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Original Research Article

The effect of uterine massage and number of embryo flushing attempts on embryo recovery in mares

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ABSTRACT

The aims of this study were to determine the effect of the embryo flushing technique and the number of flushing attempts performed by operators of different experience on embryo recovery (ER). Ten non-lactating mares were inseminated with the same stallion in six cycles each (n = 60). Embryo flushing (EF) was performed 7–9 days after ovulation by three operators (OP; 20 EF cycles each): OP1 had performed >500 EF before the study, while OP2 and 3 had performed 0 EF. Each EF was performed with 2 flushing attempts (FA) using 1L of ringer's lactate "in-and-out" using two EF techniques: 1) uterine massage (UM): continuous ballottement and massage of the uterus per rectum during ringer lactate recovery, 2) gravity flow (GF): the ringer lactate was allowed to flow back without massaging the uterus. In both groups, 20 IU of oxytocin were administered at the second FA and the ringer lactate was allowed to remain in the uterus for 3 min before recovery. An extra FA was performed in each group using 0.5 L of ringer lactate and uterine massage. More embryos ($P < 0.05$) per ovulation were recovered in the UM (17/33, 0.51) than in the GF group (8/36, 0.22). For the UM group, 16/17 embryos (94.1 %) were recovered in the first FA, while only one embryo in the second FA (1/17, 5.9 %). In the GF group, 4 embryos were recovered in each FA. No embryo was found in the extra FA in the UM group, while seven additional embryos were found in the GF group (5/7 flushed by OP1; $P < 0.05$). The overall ER per cycle was 70, 40, and 45 % for OP1, 2 and 3, respectively. In conclusion, highest embryo recovery is achieved in EF performed with UM, with the majority of embryos being flushed in the first FA.

1. Introduction

Equine embryo transfer is a common assisted reproductive technique first reported in 1972 [1,2]. It offers a wide variety of benefits which include obtaining offspring from mares during competition seasons, from older and very young (2 years old) mares and increasing the number of offspring per mare per year [3]. This procedure involves obtaining an embryo from the uterus of a donor mare through an embryo flushing (EF) and subsequently, transferring the embryo transcervically to a recipient mare in the same stage of the estrous cycle [4], which will carry the pregnancy to term.

The standard method of embryo flushing involves performing a non-surgical transcervical uterine lavage [3] using an adequate medium [5] and recovering the fluid through a tubing system connected to a filter, where the embryo is retained. There are different techniques for performing this procedure, with scarce scientific evidence on which factors are most important for recovering an embryo from a mare's uterus. Clinicians might perform a transrectal uterine massage while the medium is being flushed in or out of the uterus, aiming to create turbulence to retrieve the embryo from the endometrial folds. Also, as the embryo moves freely through the uterus before fixation [6], this technique might be helpful to ensure that the fluid is distributed throughout the entirety of the uterus and the embryo can be collected. Other operators may recover the flushing media by gravity without manipulating the uterus in some or all flushing attempts [7]. Oxytocin is a commonly used and effective drug in various reproductive procedures, such as part of the treatment for mating-induced endometritis, uterine flushes, or metritis. One of its effects is to stimulate uterine contractions, aiding in the expulsion of uterine contents [8]. Because of this effect, its use has been advocated during embryo flushing [9] to help recover the medium from the uterus and enhance embryo recovery.

In general, 3–4 flushing attempts per embryo flush with 1–2L per attempt are made [4,9], before searching for the embryo. In other ET centers, if an embryo is not found after the initial 3 flushing attempts, an additional flush is carried out [9], or no further attempts are made after the chosen number of flushing attempts deemed necessary.

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Furthermore, some clinicians may flush the mare the following day after an unsuccessful embryo recovery [7]. Some studies report an increase in embryo recovery rates after additional flushes [10]. One of the added difficulties is that at the time of embryo flushing, it is unknown whether the embryo is present in the uterus, or the mare did not conceive, or the embryo was lost before the embryo flush procedure. A previous study showed an increased color-doppler signal in the uterus of mares before the EF with the presence of an early embryo (Day 7–8) compared to barren mares [11]. However, the accuracy of this technique has not been confirmed in larger field trials.

Furthermore, inaccurate determination of the time of ovulation can negatively affect embryo recovery, as this influences the time at which the embryo enters the uterus [12]. Other factors that could potentially affect embryo recovery but have not been critically investigated include failure to fill the whole uterus with flushing media, incomplete recovery of uterine fluid at the end of each flushing attempt, and failure to identify the embryo in the filter due to the large amount of debris or its small size.

Therefore, there is a certain lack of knowledge regarding the effect of the embryo flushing technique and associated factors on the likelihood of recovering an embryo from the uterus. It is not known what the main factor influencing embryo recovery is. Furthermore, there is no reliable data on the likelihood of the embryo being flushed in each attempt. This creates stress for the veterinarian performing the embryo flush and the client and increases the cost due to the time spent and extra expense of media for successive flushes, not knowing if the embryo is still in the uterus, in the tubing system, filter, or if the mare didn't conceive. Studying the most relevant factors for embryo recovery can be useful in providing additional data to establish the most cost-effective embryo flushing technique.

The main objective of this study was to compare the embryo recovery rate obtained using two different embryo flushing techniques: uterine massage and recovery of the media by gravity flow in non-lactating mares. Secondary objectives were: 1) to determine the possibility of the embryo remaining in the tubing system after performing an embryo flushing; 2) to determine the effect of operator experience on the embryo recovery rate; 3) to study the relationship between the degree of uterine horns' expansion with flushing media, fluid left in the uterus after each flushing attempt and the recovery of the embryo; and 4) to determine the likelihood of recovering an embryo in each of the successive uterine flushing attempts performed during an embryo flushing. It was hypothesized that uterine massage would result in the recovery of more embryos in the first flushing attempt.

2. Materials and methods

2.1. Animals

Ten non-lactating horse mares (Equus caballus) of different breeds (Spanish purebred, Belgian draught, crossbreds) aged 5–20 years old (mean 13.6 ± 1.8), barren (n = 6) and maiden (n = 4) and weighing 420–670 kg were used in the study. Mares belonged to the research herd of the University, located in Náquera, Spain (39° 39′ N) and were kept in sand paddocks in groups of 3–4 animals and were fed on hay and cereal concentrate three times a day, with ad libitum access to water and mineralized salts. All mares were cycling at the beginning of the study. Mares chosen for the study had a proven fertility record and had produced embryos during the previous year. This sample was chosen based on availability of mares that fit the inclusive criteria. Furthermore, a Spanish purebred stallion aged 12 years old of proven fertility was used as semen donor for breeding. 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 reference number: 2023-VSC-PEA-085.

2.2. Experimental design

The study was carried out between September 2022 and June 2023 (Northern Hemisphere). Each mare was followed and bred to the same stallion during 6 cycles (10 × 6 = 60 cycles). In each cycle, EF were performed 7–9 days after ovulation by three different operators (OP), based on availability of the OP and to avoid weekends as much as possible. Operator 1 had previous experience in EF and had performed over 500 EF before the study. Operator 2 was experienced in reproduction procedures (over 10 years performing transrectal palpation, scanning and AI) but had never performed EF, while operator 3 was a final year vet student with no previous experience in EF and a few months experience in rectal palpation and ultrasonography. Operators 2 and 3 were trained on EF by watching three EF procedures and performing 2 sham EF in two diestrous mares before commencing the study.

To determine the effect of uterine massage on embryo recovery, the 60 EF cycles were divided into two groups: uterine massage (UM; n = 30) and gravity flow (GF; n = 30), each operator performed 10 EF of each group, and each mare's cycle was allocated to either group on alternate order (each mare had 3 EF cycles performed by UM and 3 by GF). In each EF cycle, two flushing attempts (FA) were performed either by UM or GF. Lastly, an extra flush performed with uterine massage was done in both groups to determine if an embryo had been left in the uterus or in the flushing catheter. Thus, a total of 180 flushing attempts were performed in the 60 cycles. To determine in which flushing attempt the embryo was recovered, a separate embryo filter was utilized for each FA and a fourth embryo filter was used to rinse the flushing catheters. At the end of the procedure, the four filters were thoroughly searched by two operators (one of them being the experienced operator, OP1). Mares were not administered $PGF_{2\alpha}$ on purpose so that any embryo remaining in the uterus could be identified as unwanted pregnancy 4–7 days later.

A further analysis was performed with data from flushing attempts in which the presence of an embryo in the uterus was certain (the embryo was recovered in the extra flushing attempt, or the mare became pregnant after a negative EF), so that the influence of several factors associated with the embryo flushing technique on embryo recovery could be investigated.

2.3. Ultrasound examinations and breeding management

Mares were examined by transrectal ultrasonography once daily when in estrus. When the mare presented endometrial edema (score of 1–3; 0 = no endometrial edema, 3 = maximum ingurgitation of endometrial folds/maximum score of endometrial edema), and first showed a follicle of 30–40 mm in diameter (according to previous breeding records of individual mares [13], the mare was inseminated with 1 billion of motile and freshly collected sperm. Ovulation was induced at the time of AI with 200 μg of buserelin (Suprefact 1 mg/mL, Sanofi-Aventis Deutchland GmbH, Frankfurt, Germany) 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 confirmed 24 h later by the formation of an echoic CL [14]. 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 administration of 20 IU of oxytocin (Facilpart 100 mL: oxytocin 10 UI/mL, Syva laboratories, León, Spain) intravenously if the mare presented 0–15 mm in depth of free-intrauterine fluid (IUF). If the mare showed >15 mm IUF, a uterine lavage with 1–3 L of saline (NaCL 0.9 % 1L, Braun VetCare) until the effluent was clear was performed, followed by 20 IU of oxytocin once a day for a maximum of three days.

2.4. Embryo flushing

EF was performed 7–9 days after ovulation (considering Day 0 = the

day of the first ovulation, in cycles with asynchronous double ovulations). The mare was restrained in a stock, 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 FR foley catheter (Embryo flushing catheter 32 CH, Minitube Iberica, Tarragona, Spain) connected to a Y tube closed system (Set of tubes Y Luer, Minitube Iberica, Tarragona, Spain), with one way connected to a 1L plastic bottle of ringer's lactate (Ringer lactato 1 L, Braun Vetcare, Rubí, Spain) and the other way to an embryo filter (Miniflush embryo filter, Minitube Iberica, Tarragona, Spain) 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. Then 1 L of ringer lactate was infused in the uterus for each of the first two flushing attempts. Subsequently, the uterus of the mare was scanned to determine the percentage of the uterine horns' length filled (and expanded) with ringer lactate. The uterus was scanned from the cornual bifurcation up to the tip of each uterine horn, to determine subjectively the length percentage of each horn filled with ringer lactate. An average of the degree of expansion of each horn length was taken; for example, if one horn was completely filled with ringer lactate from the bifurcation up to the tip of the horn (100 %), but the other horn was totally collapsed (0 %), the average would be 50 %. An example of a mare with a low (20 %) and high (95 %) horn expansion can be found in supplementary material (S1 and S2, respectively). The fluid was recovered either by gravity flow (GF) or assisted by uterine massage (UM) according to de experimental group:

- Uterine massage (UM): ballottement and massage of the uterus were performed transrectally from the base to the tip of each horn continuously while the medium flowed out. The mare was scanned again and if there was any fluid left in the uterus, it was emptied by further uterine massage with the aid of the scanner. Any fluid left in the uterus was measured with the electronic calipers of the scanner and recorded (the ultrasound image was frozen when the depth of pocket of free fluid was maximum, and the amount of fluid was estimated by taking two measurements with the electronic calipers at right angles and recording the mean as mm of depth of fluid).
- Gravity flow group (GF): the medium was allowed to flow out by gravity. No transrectal manipulation or uterine massage was performed at any point. Once the medium stopped flowing out, the mare was scanned again. If there was any fluid left in the uterus, it was measured and recorded, but no attempt was made to empty it.

For both groups, once the first FA was finished, the filter was removed and taken to the laboratory for later search. For the second FA, a new filter and another liter of media was infused in the uterus, with the addition (for both groups) that 20 IU of oxytocin (Facilpart 100 mL: oxytocin 10 UI/mL, Syva laboratories, León, Spain) was administered intravenously followed by a 3-min wait time as reported previously [9]. At the end of the 3 min period, horn expansion was assessed and recorded as in the first FA. The ringer lactate was recovered either by UM or GF as described previously, and any fluid left in the uterus was recorded.

Lastly, an extra flushing attempt was performed in both groups. However, only 0.5 L of ringer lactate was infused into the uterus and horn expansion was recorded as previously described. Uterine massage was performed for recovering the media of this extra FA, as described in the UM group. Once the flow stopped, the mare was scanned and if there was any fluid left in the uterus, it was emptied by further uterine massage with the aid of the scanner. If unable to empty the fluid completely, it was measured and recorded as in the previous two FA. The choice of performing uterine massage in the last attempt of the gravity flow group was made based on the hypothesis that uterine massage is more efficient, to ensure most embryos would be recovered and therefore it could be objectively reported how many embryos were left in the uterus after two attempts using gravity flow. Once the extra flush was finished, in both groups, the air in the balloon was deflated and the foley catheter removed. The third filter was disconnected, and a fourth filter was inserted for flushing the tubing system with the remaining 0.5L of media to evaluate the possibility of the embryo remaining there. The four filters were searched thoroughly by two operators, using a stereoscope (Zeiss stemi 508 doc, Zeiss). If an embryo was found, it was measured and stored for teaching purposes.

Following the EF, the mare was taken back to her paddock. No luteolytic treatment ($PGF_{2\alpha}$) was administered to the mare to avoid luteolysis and so increase the chances of survival of any unrecovered embryo. Pregnancy diagnosis was performed by transrectal ultrasound 13–16 days after ovulation. If a mare was pregnant, the embryonic vesicle was measured and reconfirmed 1–2 days later. Pregnant mares were administered 125 μg cloprostenol (Estrumate, 250 μg/mL, MSD Animal Health, Salamanca, Spain).

2.5. Statistical analyses

A binary logistic regression model was created to determine the effect of individual mare (10 levels: mares 1 to 10), Day of EF (3 levels: Day 7, Day 8, or Day 9), previous operator's experience in EF (2 levels: yes or no), and uterine massage during EF (2 levels: yes or no) on embryo recovery by the first two flushing attempts (2 levels: positive embryo flush = at least the recovery of one embryo in the first or second FA; or negative = no embryo recovered either in the first or second FA) with sample size of 60 EF cycles.

In EF cycles known to have conceived an embryo (an embryo was recovered during one of the FA or the mare became pregnant after the EF) a separate binary logistic regression was created to determine the effect of uterine massage, Day of EF, use of oxytocin and extended (3 min) media contact with endometrium (2 levels: yes or no), volume of media (2 levels: 1 or 0.5L), degree of uterine horns' expansion (4 levels: 0 = < 50 %; 1 = 50–70 %; 2 = 75–95 %; 3 = 100 %), and depth of fluid left in the uterus (4 levels: 0 = < 5 mm; 1 = 5–20 mm; 2 = 21–40 mm; 3 = > 40 mm), on the likelihood of recovering an embryo at the flushing attempt, for a sample size of 66 flushing attempts (32 positive and 34 negative recoveries). In both regression models, univariable analyses were first performed using Chi-square statistics to estimate the degree of significance of each independent variable on embryo recovery. Variables with a P value < 0.5 were included in the multivariable analysis of the final model.

The effect of UM *vs* GF on the likelihood of recovering an embryo in the first, second or extra FA was determined by Fisher's exact test. Similarly, in FA performed with uterine massage (UM group and extra flush in both groups), the effect of operator and their previous experience in EF on embryo recovery was determined by Fisher's exact test.

Continuous data were checked for normality using Anderson-Darling test. Data not normally distributed (percentage of uterine horns' expansion and depth of fluid left in the uterus) were analyzed by non-parametric Kruskal-Wallis test. All data were computed in the statistical software Systat13. Significance was set at $P \leq 0.05$.

3. Results

The overall (three flushing attempts per cycle) embryo recovery rate of this study was 51.5 % (31 positive embryo flushes out of 60 cycles), with 30 cycles resulting in the recovery of a single embryo and one cycle with the recovery of a twin embryo, adding a total of 32 embryos from 69 ovulations (0.46 embryos per ovulation). Five more embryos were apparently left in the uterus during the EF, as they resulted in 5 cycles with unwanted pregnancy.

The individual mare, Day of EF (7, 8 or 9 days after ovulation), and operator's previous experience in EF (including cycles from both uterine massage and gravity flow groups) did not influence the embryo recovery

rate by the second FA (P > 0.05); while the uterine massage did influence recovery rate by the second FA (P < 0.05; Table 1A). Embryo flushes performed with UM were more likely (P < 0.01; Table 1B and Table 2) to result in a positive embryo recovery (17/30, 56.7 %) than embryo flushes performed by GF (7/30, 23.3 %). The number of embryos recovered per ovulation was also higher (P = 0.011) in flushes performed with uterine massage (0.51 embryos per ovulation, 17/33) than in flushes performed by gravity flow (0.22 embryos per ovulation, 8/36). In the UM group, more embryos (P < 0.0001) were recovered in the first (16/17, 94.1 %; Table 2) compared with the second FA (1/17, 5.9 %). In contrast, in the GF group, embryos were as likely to be recovered in the first (4/15, 26.7 %) as in the second FA (4/15, 26.7 %). An extra FA performed with 0.5 L of ringer lactate and UM resulted in the recovery of more embryos (7/15, 46.6 %; P = 0.03) in the GF than in the UM group (0/17 embryos; Table 2). The mean embryo diameter was similar (P > 0.1) in embryos recovered from the first, second or extra flushing attempt (Table 3). No embryo was found in the tubing system (rinsed with 0.5 L of ringer lactate) in either group (Table 2). Three and two EF cycles from the GF and UM groups resulted in unwanted pregnancy, respectively (Table 2).

The likelihood of recovering an embryo during an extra flush (0.5L of media and uterine massage) was influenced by the operator's previous experience in EF (P = 0.01): unexperienced operators (OP 2 and 3) recovered fewer embryos (two embryos out of 25 flushing attempts in which an embryo had not been recovered previously, 2/25; 8 %) than the experienced operator (5/11, 45.4 %) Table 4. The embryo recovery rate for each operator is shown in Table 4. Four out of five EF cycles that resulted in unwanted pregnancy were performed by operators with no previous experience in EF.

There were 35 EF cycles in which the mares were known to have conceived: in 31 cycles at least one embryo was recovered during one of the three FA, and in four cycles with a negative embryo recovery, the mares became pregnant thereafter (unwanted pregnancy). These 35 cycles generated data from 66 FA (35 first, 17 s and 12 extra FA), in which an embryo was assumed to be in the uterus. These 66 FA resulted in either a positive (n = 32) or negative embryo recovery (n = 34 flushing attempts in which the embryo was left in the uterus). In these 66 cycles (Table 1B), the uterine massage (P = 0.001; OR = 26.47), and the degree of uterine horns' expansion by ringer's lactate infusion (P = 0.003; OR = 2.91; Fig. 1) influenced the likelihood of recovering the embryo. The median percentage of uterine horns' expansion (92.5 %) before an EF attempt that resulted in the recovery of the embryo was higher (P = 0.048) than that (45 %) of EF attempts that failed to recover the embryo (Fig. 1). In contrast, the amount of ringer's lactate left in the uterus (depth of pocket of fluid) after each FA (Fig. 2), the volume of media, or use of oxytocin and extended contact media-endometrium, did not influence the likelihood of recovering the embryo (P > 0.1; Table 1B).

Table 1A
Regression model output on the effect of different variables on the likelihood of recovering an embryo by the second flushing attempt (n = 60 cycles).

Variable	Levels	P value univariable analyses	P value multivariable analyses	Odds ratio multivariable analyses
Mare ID	1 to 10	0.667	–	–
Day of EF	7, 8 and 9	0.869	–	–
Operator's previous experience on EF	1 = yes, 0 = no	0.458	0.554	1.41
Uterine massage	1 = yes, 0 = no	0.033	0.034	3.49

The 60 EF cycles were performed either by uterine massage (n = 30) or by gravity flow (n = 30). The outcome (positive vs negative flush) was assessed by the end of the second flushing attempt.

Table 1B
Regression model output on the effect of different variables on the likelihood of recovering an embryo during a flushing attempt in mares known to have conceived an embryo.

Variable	Levels	P value univariable analyses	P value multivariable analyses	Odds ratio multivariable analyses
Mare ID	1 to 10	0.785	–	–
Day of EF	7, 8 and 9	0.179	0.442	1.37
Uterine massage	1 = yes, 0 = no	<0.0001	0.001	26.47
Oxytocin and 3 min contact	1 = yes, 0 = no	0.074	0.481	1.82
Volume of media	1L, 0.5 L	0.453	0.705	0.47
Degree of uterine horns' expansion (%)	0=< 50; 1 = 50–75; 2 = 75–95; 3 = 100	0.02	0.003	2.91
Depth of fluid left in uterus (mm)	0=<5; 1 = 5–20; 2 = 21–40; 3=>40	0.259	0.841	1.08

This set of data is composed of 35 EF cycles, and 66 flushing attempts: 32 resulting in the recovery of an embryo and 34 flushes in which the embryo was left in the uterus (negative flushes).

The order of flushing attempt influenced the degree of horn expansion (P < 0.0001; Fig. 3): the median uterine horns' expansion at the first flushing attempt (100 %) was greater than that of the second (60 %; 3 min after oxytocin administration) or extra FA (20 %, after 0.5L infusion of ringer lactate). Furthermore, the individual mare influenced (P = 0.006) the degree of uterine horns' expansion: the median horns' expansion at the first flushing attempt varied in individual mares from 20 % to 100 % (Fig. 4).

In 17 EF cycles (14 from the GF and 3 from the UM groups) one embryo was left in the uterus in at least one FA, from which 5 resulted in unwanted pregnancy. The details of these EF cycles are shown in Table 5. It is worth highlighting that from the 14 EF cycles from the GF group in which an embryo was left in the uterus after the first FA, the ringer lactate had expanded completely the uterine horns (100 % expansion) on seven occasions (7/14, 50 %) by the end of ringer lactate infusion.

4. Discussion

The main hypothesis of the study that uterine massage would result in the recovery of more embryos in the first flushing attempt compared to gravity flow is substantiated by the results of this study. The successful recovery of more embryos in the UM group compared to the GF may be the result of two actions: 1) enhanced embryo dislodgment from the endometrial folds as a result of mechanical force during uterine ballottement and squeezing of the myometrium during massage; 2) further reach of flushing media towards the tip of the horns (where some embryos could be located) as the uterus is squeezed in mares with a larger uterus in which 1L of media was not enough to fill up the horns completely. It is difficult to know which of the two actions have a higher impact on the embryo recovery. It is worth noting that in 50 % of the flushing attempts that an embryo was left in the uterus after the first flushing attempt in the GF group, the flushing media had reached both tips of the horns (100 % uterine horns' expansion), and yet the embryos remained in the uterus. On the other hand, the average horn expansion of negative flushes (45 %) was approximately half of the degree of horn expansion observed in positive flushing attempts. So, it is likely that both proposed actions contribute to an enhanced recovery rate during

Table 2
Effect of uterine massage on embryo recovery and the likelihood of recovering an embryo at different flushing attempts.

EF technique (n = cycles)	ER 1st attempt (%)	ER 2nd attempt (%)	ER by 2nd attempt (%)	ER extra attempt* (%)	Overall ER (%)	Embryos found in the tubing system	Embryos recovered in the 1st attempt (%)	Embryos recovered by the 2nd attempt (%)	Cycles with unwanted pregnancy
Uterine Massage (n = 30)	16/30 53.3[a]	1/14 7.1	17/30 56.7[a]	0/13 0.0[a]	17/30 56.7	0	16/17 94.1[a]	17/17 100.0[a]	2
Gravity flow (n = 30)	4/30 13.3[b]	3/27 11.1	7/30 23.3[b]	7/23 30.4[b]	14/30 46.7	0	4/15 26.7[b]	8/15 53.3[b]	3

EF: embryo flush; ER: embryo recovery (number of EF with at least one embryo recovered divided by the total number of EF). The uterine lavage of the extra attempt (*) was performed with uterine massage in both groups, using 0.5 L of ringer's lactate, while the remaining 0.5 L was used to rinse the tubing system. Within a column, different letters (a,b) indicate a significant ($P < 0.05$) difference in the embryo recovery.

Table 3
Relationship between embryo diameter and order of flushing attempt in which the embryo was recovered.

Embryo characteristics	Number of flushing attempt in which the embryo was recovered		
	First	Second	Extra
Embryos recovered (n)	20	5	7
Mean size ± S.E.M. (μm)	709 ± 118.4	740 ± 275.4	828.6 ± 184.5
Minimum size (μm)	150	200	200
Maximum size (μm)	1900	1700	1500

The mean embryo diameter did not differ ($P > 0.1$) amongst different flushing attempts.

uterine massage.

Over 90 % of embryos (16/17) were recovered in the first FA performed with ballottement and massage of the uterus (UM group). However, if we consider the 2 EF cycles which resulted in unwanted pregnancy in the UM group, the percentage of embryos recovered in the first FA would have been slightly lower (16/19, 84.2 %). This figure is higher than that of a previous study [9] in which the percentage of embryos recovered in the first set of flushes (three FA) was 67.8 % (59/87), with an extra flush (4th FA) resulting in the recovery of the remaining embryos (28/87, 32 %). In the previous study [9], the overall embryo recovery rate was 46 % (87/189). However, it was not clear whether every FA was performed by gravity flow or uterine massage. This difference in the EF technique may have accounted for the discrepancy in the results between the current and previous studies. In addition, in the current study the embryo filter was changed after each FA, so that there was no possibility of leaving the embryo in the filter. Nevertheless, it is clear there is always a chance of leaving an embryo in the uterus after one or several flushing attempt. Therefore, the practitioner should consider in each case the risk/benefit of continuing performing extra flushing attempts in a mare with a negative embryo recovery.

No embryo was found in the tubing system after rinsing the foley catheter and Y tube system with 0.5L of ringer lactate. This information is relevant since flushing the tubing system is a routine step of EF [3]: both in case the embryo might have remained there, and to rinse the collection filter, making it easier to search for the embryo. Given that the flushing of the system is done once the catheter has been removed from the uterus, passing through the cervix again and often touching the vaginal vestibule, it could cause contaminations in the filter and the embryo, affecting its post-transfer viability. Considering that the probability of the embryo being there is very low, the possible risks and benefits of this part of the procedure should be re-considered.

The lower embryo recovery result of the current study in the gravity flow group (without uterine manipulation per rectum) is in contrast with the results of a previous study [10] in which 17 embryos were recovered from 20 embryo flushes by the second FA, and further 5 embryos were recovered in the third flushing attempt using gravity flow (with no uterine massage). The study by Hinrichs [10], however, allowed a 3 min waiting time in every flushing attempt before recovering the media (without oxytocin administration). Furthermore, most of the embryo flushes of the previous study were performed 10–11 days after ovulation. Whether the larger embryo size (>2–3 mm) compared to the current study (<1.5 mm) or the extended period of flushing media contact with the endometrium were responsible for the higher embryo recovery by gravity flow is unknown and requires further research. The author of the previous study [10] proposed that the extended 3 min contact time of the flush fluid with the uterus would allow extra time for embryo mobility to move into the fluid (in case the flushing media had not contacted it by the end of infusion), and thus be collected. In the current study, a 3 min extended period was allowed before recovery of ringer lactate in the second FA; however, oxytocin also was used at the same time, which could have interfered with embryo mobility and uterine horns' expansion during ringer lactate infusion. On the other hand, the embryo size, or Day of EF (Day 7, 8 or 9) did not influence the likelihood of recovering the embryo in the current study. This observation agrees with the results of previous studies [7].

Since the degree of uterine horn expansion by the end of flushing

Table 4
Effect of operator performing the embryo flush on embryo recovery during three different flushing attempts (FA).

Operator ID	Experience in EF	EF technique (number of EF)	embryos 1st FA	embryos 2nd FA	embryos Extra FA	Overall ERR (%)	Overall embryos/ Ov	unwanted pregnancy (%)
1	yes	UM (n = 10)	6	0	0	14/20	14/22	1
		GF (n = 10)	3	0	5	70.0	0.63	
2	no	UM (n = 10)	5	1	0	8/20	8/24	2
		GF (n = 10)	0	1	1	40.0	0.33	
3	no	UM (n = 10)	5	0	0	9/20*	10/23	2
		GF (n = 10)	1	3	1	45.0	0.43	

EF: Embryo flush cycle. An extra FA was performed with 0.5 L of ringer's lactate and uterine massage (UM); GF: gravity flow; EF: embryo flushing. An asterisk (*) indicates that OP3 obtained a positive twin EF in the GF group with the recovery of two embryos (in the first and second FA, respectively). ERR: embryo recovery rate (number of positive EF divided by the total of EF). Ov: ovulations. The operator's previous experience in embryo flush influenced the likelihood ($P = 0.01$) of recovering an embryo during an extra flush: Operator 1 recovered five embryos out of 11 embryo flushing attempts in which an embryo had not been recovered previously, while unexperienced operators [2and3] recovered only two embryos out of 25 embryos in which an embryo had not been recovered in the previous two flushing attempts.

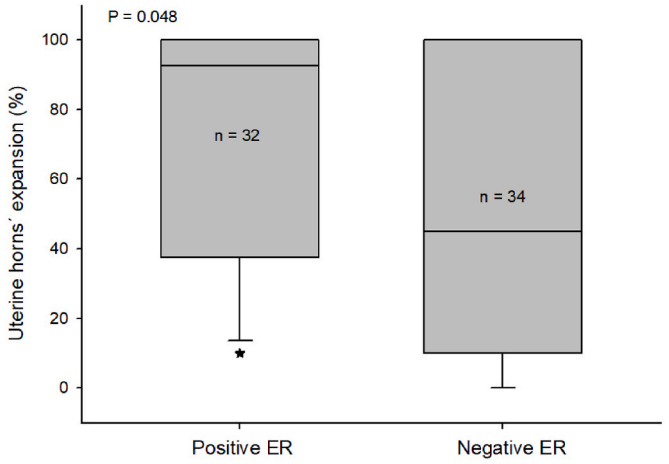

Fig. 1. Boxplot distribution of degree of uterine horns' expansion during ringer's lactate infusion in flushing attempt of mares known to have at least an embryo in the uterus. The median percentage of uterine horns' expansion was higher (P = 0.048) in flushing attempts with a positive embryo recovery (ER) than in flushing attempts with a negative ER. The number of flushing attempts in each group is shown (n = 32 and n = 34). Asterisks (*) indicate outliers.

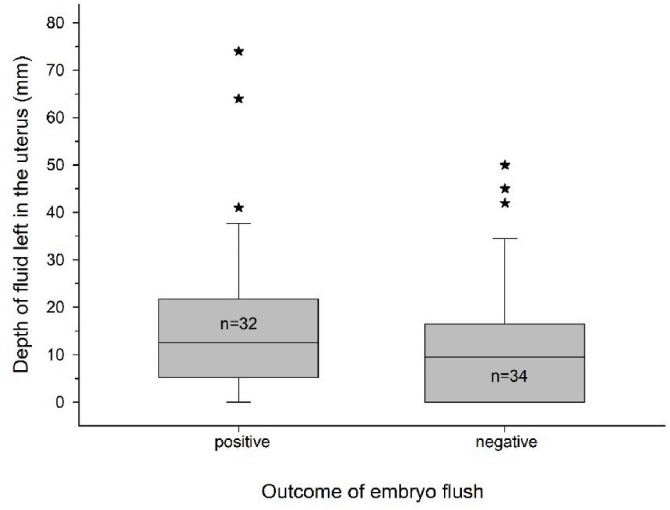

Fig. 2. Boxplot distribution of the depth of ringer's lactate left in the uterus (mm) after a flushing attempt with a positive (recovery of an embryo) or negative outcome (the embryo was left in the uterus) in flushing attempts from mares known to have at least one embryo in the uterus. Asterisks (*) indicate outliers. The median depth (mm) of fluid was not different (P > 0.05) between positive and negative flushing attempt.

media infusion affected embryo recovery, it is worth studying the factors that influenced it. Firstly, there was a high individual mare variation in the degree of uterine horn expansion after infusion of 1L of ringer lactate during the first FA, which can be explained, at least in part, to differences in the uterine volume from maiden and multiparous mares. However, it was also noted that some mares showed a variation (i.e. from 50 to 100 %) in the degree of uterine horns' expansion between consecutive EF cycles during the first FA. So other factors, different from the intrinsic uterine volume, must influence the degree of horns' expansion. Some clinicians [15] have proposed that a full bladder may interfere with the homogenous filling of the uterus with flushing media, as it can pull the uterine horn and prevent it from filling completely. In this study, no attempt to empty the bladder was made and, unfortunately, the size and degree of fullness was not recorded. Furthermore, the use of oxytocin significantly decreased the degree of uterine horns'

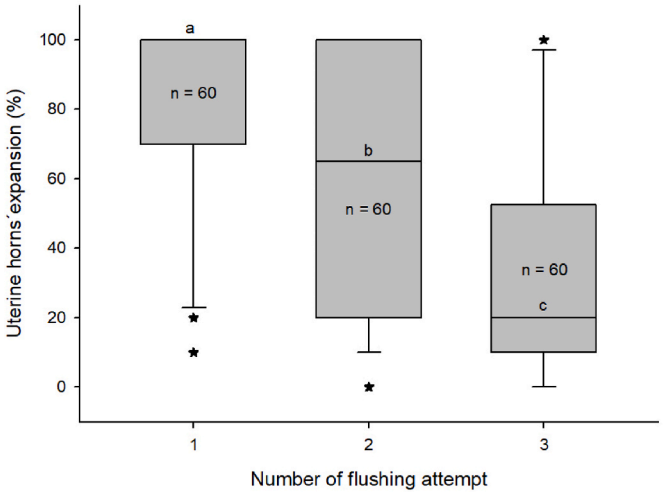

Fig. 3. Boxplot distribution of degree of uterine horns' expansion during infusion of ringer's lactate (RL) in three consecutive flushing attempts (FA). In FA 1 and 2 the uterus was infused with 1L of RL, while in FA 3 0.5L of RL was used. In FA 2, the mare received 20 IU of oxytocin 3 min before assessing the degree of horn expansion. The median percentage of uterine horns' expansion differed amongst flushing attempts (a,b, c: P < 0.0001). The number of FA in each group is 60. Asterisks (*) indicate outliers.

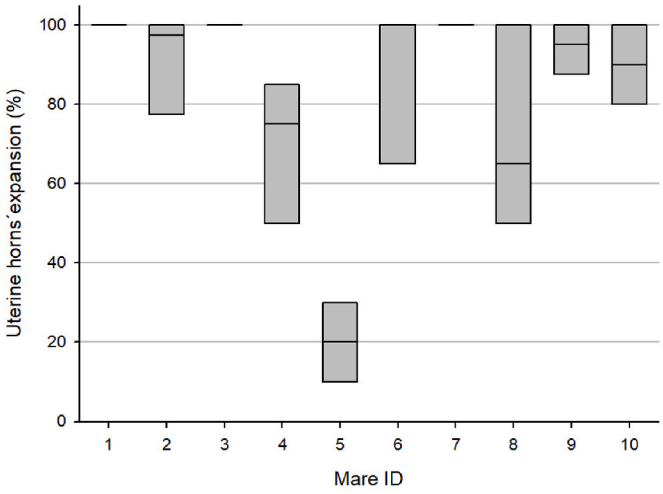

Fig. 4. Boxplot distribution of degree of uterine horns' expansion in 10 individual mares during infusion of 1 L of ringer's lactate in 6 consecutive cycles assessed before the first flushing attempt.

expansion. Uneven uterine contractions from the ecbolic effect of oxytocin may have led to partial closure of the uterine lumen and prevention of flushing media entry up the horns. High doses of oxytocin can cause tetanic contractions while lower doses produce milder and constant contractions [16] of a part of the uterus, retaining part of the fluid administered for flushing. In the second FA, contraction of a section of the horn was observed in several mares, resulting in fluid retention in the distal part and preventing its evacuation. In the current study, oxytocin did not appear to increase embryo recovery in contrast to previous studies [9]. The discrepancy between studies may be a result of differences in the study design between the current and the previous study [9]: The oxytocin was administered at a later stage during the procedure and there is no information on uterine massage [9]. Further research including different doses of oxytocin (i.e. high *vs* low) and flushing media volumes should be carried out to elucidate the effect of oxytocin on embryo recovery.

Table 5
Embryo flushing characteristics of mares in which an embryo was not recovered after the first or subsequent flushing attempts.

Mare ID	Number OV	Day of OV at EF	Operator	EF technique	1st EF attempt			2nd EF attempt (20 IU oxytocin)			Extra EF attempt (0.5 L + UM)			Unwanted pregnancy	
					Embryo size	Horns' expansion	Fluid left	Embryo size	Horns' expansion	Fluid left	Embryo size	Horns' expansion	Fluid left	Day of PD	Vesicle size
10	1	7	1	GF	–	80	10	–	60	5	200	50	0		
5	2	8	2	GF	–	20	0	–	20	12	–	20	0	15	19 mm
8	1	8	1	GF	–	100	7	–	70	0	900	50	0		
7	1	7	1	GF	–	100	0	–	100	0	450	100	5		
2	2	7	2	GF	–	70	7	–	0	16	–	0	0	14	15 mm
10	1	8	3	GF	–	80	12	–	60	8	850	30	10		
3	1	8	1	GF	–	100	13	–	90	0	500	10	0		
5	1	7	3	GF	–	30	0	–	0	26	–	0	20	14	16 mm
7	1	9	2	GF	–	100	9	1700	100	10	NA	NA	NA		
4	1	9	1	GF	–	75	42	–	20	20	1400	10	9		
6	2	7	3	GF	–	20	5	450	15	11	NA	NA	NA		
3	2	7	3	GF	300	100	16	200	90	13	NA	NA	NA		
6	1	8	2	UM	–	100	50	1000	90	74	NA	NA	NA		
1	1	9	2	GF	–	100	0	–	100	16	1500	40	13		
5	2	7&8	1	UM	250	20	18	–	10	0	–	0	15	14	15 mm
6	1	7	3	GF	–	100	27	350	100	64	NA	NA	NA		
5	1	7	3	UM	–	10	45	–	0	12	–	0	0	13	14 mm

Operators 2 and 3 had no previous experience on embryo flushing (EF); GF: gravity flow, UM: uterine massage; OV: ovulation; Embryo size is expressed in μm; Horns' expansion refers to the proportion (%) of the uterine horns that were filled with media (ringer's lactate) just before the EF attempt; Fluid left: refers to the depth (mm) of media (ringer's lactate) left in the uterus after each flushing attempt. Day of PD: Day after ovulation in which a pregnancy diagnosis was performed in mares that became pregnant after an EF; The size of the embryonic vesicle is reported in mm.

Lastly, the amount of ringer lactate used to infuse the uterus had, as expected, a significant effect on the degree of uterine horns' expansion, with 0.5L of media inducing lower horn expansion that in the FA with 1L of media. For some mares, even 1L of ringer lactate was clearly insufficient to fill the uterus completely (i.e. mares ID 4 and 5). In fact, mare ID 5 retained an embryo in the uterus on 4 different EF cycles, resulting in unwanted pregnancy. Although, a delayed oviductal descent of the embryo cannot be ruled out, it is more likely that an inadequate filling of the uterus during ringer lactate infusion was responsible for the embryo being left in the uterus, despite uterine massage. From these observations, it can be concluded that a complete uterine horn' expansion and effective uterine massage are paramount to maximize embryo recovery.

Surprisingly, the amount of fluid left in the uterus (depth of pocket of fluid) was not associated with the likelihood of leaving the embryo in the uterus. Most of the times, the pocket of fluid remained in the anterior uterine body and/or bifurcation of the horns, as it was difficult to reach the pocket of fluid with the tip of the foley catheter without excessive manipulation to drain it. This finding adds further evidence that the main reason for the embryo being left in the uterus appears to be a failure of dislodgement of the embryo from the endometrium due to either inadequate shaking forces (ballottement and massage of the uterus) and/or failure of the flushing media to reach the embryo when located in distal parts of the uterine horn.

The previous operator's experience in EF appeared to influence the embryo recovery during flushing attempts from the UM group. However, this effect was only evident during the extra flush in which 0.5L of ringer lactate was used. This may reflect a more efficient and vigorous uterine massage technique of the experienced operator to reach a larger surface of the uterine horns to dislodge the embryo from the endometrium despite using a reduced volume of ringer's lactate. A previous study [17] did not find any effect of operator on embryo recovery rate. However, all operators were trained and had sufficient previous experience in embryo flushing unlike in the current study. Operator 3 was a final year vet student, with no previous experience in EF and basic skills in rectal palpation and ultrasonography. Anecdotally, in the 10 EF that operator 3 performed with UM, the first 5 flushes resulted in only one positive recovery (1/5, 20 %), while the last 5 flushes of the study yielded 4 positive embryo recoveries (4/5, 80 %). The transrectal manipulation of the uterus when performing the uterine massage and the manipulation necessary to remove the fluid (if retained) requires skill in handling both the uterus and adjacent structures, such as the cecum, colon, or bladder. The major difficulties reported by the inexperienced operators during flushes were related to the pressure/force to move the uterus (fear of tearing the rectum during the process), the inability to grasp the uterine horns correctly, and lack of practice in manipulating intrauterine fluid (both to ensure that the fluid reaches the entire surface of the horns during the massage and creates enough turbulence, and when removing the fluid that had not come out during the flush).

Unwanted pregnancy following embryo flush has been reported previously [7,18] and is an undesired outcome. The incidence of unwanted pregnancy in the current study is relatively high because luteolysis was not induced in donor mares. Furthermore, one individual mare is overrepresented as being responsible for 4 of the 5 cycles with unwanted pregnancy. Interestingly, this mare (ID 5) had the lowest degree of uterine horns' expansion (with a median of 20 % of the total of her uterine horns being expanded after 1L of ringer lactate infusion during the first FA of 6 consecutive cycles). Other cause of unwanted pregnancy is performing the embryo flushing before the embryo has descended into the uterus, either in asynchronous twin ovulations, or when the clinician attempts to recover a small embryo for vitrification on Day 6 from a single or synchronous twin ovulation [7]. Most embryos enter the uterus between 144 and 156 h after ovulation (between Day 6 and 6.5) [12]. In the current study, all cycles that resulted in unwanted pregnancy had the EF performed on Day 7 or 8 after ovulation, and the embryonic vesicle size at pregnancy diagnosis matched with the

referenced values for the expected diameter and age [19]. Nevertheless, it cannot be ruled out that some of these 5 embryos resulting in unwanted pregnancy were still in the oviduct at the time of EF. Similarly, the fact that a mare did not become pregnant after EF, does not mean that an embryo could not have been left in the uterus. An unrecovered embryo may die following an embryo flush for different reasons: 1) endometritis from inflammation during the EF due to bacterial contamination or irritation from UM or contact with the flushing media; or 2) embryos with pre-existent abnormalities which were destined to fail anyway [20].

Therefore, these issues are limitations of the study, as it cannot be ascertained whether all mares with unwanted pregnancy had an embryo in the uterus at the time of EF and that all EF cycles with a negative recovery did not have an embryo in the uterus. Further limitations of the study were the relatively small sample size of mares with repeated use for several cycles (10 mares used for 6 cycles each), and the lack of foaling mares in the experimental herd. Foaling mares have a larger uterus with uneven size of uterine horns (gravid *vs* non-gravid horn) which is likely to affect the expansion of the uterine horns and distribution of flushing media during infusion.

In conclusion, performing uterine massage during embryo flushing resulted in a higher embryo recovery compared with the recovery of flushing media by gravity flow. Furthermore, more embryos were recovered in the first flushing attempt in the UM than in the GF group. The degree of uterine horns' expansion during infusion of flushing media was associated with the likelihood of recovering an embryo. In contrast, the use of oxytocin and the amount of unrecovered fluid after each flushing attempt did not affect embryo recovery. Despite three flushing attempts with uterine massage, it is still possible to leave an embryo in the uterus, especially if the entire uterus is not filled with flushing media.

CRediT authorship contribution statement

Laura Sala-Ayala: Writing – review & editing, Writing – original draft, Investigation, Data curation. **Rebeca Martínez-Boví:** Writing – review & editing, Investigation, Data curation, Conceptualization. **Aurora Querol-Paajanen:** Writing – review & editing, Investigation, Data curation. **Juan Cuervo-Arango:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2024.05.017.

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