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Original Research

The Effect of Different Flushing Media Used to Aspirate Follicles on the Outcome of a Commercial Ovum Pickup–ICSI Program in Mares

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ABSTRACT

The in vitro production of embryos by ovum pickup (OPU) and intracytoplasmic sperm injection (ICSI) is gaining popularity among horse breeders and veterinarians. Various collection media are available for flushing follicles during OPU. The objective of this study was to determine whether the type of flushing media used to aspirate follicles and collect oocytes influences the outcome of a commercial equine OPU-ICSI program. Two commercial embryo flushing media (EFM1 and EFM2) supplemented with heparin were compared with a flushing media designed specifically for the collection of oocytes (oocyte flushing media [OFM]) on the outcome of OPU-ICSI parameters in 234 Warmblood mares. The OPU-ICSI performed in mares using one of the EFM1 resulted in a lower ($P < .05$) blastocyst rate and blastocysts per OPU-ICSI session (11.9 ± 13.2%, 0.88 ± 1.3) than the OFM (19.2 ± 15.2%, 1.24 ± 1.2). Unlike the EFM2 solution, the heparin used to prepare the EFM1 contained preservatives including benzyl alcohol, a component known to alter the oocyte membrane, which might have been responsible for the lower developmental competence of oocytes collected with EFM1. In conclusion, exposure of oocytes (<1.5 hours) to one of the flushing medium tested in this study affected negatively the outcome of the OPU-ICSI commercial program when compared with flushing media designed for collection of equine oocytes. Care should be taken when choosing the components of the flushing media used to collect oocytes. Further research should be carried out to confirm the potential negative effect of the preservatives used in multidose heparin vials.

1. Introduction

In vitro production (IVP) of embryos by intracytoplasmic sperm injection (ICSI) is gaining popularity among horse breeders and veterinarians. The most common procedure for collecting oocytes for ICSI is ovum pickup (OPU) of immature follicles by transvaginal ultrasound-guided aspiration [1,2]. Various collection media are available for flushing follicles during OPU, with the most commonly used being commercial embryo flushing solutions [3,4]. Heparin is added to the medium to prevent coagulation of collected effluent, which would complicate the oocyte search. Commercial media specifically marketed for oocyte collection in horses are available and already contain heparin to prevent clotting [5]. Despite many types of flushing media and heparin products being reported in studies involving OPU and IVP of equine embryos [4,6–8], little attention has been paid to possible effects of the type of media used on oocyte developmental competence and subsequent IVP of embryos. However, it is difficult to assess the potential effect of different flushing media on oocyte developmental competence among different studies because the outcome of an OPU-ICSI clinical program varies greatly between laboratories and within mares [9].

In equine clinics with busy OPU-ICSI commercial programs where a large number of follicle aspirations are performed per week, the type of flushing media chosen to perform the OPU can be relevant to the management of the practice for practical and economical reasons. The commercial media specifically marketed for oocyte collection may be more convenient, as it has already the heparin included in the formulation. However, it needs to be stored at refrigeration temperature and has shorter expiration date than commercial embryo flush media. Although embryo flush media are significantly less costly than oocyte collection media, some

Animal welfare/ethical statement: The authors declare that this research article has been carried out according to the ethical guidelines of this journal. The data used belonged to the clinical program of OPU-ICSI, and therefore, all data from horses belonged to client mares handled for clinical reasons.

Conflict of interest statement: None of the authors have any conflict of interest to declare.

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commercial source of heparin (Sigma, heparin sodium salt #H3149-100KU) can increase the cost of the final preparation.

The objective of this study was to determine whether the type of flushing media used to aspirate follicles and collect oocytes influenced the outcome of a commercial equine OPU-ICSI program (blastocyst rate and number of blastocysts per OPU-ICSI session).

2. Materials and Methods

The experimental design was a prospective randomized clinical trial performed with mares enrolled in the commercial OPU-ICSI program of the Equine Clinic of the Veterinary School of Utrecht University (The Netherlands) in collaboration with the assisted reproduction laboratory of Avantea (Cremona, Italy) for in vitro maturation (IVM) of oocytes, ICSI, and embryo culture. A total of 234 Warmblood mares with a mean age of 14.1 ± 5.9 (range 2–27) years were enrolled in the study during February 2017 to May 2018. The experimental groups consisted of two different embryo flushing media (EFM) supplemented with heparin (EFM1 and EFM2) and one oocyte flushing media (OFM) specifically marketed for the aspiration of equine follicles and collection of oocytes (EquiPRO OPU recovery medium; MOFA Group LLC, Verona, WI, USA). The OFM was considered as the control group as it is designed specifically for the collection of oocytes and has heparin already included in the formulation. The OFM was compared with each of the two EFM (EFM1 and EFM2) in two different contemporary periods.

2.1. Period of Study 1

The EFM1 was the medium used in the commercial OPU program of this clinic since the start in 2014, and it was a commercial medium designed for equine embryo flushing containing bovine serum albumin (BSA) and antibiotics (EUROFLUSH 1 L; IMV Technologies, Leeuwarden, The Netherlands) supplemented with 20 IU/mL heparin sodium (Heparin Leo 5 mL multidose vials with 25,000 IU Heparin sodium, Leo Pharma, Denmark) as reported in the study by Claes et al [8]. During the period of February to November 2017, 130 mares were aspirated using either EFM1 or OFM in the following way: OPU was performed during 2 days a week (Monday and Tuesday), with 4–8 mares aspirated per day. For each day, the first half of the mares were done with one flushing medium and the second half with the other medium. The order of the collection media used for the first half of mares was changed alternatively every OPU day. In total, 130 Warmblood mares (65 for each group) were used for this period.

2.2. Period of Study 2

In this period, the OFM was compared to EFM2: a commercial embryo flushing medium with BSA and antibiotics (ViGro complete embryo flush media; Vetoquinol N.-A Inc, Princeville, Canada) supplemented with heparin sodium (Heparin sodium salt #H3149-100KU, Sigma-Aldrich, Zwijndrecht, The Netherlands) at 8 IU/mL as reported in the study by Jacobson et al [7]. During February 2018 to May 2018, follicles from 104 Warmblood mares were aspirated with one of the two flushing media in a random fashion (52 mares for each group) as in period 1.

2.3. OPU Procedure

The OPU procedure was performed by the same team at the same clinic for both study periods, regardless of the flushing media used. In brief, perioperative antibiotic treatment, analgesia, and sedation were initiated by administering broad spectrum intravenous antibiotics (gentamycin and benzyl penicillin), flunixin-meglumine, detomidine hydrochloride, and butorphanol tartrate immediately before the OPU procedure. Epidural anesthesia was induced with 8 mL of 2% lidocaine hydrochloride. After aseptic scrubbing of the perineum, the vagina was cleaned by flushing with sterile phosphate-buffered saline via a speculum, and a urinary catheter was inserted. Ovum pickup was performed by transvaginal ultrasound-guided follicle aspiration via a 12G double lumen needle using a vacuum pump (Cook Veterinary Products, The Netherlands). All antral follicles larger than 3 mm were punctured, follicular fluid was aspirated, the follicular wall scraped by twisting the needle, and each follicle was flushed 8 to 10 times with 0.5–5 mL (depending on the follicle size) with the specific flushing medium prewarmed at 37°C. Follicular fluid and flushing medium were collected into sterile 50 mL conical tubes maintained at 37°C. The collection effluent was poured through a sterile 70 μm filter immediately after the end of the OPU procedure. The contents of the filter were emptied into a sterile petri dish containing the same flushing medium (as used for OPU). Subsequently, oocytes were identified using a stereomicroscope, washed three times, transferred into a 2.2 mL CRYOVIAL (Nunc storage vials, Thermo Fisher Scientific, Waltham, MA, USA) containing 2 mL of modified Hepes Synthetic Oviductal Fluid (mH-SOF), and shipped overnight in a polystyrene box designed for transporting organs for transplantation, at 22°C to a different laboratory (Avantea, Italy) for IVM, ICSI, in vitro culture (IVC), and embryo cryopreservation. The total time during which the oocytes were in contact with the flushing media (from follicular aspiration to transfer to mH-SOF medium) ranged between 50 and 80 minutes (20–40 minutes for OPU procedure and 30–40 minutes for oocyte search). The information on which flushing medium was used for each mare was not disclosed to the IVF laboratory, so it was blinded.

2.4. IVM, ICSI, and IVC Procedures

In vitro maturation of oocytes, ICSI, and IVC to produce blastocysts was performed as described by Colleoni et al [10] with one of 73 different stallions chosen by the mare's owners.

2.5. Main Outcomes Analyzed

- Recovery rate (%): Number of oocytes recovered per number of follicles aspirated per mare.
- Maturation rate (%): Number of oocytes that reached MII within 30 hours of maturation (presence of at least one polar body, regardless of size and shape), which were submitted for ICSI, per all oocytes recovered.
- Cleavage rate (%): Number of oocytes cleaved (zygotes with 2 or more cells) by 48 hours after ICSI per the total number of injected oocytes per mare.
- Blastocyst rate (%): Number of blastocysts developed 6–8 days after ICSI per the total number of injected oocytes. A cleaved zygote was considered a blastocyst when it showed cell alignment of the presumptive trophoblast between 6 and 8 days after ICSI. Delayed blastocysts (>8 days after ICSI) were discarded.
- Blastocysts per OPU: number of day 6–8 blastocysts (day 0 = day of ICSI) per the number of OPU sessions (1 OPU per mare).
- Successful OPU-ICSI (%): Number of OPU-ICSI sessions that resulted in the production of at least one blastocyst per the total number of OPU-ICSI sessions.

2.6. Statistical Analyses

For each period of study, a separate general linear model of variance (Systat 13) was created for each dependent variable to be studied: recovery rate, maturation rate, cleavage rate, blastocyst rate,

Table 1
Effect of flushing medium used to aspirate follicles on OPU-ICSI parameters in mares submitted for OPU in a commercial program during period 1 (February to November 2017).

Flushing Medium	Aspirated Follicles	Recovered Oocytes	Recovery Rate (%)	Maturation Rate (%)	Cleavage Rate (%)	Blastocyst Rate (%)	Blastocysts per OPU	Successful OPU-ICSI (%)
EFM1 (n = 65)	22.9 ± 9.7 (6–56)	12.8 ± 6.1 (2–30)	55.5 ± 13.1 (29–100)	60.7 ± 16.3 (14–100)	67.4 ± 24.1 (0.0–100)	11.9 ± 13.2[a] (0.0–80.0)	0.88 ± 1.3[a] (0–7)	49.2[a]
OFM (n = 65)	21.9 ± 7.0 (10–45)	11.8 ± 7.2 (3–29)	54.7 ± 12.9 (31–100)	56.4 ± 17.1 (16–100)	69.0 ± 25.8 (9.8–100)	19.2 ± 15.2[b] (0.0–100)	1.24 ± 1.2[b] (0–6)	63.1[b]

Abbreviations: OPU-ICSI, ovum pickup–intracytoplasmic sperm injection; EFM1, Embryo flushing medium 1; OFM, Oocyte flushing medium.
The number of mares submitted for OPU for each flushing media group is shown in brackets (65 mares for each group).
Different superscripts (a and b) indicate a significant difference ($P < .05$).
For each parameter, the range (maximum and minimum values) is indicated in brackets.
EFM1: Embryo flushing medium 1 (EUROFLUSH, IMV Technologies, The Netherlands) supplemented with heparin sodium 20 IU/mL (Heparin Leo 25,000 IU, Leo Pharma, Denmark).
OFM: Oocyte flushing medium (EquiPRO OPU Recovery, MOFA Group LLC).

and blastocysts per OPU-ICSI session. Each model included an independent variable: the age of the mare, the number of oocytes injected per session, and the type of flushing medium used for OPU. The stallion identity was included in the models for cleavage rate, blastocyst rate, and blastocyst per OPU-ICSI session, as a random effect. All sequential data were tested for normality with Shapiro-Wilk test. Data not normally distributed were submitted for logarithmic transformation before being computed in the model. Binary logistic regression was used to test the effect of flushing media on the percentage of successful OPU-ICSI sessions. The regression model included the age of the mare, the number of injected oocytes, the type of flushing medium, and the stallion identity used as a random effect. The mean age of mare for each group was compared using unpaired student *t*-test. Data are reported as percentage (%) or mean ± standard deviation. Significance was set at $\alpha \leq .05$.

3. Results

3.1. Period of Study 1

The mean age of mare did not differ significantly in the two flushing media groups: 13.9 ± 5.1 and 13.6 ± 5.3 years old, for the OFM and EFM1, respectively. The use of EFM1 was associated with a lower ($P < .05$) blastocyst rate, blastocysts per OPU-ICSI sessions, and percentage of successful OPU-ICSI sessions (11.9 ± 13.2%, 0.88 ± 1.2, and 49.2%, respectively) compared with the OFM (19.2 ± 15.2%, 1.24 ± 1.7, and 63.1%, respectively; Table 1). The type of flushing medium did not influence ($P > .05$) the recovery rate, maturation rate, or cleavage rate (Table 1).

3.2. Period of Study 2

The mean age of mare did not differ significantly in the two flushing media groups: 14.1 ± 6.1 and 14.6 ± 5.9 years old, for OFM and EFM2, respectively. None of the OPU-ICSI parameters studied was influenced ($P > .05$) by the type of flushing medium used (Table 2).

4. Discussion

The results of this study indicated that one of the flushing media used to aspirate follicles influenced negatively the outcome of the OPU-ICSI program at the blastocyst production rate level and, in turn, the total number of blastocysts produced per OPU-ICSI session, because the number of oocytes arriving at the ICSI laboratory was similar in both groups. This negative effect of the flushing medium took place despite the relatively short time that the oocytes were in contact with the medium (under 1.5 hours). Because the two commercial EFM used in the study report similar components in their formulation (surfactant as BSA and antibiotics) and have been tested and approved for safe use in equine and bovine embryos, it is unlikely that the EFM1 itself was responsible for the lower production of blastocysts. Nevertheless, it cannot be ruled out that other unknown components in the EFM1 might also have played a role in the reduction of the outcome of the OPU-ICSI commercial program.

Different products of commercial heparin have been used to prepare flushing media to aspirate follicles during OPU [5]. Although all of these contain heparin sodium, the excipients used to manufacture the product can be different. Most studies that reported the use of EFM supplemented with heparin used heparin sodium diluted in water for injection (without preservatives): commercial monodose vials of 1 mL with heparin sodium EDTA (Clarisco Forte, Happyfarma, Italy) [6] or heparin sodium from pig intestinal mucosa (Sigma, heparin sodium salt #H3149-100KU) [7]. However, there is also commercially available multiple-dose 5-mL vials containing heparin sodium and preservatives such as benzyl alcohol and parabens (methyl parahydroxybenzoate and propyl

Table 2
Effect of flushing medium used to aspirate follicles on OPU-ICSI parameters in mares submitted for OPU in a commercial program during period 2 (February to May 2018).

Flushing Medium	Aspirated follicles	Recovered Oocytes	Recovery rate (%)	Maturation rate (%)	Cleavage rate (%)	Blastocyst rate (%)	Blastocysts per OPU	Successful OPU-ICSI (%)
EFM2 (n = 52)	22.2 ± 7.5 (9–51)	12.3 ± 5.6 (3–27)	55.7 ± 12.1 (33–89)	60.5 ± 18.9 (14–100)	70.8 ± 28.1 (0–100)	15.6 ± 17.1 (0–62.5)	1.27 ± 1.2 (0–6)	57.7
OFM (n = 52)	23.5 ± 7.7 (12–43)	13.3 ± 4.7 (3–30)	56.0 ± 12.2 (31–90)	57.0 ± 25.6 (15–100)	64.5 ± 21.6 (0–100)	18.9 ± 16.8 (0–100)	1.44 ± 1.3 (0–10)	63.5

Abbreviations: OPU-ICSI, ovum pickup–intracytoplasmic sperm injection; EFM2, Embryo flushing medium 2; OFM, Oocyte flushing medium.
The number of mares submitted for OPU for each flushing media group is shown in brackets (52 mares for each group).
None of the parameters studied differed significantly ($P > .05$).
For each parameter, the range (maximum and minimum values) is indicated in brackets.
EFM2: embryo flushing medium 2 (ViGro complete embryo flush, Vetoquinol, Canada) supplemented with heparin sodium at 8 IU/mL (Heparin sodium salt #H3149-100KU, Sigma-Aldrich, The Netherlands).
OFM: oocyte flushing medium (EquiPRO OPU Recovery, MOFA Group LLC).

parahydroxybenzoate) (Heparin sodium 5,000 IU/mL, Heparin Leo; Leo Pharma, Denmark) [8]. In our clinic, we used this type of heparin (Heparin Leo with preservatives) to supplement the EFM1 since the start of our OPU commercial program in 2014, for no other reason that it was readily available in the Hospital's Pharmacy.

Because the heparin containing preservatives was only used to supplement the EFM1 but not the EFM2, it is plausible to think that some of the preservatives present in the multidose vials (and not the components of the embryo flushing medium) may have been responsible for the lower blastocyst rate observed in the first period of this study. It has been shown that benzyl alcohol has a strong depolarizing potential activity in the membrane of oocytes from mice, inducing a dramatic rise in intracellular $Ca2+$ after exposure to 0.3% solution [11,12]. According to the data sheet information of the manufacturer, the heparin product used in EFM1 added a total of 40 mg of benzyl alcohol to 1L of the flushing solution. Whether this quantity was able to interfere with the developmental competence of the oocytes is unknown. Furthermore, there is evidence of the toxicity of parabens in mitochondrial activity even at low concentrations in testicular and hepatic cells [13]. Further research should be carried out using experimental oocytes to test the specific effects of these preservatives (benzyl alcohol and parabens) on oocyte quality and IVP of equine blastocysts.

In the second period of the study, the specific embryo flushing medium (EFM2) and the heparin was chosen based on previous reports which already used these products successfully [4,7]. According to the manufacturer's information (Sigma), this heparin sodium was obtained from pig intestinal mucosa and was diluted in water for injection, without the addition of any preservatives. Despite being around 10 times more expensive than the heparin in multidose vials, its use is justified because the addition to EFM2 did not affect the IVP of blastocysts compared with that of mares aspirated with OFM.

In conclusion, a short exposure of the oocytes (<1.5 hours) to one of the flushing media supplemented with heparin sodium and preservatives tested in this study affected negatively the outcome of the OPU-ICSI commercial program when compared with the flushing medium specifically designed for collection of equine oocytes. Care should be taken when choosing the components of the flushing medium used to collect oocytes. Further research should be carried out to confirm the potential negative effect of the preservatives used in multidose heparin vials on oocyte quality.

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