 7–12). There were 5 cycles in which the donor mare remained pregnant after the EF, although four were from a single mare.

Main limitations: The mare population was limited to barren and maiden mares. The cycle order and operator allocation to each EF were not randomised.

Conclusions: EF induces a subtle, but significant reduction in progesterone concentrations compared with non-EF cycles. However, the percentage of mares with EF-induced full luteolysis is low (9.3%). The fertility of mares after repeated EF without administration of $PGF_{2\alpha}$ was unaffected; however, there is a considerable risk of

unwanted pregnancy (5/27 = 18.5%) in donors from which an embryo was not recovered.

KEYWORDS
embryo flushing, horse, luteolysis, progesterone, unwanted pregnancy

1 | INTRODUCTION

Embryo flushing (EF), recovery and transfer of in vivo derived embryos is a common assisted reproductive technique performed worldwide in the mare.[1–3] Most veterinary surgeons administer a luteolytic drug, prostaglandin $F_{2\alpha}$ (PGF), at the end of EF to induce oestrus.[4–6] The proposed advantages of inducing oestrus by PGF after EF are the following: (1) to shorten the interval to the next oestrus and ovulation, so more EF can be performed in one breeding season[6]; (2) to avoid unwanted pregnancy in case the embryo is left in the uterus or oviducts[4–6]; and (3) to decrease the risk of endometritis.[4] While the first reason is obvious, the likelihood of donor mares developing endometritis or becoming pregnant after EF without the concurrent administration of PGF is unknown. Furthermore, the factors that influence these possible outcomes have not been investigated. Possible explanations of unwanted pregnancy may be failure of complete distension of endometrial folds with flushing media, poor uterine massage technique, failure to recover all flushing media and flushing before the embryo has entered the uterus.

There may be some occasions on which the veterinary surgeon may choose not to give PGF after the EF. It is possible that the mare's owner asks that their mare does not receive PGF for several reasons; furthermore, some mares may present a large pre-ovulatory follicle (i.e., >35 mm in diameter) at the time of EF.[7] The fate of these large dioestrous follicles is difficult to predict,[8] as they can either ovulate rapidly or regress following PGF administration, resulting in longer intervals to subsequent ovulation.[7–10] This uncertainty might be challenging for the practitioner when facing the decision of when to order semen for the next oestrus. This is even harder if there is a weekend soon after the EF. In addition, the embryo recovery of mares bred and ovulating within 5 days of the previous EF (plus PGF administration) has been reported to be reduced.[7,9]

It has been shown that manipulation and dilation of the cervix during dioestrus may result in release of endogenous oxytocin but not PGF (measured via its circulating metabolite; PGFM), resulting in an earlier decrease in progesterone concentration and shortening of the cycle.[10] Conversely, other authors have proposed no reduction of cycle length following EF, despite release of PGF[11] and/or cervical dilation alone.[12,13] Furthermore, a recent study did not find any significant decrease in progesterone concentration 48 h after EF in 9 mares flushed during 17 cycles 8–9 days after ovulation.[14] The discrepancy in the results amongst studies may be related to differences in the degree and time of cervical dilation and/or accidental occurrence of bacterial contamination of the uterus during EF which may induce a more rapid luteolysis in infected mares.[14]

The objective of this study was to determine the effect of repeated EF on plasma progesterone concentration, percentage of mares with endometritis, incidence of unwanted pregnancy and subsequent fertility in mares flushed without the use of PGF. The main hypothesis was that the EF procedure would not induce full luteolysis. The second hypothesis was that repeated EF without the administration of PGF during a breeding season would not affect mare fertility.

2 | MATERIALS AND METHODS

2.1 | Animals

Nine non-lactating mares of different breeds (Spanish purebred, Belgian draught, crossbreds), from a total research herd of 11 mares, aged 5 to 20 years old (mean 13.0 ± 1.6), barren ($n = 5$) and maiden ($n = 4$) and weighing 420 to 670 kg were used in the study. Mares belonged to the research herd of the Veterinary School, Universidad CEU Cardenal Herrera. The inclusion criteria for the animals in the study were: any mare belonging to the research herd that had produced a pregnancy in the previous 2 years during routine breeding procedures, and was therefore considered capable of conceiving ($n = 9$). All mares with a history of infertility or chronic clinical endometritis due to cervical problems ($n = 2$) were excluded from the study. One mare (ID 5) had only one ovary. The reason for this is unknown, because there was no reproductive history available when the mare was purchased, other than she had at least one foal (she was recently weaned). Mares were kept in sand paddocks in groups of 3 to 4 animals and were fed on hay and cereal concentrate three times a day, with ad libitum access to water and mineralised salts. All mares were cycling at the beginning of the study. Furthermore, a Spanish purebred stallion aged 12 years old of proven fertility was used as semen donor for breeding.

2.2 | Experimental design

The study was carried out during the 2022 breeding season in Valencia, Spain (Northern Hemisphere) and took 6 months. Each mare was followed and bred to the same stallion during 7 cycles ($9 \times 7 = 63$ cycles in total): six cycles for EF (EF; $9 \times 6 = 54$) and one cycle to carry the pregnancy until the first pregnancy diagnosis 12–15 days after ovulation (non-EF cycles, $9 \times 1 = 9$). For each mare, the allocation of cycle order to the type of breeding technique (non-EF or EF) was chosen by assigning the mare ID number (1 to 9) in

decreasing alphabetical order to the name of the mare (i.e., ID number 1 to the mare name starting with an 'a', ID number 9 to the mare name starting with a 'z'). Thus, for mare 1, the first cycle was a non-EF; for mare 2, the second cycle was non-EF, and so on (Table 1). Whether each EF was performed on Day 7, 8 or 9 after ovulation was decided depending on the operators' availability and to avoid weekends and bank holidays as much as possible. If more than one possibility was available, the Day of EF and the operator who performed the EF was chosen in alternate order of replicates (operators and days of EF) so that each mare was flushed twice by each of the three operators and twice on each Day after ovulation (7, 8 and 9). By the end of the study, each operator had performed EF in 18 cycles (Day 7 EF = 6; Day 8 EF = 6; and Day 9 EF = 6).

Two operators had no previous experience of EF (O1 and O2), but they were experienced in AI and transrectal palpation and ultrasonography. They were trained in EF by watching three EF procedures and performing two sham EF in two dioestrous mares before commencing the study. The third operator (O3) had performed at least 500 EF before the study. No attempt to blind the operators for the Day of EF for each mare was carried out. This was due to the fact that the operators performing the EF were the same operators in charge of the breeding management of mares, and therefore it was not possible to have them blinded.

To determine the effect of EF on luteal function, circulating progesterone concentrations were compared pre-EF and 72 h post-EF in each EF cycle. Furthermore, progesterone concentrations were compared on equivalent days after ovulation between EF and non-flushed cycles: Blood samples were taken every 24 h from Day 7 to Day 12 in non-flushed cycles (six samples per mare), while in EF cycles blood samples were obtained just before the EF and 72 h after.

The overall fertility of mares (either positive embryo recovery or positive pregnancy diagnosis in non-EF cycles) was calculated during the period studied and broken down per order of consecutive cycle (first to seventh), so that a possible negative effect of repeated EF without the administration of PGF to induce oestrus after the EF could be investigated.

Finally, the number of cycles in which the mare had early luteolysis or remained pregnant after EF was registered by daily ultrasonography, so that the percentage of these negative outcomes could be calculated and presented in a descriptive manner.

2.3 | Ultrasound examinations and breeding management

Mares were examined by transrectal ultrasonography once daily when in oestrus. When the mare presented endometrial oedema (score of 1 to 3; 0 = no endometrial oedema, 3 = maximum engorgement of endometrial folds/maximum score of endometrial oedema[15]) and first showed a follicle of 30 to 40 mm in diameter (according to previous breeding records of individual mares[16]), the mare was inseminated with one billion motile, freshly collected sperm. Ovulation was induced at the time of AI with 200 μg of buserelin (Suprefact 1 mg/mL, Sanofi-Aventis) administered subcutaneously. If the mare had not ovulated by 72 h, AI was repeated. Ovulation(s) was diagnosed by daily ultrasonography after AI (Day 0 = Day when ovulation was first diagnosed) and

TABLE 1 Distribution of type of cycle (embryo flushing: EF or non-flushed: NF [mare was left to carry the pregnancy]; and Day of EF: D7, D8 and D9 post-ovulation) for each individual mare (1 to 9) during the seven consecutive cycles (first to seventh) of the experiment.

	Mare ID									
Order of cycle	1	2	3	4	5	6	7	8	9	Positive cycles
First	**NF**	EF-D8 O1	EF-D9 O2	**EF-D7 O3**	EF-D9 O3	EF-D9 O1	EF-D7 O2	**NF**	**EF-D7 O3**	4
Second	**EF-D7 O1**	NF	**EF-D7 O3**	EF-D8 O2	***EF-D8 O2***	**EF-D7 O3**	EF-D8 O1	**EF-D8 O2**	EF-D9 O3	5
Third	EF-D8 O2	**EF-D7 O2**	NF	**EF-D9 O3**	**EF-D9 O2**	**EF-D8 O1**	**EF-D9 O2**	***EF-D7 O2***	EF-D8 O1	6
Fourth	**EF-D9 O3**	EF-D9 O1	**EF-D7 O1**	**NF**	***EF-D7 O1***	**EF-D9 O2**	**EF-D7 O3**	**EF-D9 O1**	**EF-D9 O1**	8
Fifth	EF-D7 O2	**EF-D8 O2**	EF-D9 O1	**EF-D7 O1**	**NF**	**EF-D7 O2**	**EF-D8 O3**	**EF-D8 O3**	EF-D7 O2	6
Sixth	EF-D8 O3	**EF-D9 O3**	EF-D8 O2	**EF-D8 O1**	**EF-D8 O3***	**NF**	**EF-D9 O1**	EF-D9 O3	EF-D8 O2	5
Seventh	EF-D9 O1	**EF-D7 O3**	**EF-D8 O3**	**EF-D9 O2**	***EF-D7 O1***	**EF-D8 O3**	**NF**	EF-D7 O1	NF	6
Positive cycles	3	4	3	6	6	6	5	5	2	40

Note: The EF were performed by one of three operators (O1, O2 and O3). The cycles that resulted in a pregnancy are shown in bold, while the EF cycles in which no embryo was recovered but the donor remained pregnant are shown in bold and italicised font. The sixth cycle of mare ID 5 (*) resulted in the flushing of an embryo and an unwanted pregnancy.

confirmed 24 h later by the formation of an echoic CL.[17] If a second dominant follicle was present at the time of the first ovulation, ultrasound examinations continued daily until ovulation or regression of the follicle up to 3 days after the first ovulation.

Post-breeding management consisted of the administration of 20 IU of oxytocin (Facilpart 10 IU/mL, Laboratorios Syva SA) intravenously if the mare presented 0 to 15 mm in depth of free-intrauterine fluid (IUF). If the mare showed >15 mm IUF, a uterine lavage with 1 to 3 L of saline (NaCL 0.9% 1 L, Braun VetCare SA) until the efflux was clear was performed, followed by 20 IU of oxytocin once a day for a maximum of 3 days.

In the non-flushed cycle, the mare was scanned for pregnancy diagnosis 12 to 15 days post-ovulation. If pregnant, the embryonic vesicle(s) was squeezed and the mare was administered a synthetic PGF analogue: 125 μg of d,l-cloprostenol (Estrumate 250 μg/mL d-l cloprostenol, MSD Animal Health) to induce luteolysis. If the mare was neither in oestrus nor pregnant by Day 19 after ovulation, the mare was considered to be in prolonged dioestrus,[18] and luteolysis was induced with 125 μg of d,l-cloprostenol.

2.4 | EF, embryo searching and post-EF ultrasound examinations

In the EF cycles, 7 to 9 days after ovulation (considering Day 0 = the Day of the first ovulation, in cycles with asynchronous twin ovulations), the mare was restrained in a stock, a blood sample was taken and the uterus and ovaries were scanned to record the number of CL, presence of free-intrauterine fluid and endometrial oedema, if any. The perineum and vulva were washed and scrubbed three times with neutral soap and rinsed with tap water. After that, the area was dried with paper towels and the entrance to the vestibule was cleaned with cotton wool soaked with sterile distilled water. A 32 French (CH) catheter (Embryo flushing catheter 32 CH, Minitube Ibérica SL) connected to a Y tube closed system, with one line connected to a 1 L plastic bottle of ringer's lactate (Ringer lactate 1 L, Braun VetCare SA) and the second line to an embryo filter (Miniflush embryo filter, Minitube Ibérica SL) was used to perform the EF. The foley catheter was passed through the cervix using a sterile glove and, once in the uterus, the balloon was inflated with 40 mL of air and the catheter was pulled backwards slightly to seal the internal os of the cervix. The uterus was lavaged three times (1 L each, in and out) with ringer's lactate by gravity flow and uterine massage. At the end of the last flushing attempt, the uterus was scanned to aid in the recovery of any fluid left. Once finished, the air in the balloon was deflated and the catheter removed. The filter was disconnected from the system and searched thoroughly by two operators (which always included the operator with most experience), using a stereoscope (Zeiss stemi 508 doc, Zeiss Iberia). If an embryo(s) was found, it was measured and saved for teaching purposes.

Following the EF, the donor mare was put back in her paddock (without administering PGF) and re-examined 3 days later: a blood sample was taken, and the uterus and ovaries of the mare were scanned. The uterus was assessed and the degree of endometrial oedema and presence (depth and echogenicity) or absence of IUF were recorded. If the uterus had >15 mm in depth IUF, the mare was lavaged with 1 to 3 L of saline (until the recovered efflux was clear). Uterine lavage was repeated daily if needed. All mares were scanned daily until the following oestrus. Mares in which fewer embryos were recovered than CL present at the time of the EF, were scanned thoroughly for the presence of an embryonic vesicle from Day 12 to 15 after ovulation. If present, the embryonic vesicle was measured and confirmed 1 to 2 days later. Thereafter, the vesicle was squeezed, and the mare was administered 125 μg of d,l-cloprostenol. If the mare was neither in oestrus nor pregnant by Day 19 after ovulation, it was considered to be in prolonged dioestrus, and luteolysis was induced with 125 μg of d,l-cloprostenol. In the following oestrus, mares were bred again until 7 cycles were completed.

2.5 | Progesterone determination

Blood was collected from the jugular vein into heparinised tubes and centrifuged (2000 g for 10 min). Plasma was decanted and stored at −20°C until it was assayed. The plasma progesterone concentrations were assayed using a competitive solid-phase ELISA (DRG Instruments GmbH). It was determined without extraction from plasma, in duplicates. The assay sensitivity was 0.02 ng/mL, and the intra-assay coefficient of variation was 3.9%.

2.6 | Statistical analyses

The percentage of pregnant donor mares after an EF and mares with early luteolysis (progesterone concentration <2 ng/mL 72 h after EF) were reported using descriptive statistics. The threshold for early luteolysis of 2 ng/mL of progesterone was chosen since mares can develop endometrial oedema when their progesterone circulating concentration falls below that value.[19] Sequential data are reported as mean ± S.E.M., unless stated otherwise. A probability of $p \leq 0.05$ indicated that a difference was significant, whereas probabilities between $p > 0.05$ and $p \leq 0.1$ indicated that a difference approached significance. All data were computed in a statistical software Systat13 (Systat). Progesterone data were tested for normality by Anderson–Darling test. Data not normally distributed were ranked before being computed in the general linear model of variance. Potential for clustering of progesterone data was checked by scatter plot and visual examination of the scattered data. Furthermore, the change in progesterone concentration between Day 7 and 10, Day 8 and 11 and Day 9 and 12 from EF and non-EF cycles were compared by student unpaired t-test. The effect of Day of EF on the inter-ovulatory interval (IOI) was determined by one-way ANOVA.

A binary logistic regression model was created to determine the effect of order of breeding cycle (seven levels: first to seventh as independent variable), and breeding technique (two levels: EF vs. Non-EF, as independent variable) on mare fertility (1 = positive EF or positive

pregnancy diagnosis; 0 = negative EF or pregnancy diagnosis, as dependent variable). A separate binary logistic regression model was created to test the effect of EF variables (independent variables): Day of EF (three levels: Day 7, Day 8 and Day 9), operator performing the EF (three levels: operators 1, 2 and 3) and individual mare (mares ID 1 to 9) on the likelihood of having a positive EF (1 = at least one embryo recovered; 0 = no embryo recovered, as dependent variable). For each variable the different levels were computed in the regression models as numerical data, setting the lowest value as reference for the dependent variable. For both models, univariable analyses were first performed using Chi-square statistics to estimate the degree of significance of each independent variable on mare fertility (Model 1) and EF outcome (Model 2). Variables with a p value <0.5 were included in the multivariable analysis of the final model. The output of the univariable and multivariable models are shown in Table S1.

The effect of EF on progesterone concentration was determined by a general linear model of variance in which the dependent variable was the difference in progesterone concentration between the pre- and post-EF (72 h later), while the Day of EF (Day 7, 8 and 9), the individual mare (mare ID 1 to 9), and the operator performing the EF (1, 2 and 3) were computed as independent variables. Each independent variable was tested individually via univariable analysis before deciding whether to include it or not in the multivariable model. Variables showing a $p < 0.5$ were included in the multivariable analysis.

3 | RESULTS

The overall positive per cycle embryo recovery was 30/54 = 55.5% 95% CI [0.41, 0.69], while the likelihood of pregnancy in non-EF cycles was 6/9 = 66.7%, 95% CI [0.29, 0.92]. In the EF cycles, the mean IOI was 22.1 ± 1.1 days (15 to 29 days; $n = 46$). The IOI of the remaining eight cycles was removed from analyses because dioestrus was shortened with PGF due to prolonged dioestrus ($n = 3$) or unwanted pregnancy ($n = 5$). The median interval between two consecutive EF in the same mare was 24 days (14 to 47 days). There were three non-pregnant mares in the non-EF cycles, one entered prolonged dioestrus and the other two mares had an IOI of 28 and 22 days, respectively. Overall, from the 63 bred cycles, 40 (63.5% per-cycle pregnancy outcome, 95% CI [0.50, 0.75]) resulted in the recovery at least of one embryo 7 to 9 days post-ovulation ($n = 30$ cycles) or identification of an embryonic vesicle in the mare 12 to 15 days post ovulation ($n = 10$ cycles: 6 mares from non-EF cycles, and 4 cycles from negative EF that subsequently resulted in pregnancy in the donor mare).

A 55.5%, 95% CI [0.35, 0.74], likelihood of pregnancy was encountered in the first 3 cycles ($n = 27$), compared with a 69.4%, 95% CI [0.51, 0.83] likelihood in the last 4 cycles (Table 2), demonstrating that the order of breeding cycle did not influence mare fertility ($p = 0.52$). The individual mare per cycle likelihood of pregnancy varied from 28.5% (2/7), 95% CI [0.04, 0.71] to 85.7% (6/7), 95% CI [0.42, 0.99]. For EF cycles, the Day of EF did not influence ($p > 0.1$; Table 3) embryo recovery, while the operator performing the EF ($p = 0.06$: OR = 0.51) tended to influence embryo recovery. The operators with lower previous experience on EF (O1 and O2) had a percentage of positive embryo recovery cycles of 44.4% each (8/18, 95% CI [0.21, 0.69]). This was lower than the 77.8% embryo recovery of the more experienced operator (14/18, 95% CI [0.52, 0.93]).

The change in progesterone concentration (−1.5 ± 0.5 ng/mL) in mares between pre-EF and 72 h later was greater ($p = 0.03$; Table 4) than in non-EF cycles (−0.1 ± 0.3 ng/mL) for the equivalent days post-ovulation. In EF cycles, the Day of EF tended to influence the change in progesterone concentration by 72 h ($p = 0.09$): The change in progesterone for mares flushed on Day 7, 8 and 9 were −1.0 ± 0.8, −1.3 ± 1.1 and −2.3 ± 0.7 ng/mL, respectively (Table 4). The change in progesterone concentration in mares flushed by operators 1, 2 and 3 were −2.2 ± 0.9 ng/mL, −1.4 ± 0.8 ng/mL, and −0.9 ± 1.0 ng/mL, respectively. The individual mare did not influence the change in progesterone concentration ($p > 0.1$; Table S2). EF performed on Day 9 post-ovulation induced a significant decrease in progesterone concentration by 72 h ($p = 0.008$), with a concentration of 7.2 ± 0.4 ng/mL before EF and 4.9 ± 0.6 ng/mL 72 h after, while the EF performed on Day 7 or 8 did not affect progesterone concentration ($p > 0.1$; Table 3; Figure 1). A further analyses of progesterone data from Day 9 EF in which the mares with early luteolysis ($n = 2$) were removed, also showed a significant ($p = 0.007$) decrease in progesterone concentration between pre-EF levels 7.2 ± 0.5 ng/mL and 72 h after EF: 5.7 ± 0.4 ng/mL. The progesterone concentration in non-EF cycles

TABLE 2 Effect of order of consecutive cycle on mare per-cycle pregnancy (likelihood of recovering an embryo 7 to 9 days post-ovulation or finding an embryonic vesicle 12 to 15 days post-ovulation).

Order of cycle	*n*	Pregnancy %	Pregnancy 95% CI	IOI
First	4/9	44.4	[0.13, 0.78]	21.3 ± 1.3
Second	5/9[a]	55.5	[0.21, 0.86]	22.3 ± 1.5
Third	6/9[a]	66.7	[0.29, 0.92]	22.3 ± 1.8
Fourth	8/9[a]	88.9	[0.51, 0.99]	21.7 ± 1.5
Fifth	6/9	66.7	[0.29, 0.92]	21.9 ± 1.1
Sixth	5/9	55.5	[0.21, 0.86]	22.6 ± 2.1
Seventh	6/9[a]	66.7	[0.29, 0.92]	21.5 ± 0.8
All cycles	40/63	63.5	[0.50, 0.75]	22.1 ± 1.1

[a]A donor remained pregnant after a negative embryo flush. The order of cycle did not affect ($p > 0.1$) pregnancy outcome.

TABLE 3 Characteristics of embryo flushing (EF) cycles according to the Day of EF.

Day of EF	Cycles (*n*)	OV (*n*)	Positive f lushes (%)	Recovered embryos (*n*)	Embryo diameter	P4 pre-EF (ng/mL)	P4 72 h post-EF (ng/mL)	Early luteolysis (*n*)	Pregnant donors (*n*)	IOI (days)	Prolonged dioestrus (*n*)
7	18	22	61.1	12	317.5 ± 31.8[a]	7.1 ± 2.1	6.1 ± 2.3	1	3	23.0 ± 1.2	2
8	18	22	50.0	9	616.7 ± 86.3[b]	8.0 ± 2.4	6.7 ± 3.2	2	2	22.0 ± 1.2	0
9	18	19	55.5	10	1395 ± 102.1[c]	7.2 ± 1.6	4.9 ± 2.2*	2	0	21.3 ± 0.6	1
All	54	63	55.5	31	746.1 ± 526.5	7.5 ± 2.1	5.9 ± 2.5*	5	5	22.1 ± 1.1	3

Note: Ovulation (OV); Progesterone (P4); inter-ovulatory interval (IOI); Early luteolysis (mares with progesterone concentration below 2 ng/mL 72 h after the EF).
*Indicates a significant ($p < 0.01$) decrease in progesterone concentration between pre-EF and 72 h later. Within column, different superscripts (a, b, c) indicate a significant difference in embryo diameter ($p < 0.0001$).

did not vary ($p > 0.1$) between any day of the sampled period (Day 7 through Day 12; Figure 1). The mean progesterone concentration of mares from non-EF cycles on Day 12 post-ovulation that became pregnant was 7.2 ± 1.6 ng/mL ($n = 6$). This was not significantly different from that of non-pregnant mares: 6.5 ± 0.4 ng/mL ($n = 3$).

In the EF cycles, five mares presented progesterone concentrations below 2 ng/mL by 72 of EF (Table 5). All these mares showed signs of early luteolysis with obvious endometrial oedema and/or a non-visible or regressed (<15 mm in diameter) CL on ultrasonography, while four of them also presented accumulation of IUF 72 h after the EF. One of these mares (mare ID 6) needed antibiotic treatment because the amount of IUF and quality (echogenic) had not resolved by the second day of treatment with uterine lavage and oxytocin. The fluid recovered from the uterine lavage was plated on blood agar, and 24 h later, a pure growth of beta haemolytic *streptococci spp* was obtained (Figure S1). Mare ID 6 was infused with 3 g procain-penicillin (Depocillin 300 mg/mL, Intervet) into the uterus for three consecutive days. The other mares with early luteolysis and IUF did not require antibiotic treatment because the uterine lavage or oxytocin were enough to remove and stop the IUF production by the next day. No mare from the non-EF group showed signs of early luteolysis or had a progesterone concentration below 2 ng/mL during the period examined (Day 7 to Day 12).

In five EF cycles, the donor mare became pregnant following the EF. These EF cycles were performed on Day 7 ($n = 3$) and Day 8 ($n = 2$). No EF on Day 9 resulted in an unwanted pregnancy. Four of the five unwanted pregnancies occurred in the same donor mare. She was a multiparous 17-year-old Spanish purebred mare weighing 550 kg. The characteristics of these five EF cycles with unwanted pregnancies are shown in Table 6. Thus, the likelihood of unwanted pregnancy in this study for EF negative cycles was 16.7% (4/24), 95% CI [0.05, 0.37]. While the percentage of unwanted pregnancy for EF cycles in which the number of embryos recovered was less than the number of CL present in the donor mare at the time of the EF was 18.5% (5/27), 95% CI [0.06, 0.38].

4 | DISCUSSION

The results of the present study confirm that EF itself does not induce full luteolysis. This finding is in agreement with previous work.[11,14] Only a small percentage of mares showed signs of luteolysis following EF, which appeared to be more related to bacterial contamination or post-flush inflammation and development of endometritis, rather than prostaglandin release from cervical dilation and manipulation of the uterus during EF. Handler and coworkers[13] experimentally induced cervical dilation to inseminated mares 7 days after ovulation and showed no difference in progesterone concentration profile or likelihood of pregnancy between mares with or without cervical dilation. However, they did report that two of the eight cervical dilation mares showed signs of bacterial endometritis with a sudden drop in progesterone concentration to basal levels and signs of endometritis (endometrial oedema and accumulation of IUF) following cervical

TABLE 4 Change in progesterone concentration between the Day of embryo flushing (Day 7, Day 8 or Day 9; $n = 18$ cycles each Day) and 72 h later (Day 10, Day 11, Day 12).

		Difference in progesterone concentration (ng/mL)			
Type of cycle	*n*	Day 10–Day 7	Day 11–Day 8	Day 12–Day 9	All cycles
Non-flushed	9	−0.3 ± 0.5	−0.2 ± 0.5	0.0 ± 0.7[a]	−0.18 ± 0.3[a]
EF	18	−1.0 ± 0.8	−1.3 ± 1.1	−2.3 ± 0.7[b]	−1.54 ± 0.5[b]

Note: A comparison from the non-EF cycles is given showing the change in progesterone concentration at the equivalent days post-ovulation. Within column, different superscripts letters (a, b) indicate a significant difference ($p < 0.05$) in the change in progesterone concentration between EF and non-flushed cycles. In EF cycles, all progesterone concentration values resulted from the calculation between the progesterone concentration values 72 h after EF minus the concentration values before the EF.

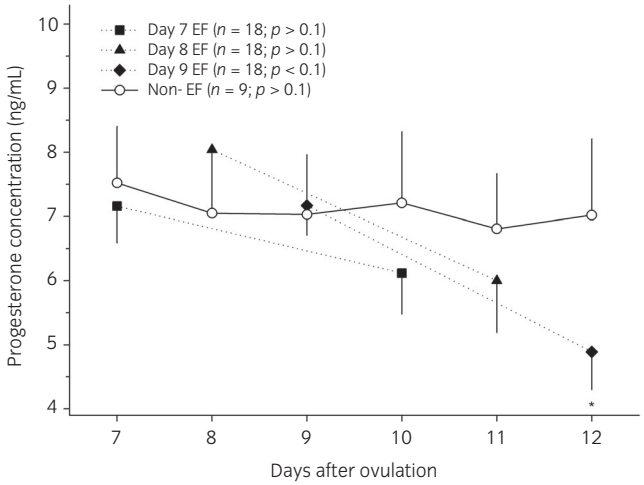

FIGURE 1 Mean (±S.E.M.) progesterone concentration (ng/mL) from mares in two types of cycle. Embryo flushing (EF) cycles in which an embryo flush was performed either on Day 7, Day 8, or Day 9 after ovulation. Progesterone concentration was determined just before EF and 72 h later. In non-flushed cycles, each mare had progesterone concentration determined every day from Day 7 to Day 12 after ovulation. An asterisk (*) indicates a significant difference ($p < 0.01$) in progesterone concentration at Day 12 after ovulation between non-EF and Day 9-EF cycles. The progesterone concentration of mares from non-EF and Day 7 and Day 8 EF cycles did not vary significantly ($p > 0.1$) between any day.

manipulation,[13] similar to the five cycles with early luteolysis reported in the present study. A shortened luteal phase is a common sign of bacterial endometritis,[20] as endometrial inflammation caused by bacterial contamination during breeding or breaching of the cervix during dioestrus[21] can cause bacterial multiplication and sufficient prostaglandin release to lyse the CL.[20] On the other hand, EF did affect to a certain extent luteal function of flushed mares. The results of the present study showed a subtle but significant reduction of progesterone concentration within 3 days of EF compared with non-flushed mares. This significant reduction in progesterone concentration was observed even when the mares with early luteolysis were excluded from the analysis. An interesting finding was that the degree of progesterone reduction following the EF was dependent on the Day post-ovulation on which the EF was performed. That is, mares flushed on Day 9 post-ovulation showed a significant decrease in progesterone concentration, while mares flushed earlier did not (Day 7 and Day 8). This relationship could be explained by the fact that the sensitivity of the CL to a luteolytic stimulus increases with the age of the CL.[22,23] The sub-luteolytic stimulus could be the prostaglandin[11] and/or oxytocin[10] release associated with the cervical dilation and uterine manipulation induced during EF, or from low level inflammation from flushing that could stimulate PGF release. A sub-luteolytic dose of PGF can induce a partial luteolysis in mares,[22] in which the progesterone concentration drops significantly, but does not reach basal levels and the mare remains in dioestrus.[23] The magnitude of a PGF dose required to be luteolytic depends on the type of PGF, age of CL and mare individual variation.[22]

Most of the mares with EF cycles in the present study showed absence of luteal regression, which allowed a more realistic determination of the percentage of unwanted pregnancy, that is, donor mares that remained pregnant after a negative EF. Although this finding is not novel, several authors have warned about this possible undesired outcome,[4–6] the expected percentage had not been determined critically. In the present study 5 unwanted pregnancies were identified, which makes up 18.5% of unwanted pregnancy per EF in mares with fewer embryos recovered than CL present at EF (5/27). This percentage is probably overestimated due to the relatively small sample size (nine mares) and the fact that four of the five unwanted pregnancies belonged to the same mare. On the other hand, it sheds light on possible causes related to this undesired outcome. Although not assessed critically, this multiparous mare had a relatively large uterus. It is possible that the use of 1 L of media to flush the uterus was not enough to fill it completely. If the embryo happened to be at the tip of one of the horns at the time of EF, it could have been left in the uterus. Furthermore, it is possible that a poor uterine massage technique (three of the four EF cycles with unwanted pregnancy were flushed by the operators with no previous experience on EF) may have contributed to the failure of the flushing media to reach all endometrial surface. In the five EF cycles in which mares ID 5 and 8 produced unwanted pregnancies, the uterus was completely emptied during the last flushing attempt in four cycles while in only one cycle a small pocket of flushing media (15 mm in depth) could not be recovered and was left in the uterus. So, it seems that emptying completely the uterus after the EF does not preclude leaving an embryo behind. The detailed analyses of the EF cycles with unwanted pregnancy showed that it was unlikely for the embryos to be still in the oviducts at the time of EF, however, this cannot be ruled out. It has been shown that the equine embryo enters the uterus between 6 and 6.5 days post-ovulation,[24] but anecdotical reports suggest that in some older mares the oviductal

TABLE 5 Cycle characteristics of mares that showed early luteolysis (progesterone concentration <2 ng/mL) 72 h after embryo flushing (EF); IUF (free intrauterine fluid); Regressed: CL < 15 mm in diameter, and increased in echogenicity; IOI (inter-ovulatory interval); Oxy (20 IU oxytocin); Lavage × (number of days in which a uterine lavage was performed followed by oxytocin administration); AB (intrauterine administration of 3 g procaine penicillin for 3 days); Embryo+ (recovery of at least one embryo); Pregnant (positive pregnancy diagnosis in non-flushed cycles).

Mare ID	Age	Cycle order	Day of EF	P4 72 h after EF (ng/mL)	Endometrial oedema score	IUF depth (mm)	IUF quality	CL appearance (mm)	Treatment	IOI (days)	Next cycle outcome
3	9	Fifth	9	0.45	2	5	Anechoic	Regressed	Oxy	17	Embryo−
6	17	Seventh	8	0.54	1	25	Echoic	Regressed	Lavage × 3 + AB × 3	20	Not bred
2	14	Seventh	7	1.62	0	18	Anechoic	Regressed	Lavage × 1	21	Not bred
4	20	Third	9	0.78	1	0	NA	Non-visible	Nothing	22	Pregnant
1	5	Third	8	1.81	2	22	Echoic	Regressed	Lavage × 2	15	Embryo+

TABLE 6 Cycle characteristics of embryo flushing (EF) that resulted in the donor mare remaining pregnant after the EF.

Mare ID	Order of cycle	Operator	CL number at EF	Age of CL at EF (days)	EF outcome	Day of first PD	Number of EV	EV size (mm)	Day of second PD	EV size (mm)
5	Second	2	2	8	Negative	Day 15	1	19.0	Day 16	24.0
8	Third	2	2	7	Negative	Day 14	1	15.3	Day 16	23.2
5	Fourth	1	1	7	Negative	Day 12	1	9.1	Day 14	16.1
5	Sixth	3	2	8 & 7	1 embryo 250 μm	Day 13	1	11.5	Day 14	15.3
5	Seventh	1	1	7	Negative	Day 13	1	14.2	Day 15	25.4

Abbreviations: EV, embryonic vesicle; PD, pregnancy diagnosis.

descent could take much longer. In the five EF cycles with unwanted pregnancy of the present study, all CL present at the time of EF had originated from ovulations occurring at least 7 days earlier. However, mare ID 5 might have had a longer oviductal descent for their embryos, taking longer than 7 days to reach the uterus (most missed embryos were Day 7 at the time of EF). In addition, the diameters of the embryonic vesicles identified at the first pregnancy diagnosis were within the expected referenced values[25] for the recorded ovulation dates, which is further evidence to support the presence of the embryos in the uterus at the time of the unsuccessful EF. Nevertheless, and regardless of the reason, being pregnant after EF would likely be considered clinically undesirable and we demonstrate there is a risk of this outcome when not administering PGF and therefore this should be considered when deciding whether to administer PGF at the time of EF.

The repeated EF attempts without the subsequent administration of PGF did not appear to affect mare fertility during the study. The likelihood of obtaining a pregnancy or an embryo in mares bred the first cycles of the study was similar to that of the last cycles (5 to 6 breedings later). The results of the current study are in agreement with a previous study[26] in which healthy research mares were inseminated and flushed for 7 to 26 consecutive cycles. The current study used much older mares (mean age of 13 years old) compared with the previous study (mean aged 5.8 years old), and despite not administering PGF, the fertility did not appear to decrease in the current study. On the other hand, a field trial reporting data on repeated embryo collection attempts in a commercial ET programme have shown a decrease in the likelihood of recovering embryos as the number of embryo collections increases.[27] However, in a commercial ET programme, the reasons to obtain multiple embryos in donor mares may be different. Subfertile mares may be flushed for more consecutive cycles than fertile mares, to meet the owner's desired number of pregnancies, which could bias the results on the effect of repeated embryo collection on fertility.

Only a small percentage of mares showed signs of endometritis with accumulation of purulent IUF following the EF, which responded favourably to treatment. It is likely that the contamination of these mares was introduced during the placement of the foley catheter through the cervix. Whether the subsequent bacterial multiplication and infection of the endometrium was favoured by the lack of oestrus induction with PGF cannot be ruled out. However, in the authors' experience, it is not rare to find a donor mare with signs of endometritis following EF even if oestrus has been induced with PGF. Nevertheless, this finding highlights the relevance of preparing the perineum and vestibule entrance of the donor mare as aseptically as possible to reduce the chances of bacterial contamination during the EF procedure. A previous study showed that repeated inseminations and embryo collections in mares induced chronic inflammatory changes in the uterus despite using PGF following each EF cycle.[28] Unfortunately, uterine biopsies or cytologies were not performed in the current study, so the effect of not administering PGF after each EF on uterine health could not be compared with previous data. However, even if the repeated embryo collection attempts induced some sort of

uterine inflammation in the mares of the current study, their ability to produce embryos did not change over the course of the study.

The main limitations of the study are the relatively small sample size of mares, and the repeated use of the same mares for several cycles, likely producing an overestimation of unwanted pregnancies due to mare ID 5 (4 of the 5 unwanted pregnancies belonged to this mare). Despite not finding indication of clustering of data points, the fact that the present study used repeated samples from the same horses and at closer time points, data are more likely to be similar than ones from different horses and/or at more distant time points. On the other hand, progesterone output is so variable, as it may be influenced by different factors (i.e., number of corpora lutea,[29] Day of cycle relative to ovulation,[29] time of season[30] and presence of inflammatory products in the uterus as evidenced by the results of the present study) that the cycle itself is more likely to have an impact on the progesterone concentration profile during dioestrus than the individual mare. Therefore, the impact of repeated use of animals in different cycles on clustering of data or any bias on progesterone profile is unlikely to have affected the progesterone results. Furthermore, the animals did not include foaling mares, which are known to have a larger uterus. This could have affected the overall EF outcome. In addition, the allocation of cycle order and operator to each EF was chosen by convenience, and therefore it was not randomised. And lastly, the study did not have a control group in which mares received PGF after the EF to compare the percentage of unwanted pregnancy and mares with signs of endometritis.

In conclusion, EF induces a subtle, but significant reduction in progesterone concentration within 3 days of EF compared with non-EF cycles. The Day of EF (Day 7, 8 and 9), influences the degree of reduction in progesterone concentration, with mares flushed 9 days post-ovulation having a greater drop in progesterone concentration within 3 days of EF. The fertility of mares after repeated EF without administration of PGF appears to be acceptable, however, there is a risk of unwanted pregnancy in donor mares following a negative EF (4/24 = 16.7%) or EF cycles in which the number of embryos recovered is less than the number of CL present in the donor mare at the time of the EF (5/27 = 18.5%), although this could be biased by individual mares.

AUTHOR CONTRIBUTIONS

Rebeca Martínez-Boví: Investigation; methodology; writing – review and editing. **Laura Sala-Ayala:** Investigation; methodology; writing – review and editing. **Aurora Querol-Paajanen:** Investigation; methodology; writing – review and editing. **María Plaza-Dávila:** Investigation; methodology; writing – review and editing. **Juan Cuervo-Arango:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

No competing interests have been declared.

DATA INTEGRITY STATEMENT

Juan Cuervo-Arango had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

ETHICAL ANIMAL RESEARCH

Animal procedures were approved by the local animal welfare committee of the Universidad CEU Cardenal Herrera and authorised by the regional official authority (*Conselleria de agricultura, desarrollo rural, emergencia climática y transición ecológica de la Generalitat Valenciana*), for the use of animals in research: Licence ref. 2023-VSC-PEA-085.

INFORMED CONSENT

Not applicable.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/evj.14073.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Zenodo at https://zenodo.org/records/10714694.

[Correction added on 01 March 2024, after first online publication: Data Availability Statement was updated in this version.]

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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