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Original Research Article

Use of excised ovaries for oocyte recovery by ultrasound guided follicular aspiration – Validation of an experimental model for research purposes in live mares ovum pick up

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ABSTRACT

Ovum pick-up (OPU) by transvaginal ultrasound guided follicle aspiration in mares is a common assisted reproductive technique used for oocyte recovery and *in vitro* production of horse embryos. There has been relatively little research into the factors influencing oocyte recovery in OPU from live mares. The objective of this study was to compare oocyte recovery and morphology of ultrasound-guided follicle puncture and aspiration in live mares and in *postmortem* excised ovaries, in order to validate an experimental model for research purposes of the efficiency of OPU in mares. Data from OPU performed in 12 mares from a commercial program (follicle numbers, oocyte recovery and oocyte morphology) were compared to that obtained from ultrasound-guided follicle puncture of 13 postmortem excised ovaries from slaughtered mares processed within 2 h of slaughter. In both groups, the OPU was performed by the same operator using the same equipment and OPU technique. The recovered oocytes per aspirated follicle was higher ($P < 0.05$) in the *postmortem* group (105/166, 63.2 %) than in live mares (138/261, 52.9 %). There was more ($P < 0.05$) expanded cumulus oocyte complexes in the *postmortem* than in the live mares (18 % vs. 2.9 %). Several oocytes (5 oocytes from 81 aspirated follicles) were found in the leaked fluid which overflowed during follicle flushing of *postmortem* ovaries. In conclusion, the higher recovery rate obtained in the excised ovaries and the finding of oocytes in the leaked fluid during OPU, suggests that there is still room for improvement in the *in vivo* OPU technique. Utilizing *postmortem* excised ovaries could offer an alternative for further research into factors affecting oocyte recovery and oocyte leakage during OPU procedures.

1. Introduction

The technique of ovum pick-up (OPU) in mares has significantly evolved [1] since its inception, adapting methods initially used in human [2] and cattle [3] reproductive medicine. The method of transvaginal ultrasound-guided oocyte retrieval, first described in the early 90' [4–6] and now commonly employed in equine reproduction, was a significant leap from more invasive techniques previously used, which involved aspiration of pre-ovulatory sized follicles via colpotomy [7] and laparotomy [8]. The methods have been refined to ensure higher oocyte recovery and better quality, which are crucial for successful *in vitro* embryo production. Obtaining embryos from OPU allows for multiple pregnancies per year from valuable mares, including those that are older, competing, or subfertile.

The *in vitro* production of horse embryos by OPU of immature oocytes and intracytoplasmic sperm injection (ICSI) is currently the most efficient assisted reproductive technique available in the equine breeding industry in order to obtain the highest number of embryos per mare and year with an average of 0.9–2.1 embryos per OPU-ICSI session [9–14], compared to the more classical alternative ART of artificial insemination, embryo flushing and embryo transfer in which approximately 0.5–0.88 embryos are obtained per flushing attempt [11,15]. A previous study found a positive correlation between the number of oocytes obtained in an OPU session and the likelihood of success [12] suggesting that the chances of producing at least one embryo by OPU-ICSI increase when more oocytes are recovered. This finding highlights the importance of obtaining more oocytes, and therefore studying the factors involved in oocyte recovery holds clinical

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relevance.

Other factors that also influence the number of embryos produced in a session and should be further studied include quality of the oocytes, mare genetics, follicular dynamics, and donor mare uterine abnormalities [10,12,13,16]. It has been recently reported that age, breed, season and phase of the estrous cycle also influences oocyte recovery and embryo production [17]. Although the ultrasound-guided OPU has been described since the late 20th century [18–20], many factors about the technique and recovery rates are yet to be studied. Some of these factors which have not yet been researched critically in literature involve specifics about the technique. These might include aspects regarding the follicle flushing: i.e. number of times a follicle needs to be flushed for optimal oocyte recovery, different aspiration or injection pressures, volume of media injected in each flushing, twisting of needle, etc. Some factors regarding the dislodging of the oocytes from the follicular wall, in addition to the effects of OPU on oocyte morphology and functionality, remain to be carefully studied. Most aspects of the OPU technique have been adapted from cattle reproduction medicine, and slightly changed to fit mare reproduction dynamics [21], so they may not be totally refined for optimal oocyte recovery. Moreover, since the OPU technique in mares has gained popularity in the recent years and it continues to expand [10], researching novel factors affecting the efficacy of the technique could serve to ultimately obtain a larger number of oocytes – and higher quality oocytes – which can lead to a higher production of embryos per OPU-ICSI session. Investigating the contribution of novel factors would allow the design of an improved procedure to increase the overall efficiency (time spent per procedure and overall number of recovered oocytes).

Because of the nature of the technique, it presents several challenges that can limit the scope and depth of research. Firstly, mares undergoing OPU procedures are typically part of commercial breeding programs, which prioritize reproductive success over experimental investigations. Consequently, accessing these animals for research purposes can be difficult due to commercial interests and logistical constraints. Secondly, OPU in live mares is a resource-intensive procedure, necessitating mares, specialized materials, and skilled personnel. This requirement for extensive resources results in OPU studies being difficult and time-consuming, particularly within the confines of academic research projects that often operate under strict budgetary and temporal limitations. Additionally, OPU is a relatively invasive procedure that poses potential risks and stress to the mares, necessitating careful ethical considerations and comprehensive welfare protocols. These factors collectively complicate the design and execution of rigorous scientific studies on OPU in mares, thereby hindering the generation of data and limiting advancements in the technique's optimization and application.

Slaughterhouse ovaries are a byproduct of the meat processing industry. Utilizing these tissues, which would otherwise be discarded, is an efficient use of existing biological resources. This approach aligns with the principles of reducing waste and enhancing sustainability within scientific research, ensuring that the organs are used for valuable scientific inquiry instead of being disposed of without any further utility. Using these tissues for further research also supports the Three Rs principle—Replacement, Reduction, and Refinement—of humane animal research. These organs can provide ample material for studying various factors affecting OPU outcomes. So far, there is no available data on obtaining oocytes from *postmortem* mare ovaries using ultrasound-guided OPU, since there are more efficient and well-established ways of collecting these oocytes [22–25], when needed for other commercial procedures like cloning [22].

The objective of this study was to compare the efficacy of the ultrasound-guided ovum pick-up technique between live mares and *postmortem* ovaries to determine if the latter can serve as a viable alternative for research purposes. The hypothesis of this study was that the ultrasound-guided follicle puncture and aspiration of follicles from live mares and *postmortem* excised ovaries would yield comparable oocyte recoveries, establishing it as a valuable experimental model for investigating novel and specific factors of the technique to enhance overall efficiency that can be later transferred to live procedures in commercial programs.

2. Materials and methods

2.1. Experimental design

This study was composed of a controlled experiment (*postmortem*) and retrospective data (OPU from live mares in a commercial OPU-ICSI program) to compare the oocyte recovery rate after follicle aspiration using the same ovum pick up technique in two different samples: live mares and *postmortem* ovaries. For both groups, OPU was performed by the same operator using the same technique (only one operator managing the aspiration and injection, needle and ovary), flushing system and equipment. The protocol involved aspirating all antral follicles seen in the ovary, flushing each follicle 10 times and rotating the needle once the follicle had collapsed. In both experiments, the effluents were searched by the same experienced operators.

- *Postmortem* (excised ovaries): this part of the study was conducted during two consecutive days in a commercial laboratory in March 2024 in Poland. A total of 13 ovaries from slaughtered mares in a nearby (20 min drive from the laboratory) abattoir was obtained and used within 2 h of slaughter. The ovaries were collected into plastic bags, placed in a Styrofoam box and allowed to cool down passively during transport. The arrival temperature of the ovaries at the laboratory was 32 °C. Reproductive history, age and breed from the slaughtered mares was not available. Ovaries were divided into replicates. Each replicate contained from 1 to 3 ovaries based on availability of ovaries at each OPU session. No attempt to remove the tunica albuginea from the ovaries was made. So, the OPU was performed directly in the ovaries as they came from the abattoir.
- *In vivo* (live mares): retrospective data from 12 live mares OPU procedures performed on three different days were recovered from a commercial program performed by the same operator in February 2024, in Poland. Mares were sport (showjumping warmbloods) and quarter horses. Age and reproductive data of these mares were not available. The inclusion criteria for these mares were that they were aspirated by the same operator, using the same equipment (scanner, probe and vacuum pump), aspiration flow rates and flushing media. Mares were not selected based on the number of antral follicles presented in the ovary prior to the day of OPU. Therefore, regardless of quantity of follicles, all mares were aspirated (and all visible antral follicles were punctured and aspirated).

2.2. Postmortem OPU technique

The OPU procedure was performed by one single experienced operator. Follicles were visualized using a transvaginal probe (Draminski Monoblock - OPU guide, Ząbruk, Poland) connected to the scanner (Draminski ultrasound scanner BLUE, Ząbruk, Poland). Follicle size was estimated using the scanner scale. The injection and aspiration of the follicles was performed by ultrasound-guided follicle aspiration via a double lumen needle (Minitube, double lumen needle 12G x 25" for transvaginal oocyte aspiration in mares, Tiefenbach, Germany) using a vacuum pump (Minitube aspiration and flushing pump for equine OPU, 230 V, Tiefenbach, Germany). The aspiration pump was set to an aspiration pressure of 50 mmHg (0.8 mL/s) and an injection pressure of 465 mmHg. The pump was positioned 80 cm below the needle. Flow rate for each pressure was calculated right before starting the procedure using a falcon tube.

Each ovary was held tightly by the operators' right hand on top of a metal tray positioned on a bench table. The probe was then held against the ovary simulating the position of an *in vivo* OPU. A second operator helped holding the probe in place so the OPU operator could hold the

ovary and manipulate the needle, since the probe was more mobile and unstable than it is inside the mare's vagina.

For each aspirated follicle, the estimated diameter was recorded according to the following size groups (follicles <5 mm, 5–10 mm, 11–20 mm and >20 mm). All antral follicles visible on the ovary were punctured and then flushed 10 times with a commercial flushing media containing heparin for oocyte recovery (IVF Bioscience, Cornwall, United Kingdom). In each flushing, a volume of media enough to expand the follicle visually to the original size was used, using the injection and aspiration pedals from the pump. The needle was twisted for a few seconds once the follicle was collapsed, to scrape the follicle's wall and facilitate oocyte dislodgment. Follicular fluid and flushing media were collected into a 500 mL bottle. After aspirating all follicles of a replicate, the system was then thoroughly flushed before removing the collecting bottle and inserting a new sterile collecting bottle before repeating the process with a new replicate. The effluent of the aspiration and flushing media obtained in the collection bottle was filtered through an embryo filter (EmCon™ filter, Kansas, USA) of 75 μm nylon mesh, and rinsed with new flushing media until the effluent was clear. Oocytes were searched using a stereomicroscope (Olympus SZX7), by two experienced operators. Oocytes found were photographed and evaluated as previously described [13]. In brief, cumulus oocyte complexes (COCs) were divided into 4 different categories for further analysis: degenerated (shrunken and misshapen cytoplasm). Denuded: COCs with only corona radiata or partial layer of cumulus. Compact (Cp): COCs with compact cumulus. Expanded (Ex): COCs with well-expanded cumulus (Fig. 1).

Analysis of leaked fluid: During the first day of the *postmortem* OPU experiment, it was noted that a significant amount of fluid was being leaked out of the ovary during OPU. Therefore, in the second Day of the *postmortem* experiment, it was decided to collect that fluid for oocyte searching, in case oocytes were being lost along with the leaked fluid: once the ovary(s) from one replicate had been aspirated, the operators' hand, end of the probe (that had been in contact with the ovary), and tip of the needle were rinsed with flushing media. The surface of the ovary was also rinsed with flushing media, using a 20 mL syringe connected to a 21 G needle. This process was performed on top of the metal tray where the ovary had been held for the OPU (Fig. 2). All the fluid on the tray (the fluid leaked from the ovary during the OPU and the fluid from rinsing all surfaces) was then filtered in a new filter and searched as described before.

2.3. In vivo OPU technique

Retrospective data from an OPU commercial program in live mares were identified. Inclusive criteria for data used in this group included mares that had been aspirated by the same operator as in the *postmortem* group, using the same equipment and OPU technique, and oocytes searched by the same operators.

Mares underwent sedation with detomidine hydrochloride (Dormosedan, 0.01 mg/kg i.v, Zoetis, USA) and butorphanol tartrate (Torbugesic, 0.01 mg/kg i.v, Zoetis, USA). After aseptic preparation of the vulva, the urethra was catheterized and then the transvaginal probe was inserted into the vagina. The ovary was then manipulated and fixed by rectal palpation against the vaginal wall. The aspiration process proceeded as described for the *postmortem* group. The vacuum pump was positioned 60 cm below the needle (mare's vulva) and set to an aspiration pressure of 75 mmHg and an injection pressure of 465 mmHg. The aspiration flow rate (flow rate 0.8 mL/s) was calculated right before starting the procedure using a falcon tube. All visible follicles were punctured, aspirated, and flushed 10 times as in the *postmortem* group. The needle was twisted as described for the *postmortem* group, and the collection bottles (one for each mare) processed in the same way. The OPU was performed with a "single operator" technique: the same operator performing the OPU held the ovary through the rectum (right hand) while holding the probe against the vagina using the palm of the left hand and handling the needle for follicle puncture and scraping (thumb and index finger of left hand). The vacuum pump was controlled by foot (pump pedal) for the aspiration and injection modes.

2.4. Statistical analyses

Oocyte recovery data were tested for normality of distribution using the Shapiro-Wilk test. Since the data had a normal distribution, an unpaired T-test was used to compare the mean oocyte recovery rates in each group (per ovary and per replicate). Furthermore, a Chi-square test was used to compare 1) the difference in the overall oocyte per follicle recoveries 2) the differences in the proportions of follicles aspirated in each follicle size group; and 3) the differences between oocyte morphology in the *postmortem* and *in vivo* group. The significance was set at 0.05. Lastly, the ease of performing the OPU (rotating the ovary to visualize all antral follicles and moving the needle during follicle scraping) was subjectively scored for the *postmortem* and *in vivo* groups based on the frequency of dropping the ovary to reposition it for a better image of the follicles and the difficultness of holding the ovary (cramps in the forearm and numbness in the fingers of the hand holding the ovary.

3. Results

The ease which the ovaries from the *postmortem* group could be held and rotated to image every follicle and the ease in which the needle could be rotated for follicle scraping were subjectively greater than in the live mares: In 4/12 mares, the ovary had to be dropped to rest the arm, as the fingers of the hand holding the ovary turned numb. This never happened during the holding of excised ovaries. Similarly, the needle during the OPU in live mares could only be partially rotated (45-60°). However, as a second operator held the probe during OPU in excised ovaries (Fig. 2, lower panel), the needle could be rotated more

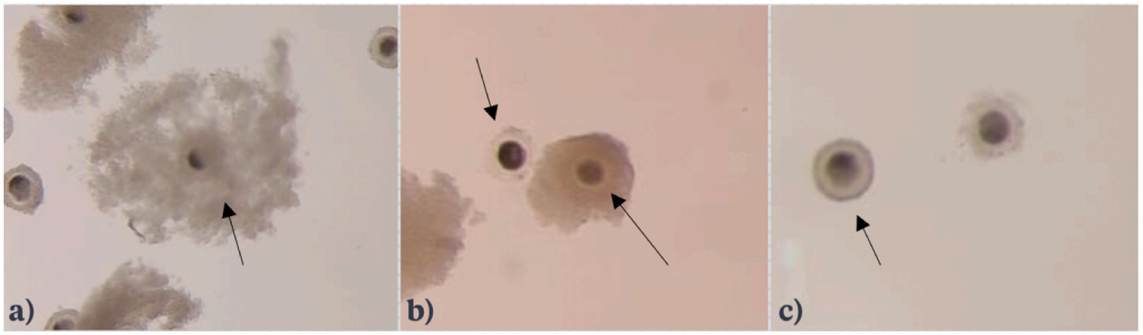

Fig. 1. Images of equine oocytes obtained during the study. (a) Expanded cumulus: multiple layers of granulose or mucosae-like cumulus (the arrowhead points to the expanded cumulus cells). (b) Degenerated oocyte (left arrowhead): exhibiting a shrunken and misshapen cytoplasm. Compact cumulus (right arrowhead). (c) Denuded oocyte with corona radiata only (arrowhead pointing to the single layer of cumulus cells/corona radiata).

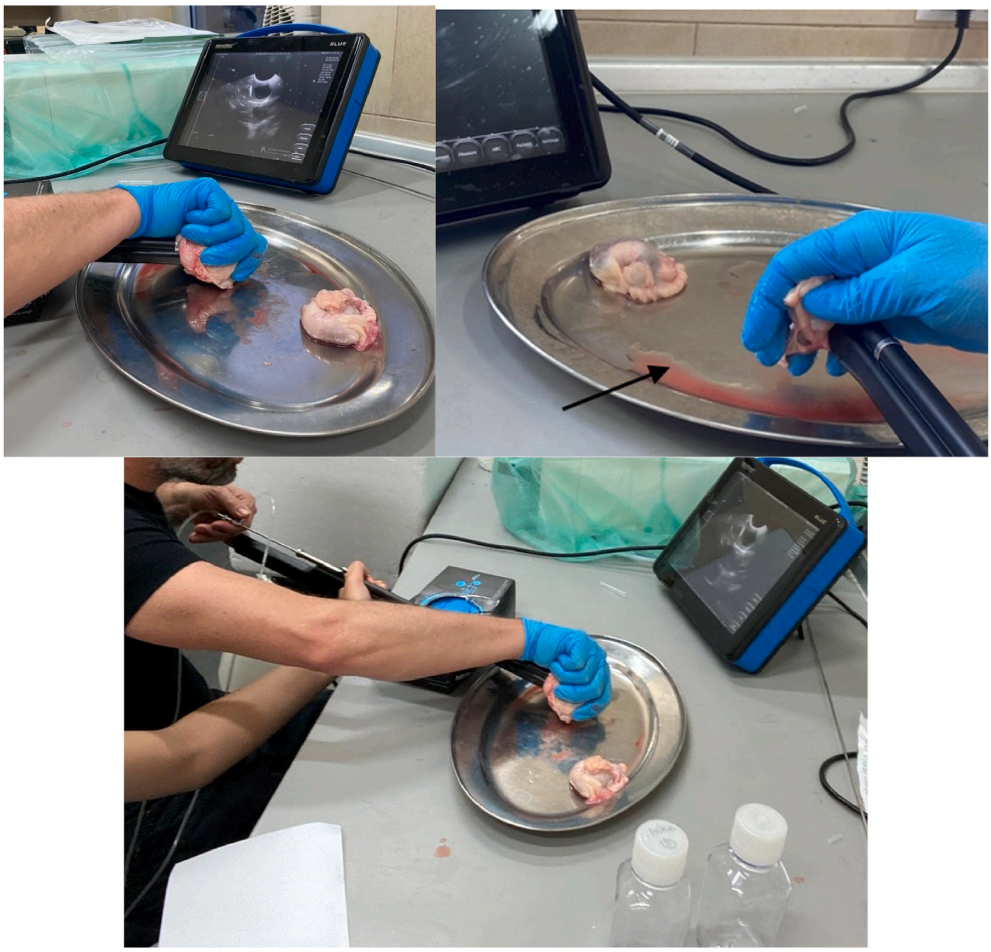

Fig. 2. OPU setup and overflowed fluid from the ovary during the OPU process in excised ovaries. Arrowhead points to leaked fluid (right panel). A second operator holds the probe in place, so that the first operator can twist the needle using the whole hand for a wider degree of rotation (lower panel).

energetically and for a higher degree (around 180°) However, the images obtained in the ultrasound scanner during puncturing and flushing (follicle collapse and expansion) of follicles appeared similar for both groups. Furthermore, the tubing system was blocked in both groups during the OPU procedure (during four occasions in the live mares and in three in the *postmortem* group). Most of the times the blockage was identified in the cork lid in the collection bottle (3/4 and 2/3 in the live mares and *postmortem* groups, respectively), where the narrowest part of the aspiration tube is found. The remaining blockages were identified in the lumen of the inner needle (aspiration needle). As an observation, the fluid recovered in the *postmortem* ovaries had a clear yellow color, whereas the one from the *in vivo* OPU had a dark reddish color and contained varying amount of blood clots (Fig. 3).

The mean (±SD) number of follicles aspirated, and oocytes recovered

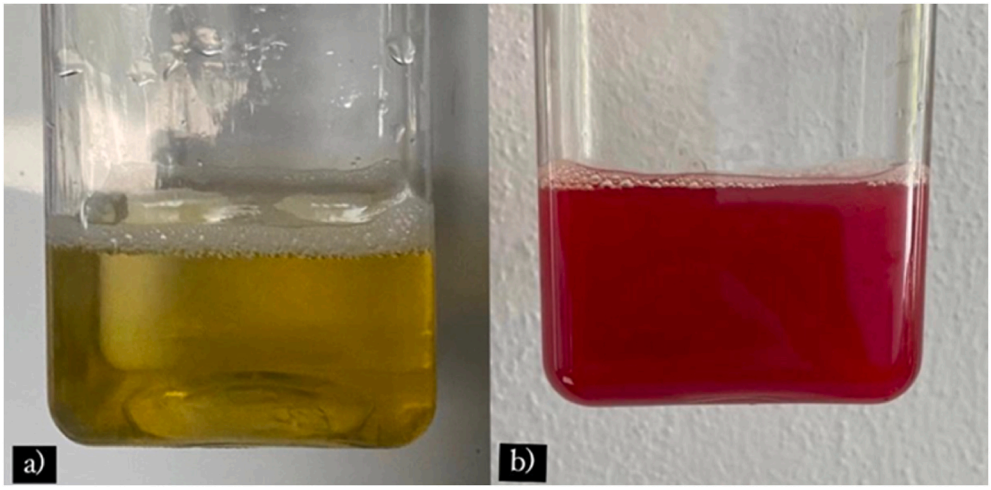

Fig. 3. Fluid recovered from the *postmortem* (excised ovaries) (a) and live mare (b) OPU aspirations.

per ovary for the *postmortem* (13.4 ± 5.7 and 8.8 ± 4.9, respectively) and *in vivo* (10.9 ± 4.8 and 5.8 ± 3.6, respectively) groups were not different (P > 0.05). The overall recovery rate from *postmortem* ovaries (105/166, 63.2 %) was greater (P = 0.03) than that of live mares from the *in vivo* group (138/261, 52.9 %), Table 1. The mean number (±SD) of antral follicles aspirated, recovered oocytes and oocyte recovery rates per replicate was similar (P > 0.05) in the *postmortem* and in the *in vivo* group (Table 2 and Fig. 4).

Within columns, different superscripts (a,b) indicate significant differences (Chi-square test; P = 0.03).

The different proportions of follicle size categories are shown in Table 3, where no significant differences were noted between groups of follicles sized <5 mm, 5–10 mm, and >20 mm. However, a significant difference (P < 0.05) was found in the number of follicles sized 11–20 mm that were aspirated between the two groups (19.3 % vs 33 %; Table 3). Overall, a higher (P = 0.017) proportion of small follicles (≤10 mm) were aspirated in the *postmortem* ovaries (121/166; 72.9 %) than in the ovaries from live mares (161/266; 60.5 %).

Oocyte morphology assessments indicated comparable levels of degenerated, denuded, and compacted COCs between the groups (Table 4). However, a significantly higher quantity of expanded COCs was found in the *postmortem* group (18 %) compared to the *in vivo* group (2.8 %; P < 0.05).

Overflowed fluid was analyzed for 3 replicates (6 ovaries). A total of 5 oocytes were recovered from this fluid, making it and average of 0.83 oocytes per ovary. A total of 81 follicles were aspirated in these ovaries, and a total of 52 and 5 oocytes were recovered during the collected effluent during OPU and in the leaked fluid, respectively (Table 5).

In the *in vivo* group, free fluid around the ovaries during and after OPU was observed in every mare. The fluid was slightly echoic, which may indicate presence of blood mixed with flushing media (Fig. 5).

4. Discussion

In this study, ultrasound-guided OPU was used to retrieve oocytes from excised ovaries, successfully demonstrating the technique's applicability in *postmortem* settings to obtain a comparable number of oocytes to live mares. The findings indicate a significantly higher oocyte recovery rate in the *postmortem* group compared to the *in vivo* group. These results are equivalent to the ones described in another study, which obtained a significantly higher recovery rate when aspirating *postmortem* ovaries than live mare ovaries (31.2 % and 19.3 %, respectively) [25], although they did not use ultrasound during the procedure, only aspirating the superficial visible follicles.

Several factors may have contributed to the enhanced efficiency observed in *postmortem* oocyte recovery. It is important to note that mares included in the live mare group didn't necessary present more follicles, as they were not specially selected as suitable for OPU. They presented different kinds of follicular states at the time of OPU.

Firstly, the manipulation of ovaries in a *postmortem* setting allows for a more thorough handling and positioning of the ovary, which is evidently more difficult in live mares, especially with a single operator technique (same operator holding the ovary, probe and handling the needle). This enhanced manipulation may have facilitated a more precise needle insertion and follicle scraping, which could potentially lead to increased oocyte recovery rates.

Moreover, the apparent absence of blood in the fluid recovered from *postmortem* ovaries seems to provide a clearer effluent in which to identify and collect oocytes. It has been anecdotally reported by practitioners that blood can obscure visibility during oocyte search, making it challenging to ensure that all oocytes are accurately retrieved from the aspirate. Furthermore, oocytes could be trapped and stuck to blood clots formed within the recovered fluid. The clear fluid obtained in *postmortem* ovaries might be simplifying the process of identifying and collecting oocytes, potentially increasing the number of oocytes recovered in this group.

Table 1
Comparison of oocyte recoveries after OPU from *postmortem* (excised ovaries) and *in vivo* ovaries.

Group	Number of ovaries	Total follicles aspirated	Total oocytes recovered	Oocytes per follicle (%)
Postmortem	13	166	105	63.2[a]
In vivo	24	261	138	52.9[b]

Table 2
Mean number of follicles, oocytes and recoveries per replicate from the *postmortem* (excised ovaries) and *in vivo* groups.

Group	Number of replicates	Number of ovaries	Mean (±SD) follicles per replicate	Mean ± SD oocytes per replicate	Mean ± SD oocyte recovery per replicate
Postmortem	8	13	20.8 ± 10.3 (9–42)	13.1 ± 6.6 (4–25)	62.8 ± 12.3 (44.4–84.6)
In vivo	12	24	21.8 ± 9.7 (13–45)	11.5 ± 7.3 (2–26)	50.7 ± 21.5 (22.2–88.2)

For each variable, the minimum and maximum observation is shown in brackets. No significant differences (P > 0.05) were found in any of the variables analyzed between groups.

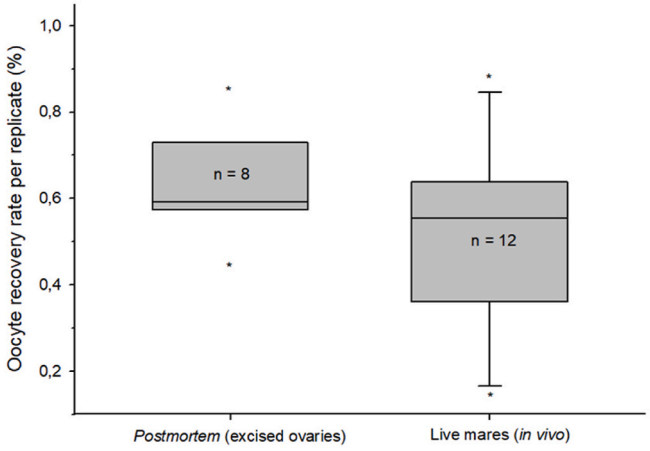

Fig. 4. Boxplot distribution of oocyte recoveries of replicates from the *postmortem* and *in vivo* groups. The number of replicates in each group is shown within each box. The asterisks (*) indicate outliers. The mean and median oocytes recoveries were not different (P > 0.05) between groups. The 8 replicates (with 8 separate oocytes searches) from the *postmortem* group included 13 ovaries, while the 12 replicates (live mares) of the *in vivo* group included 24 ovaries.

Table 3
Distribution of aspirated follicles based on size from the *postmortem* (excised ovaries) and the *in vivo* groups.

Group	Follicle diameter groups			
	>20 mm	11–20 mm	5–10 mm	<5 mm
***Postmortem* (n = 166)**	13	32	61	60
	7.8 %	19.3%[a]	36.8 %	36.1 %
***In vivo* (n = 261)**	14	86	80	81
	5.3 %	33%[b]	30.7 %	31.0 %

Within column, different superscripts (a,b) indicate significant differences (P < 0.05). When comparing follicles of less than 10 mm (groups <5 mm and 5–10 mm combined), a significant difference was found between groups (P < 0.05).

Table 4
COCs (cumulus oocyte complexes) morphology classification of recovered oocytes from *postmortem* and *in vivo* groups.

	Degenerated	Denuded	Cp	Ex
Excised ovaries (n = 105)	8	39	39	19
	7.6 %	37.2 %	37.2 %	18%[a]
Live mares (n = 138)	6	69	59	4
	4.3 %	50 %	42.8 %	2.9%[b]

Characteristics of oocytes in each group. Cp: COCs with compact cumulus. Ex: COCs with well-expanded cumulus. Degenerated: shrunken and misshapen cytoplasm. Denuded: COCs with only corona radiata or partial layer of cumulus. Within column, different superscripts (a,b) indicate significant differences ($P < 0.05$).

In addition, the degeneration of ovarian tissue *postmortem* might also play a role in facilitating oocyte dislodgement from the follicle walls [26], making it easier to aspirate them. In the latter study [26], a lower oocyte recovery rate (35 %) was obtained in aspirated follicles from excised ovaries processed within 1.5–4 h of slaughter, compared to that (48 %) in ovaries stored for 6–8 h before follicle aspiration. Histological examinations of follicle walls have shown that immature oocyte complexes are tightly fixed to the follicle wall by the broad cumulus cell hillock [27]. Tissue breakdown could alter the structural integrity of the follicle, reducing the adherence of oocytes to the follicle wall and thereby increasing the likelihood of their release during aspiration. Therefore, the timing of OPU after slaughter should be taken into account to standardize results of different studies. However, the excised ovaries of the current study were processes for OPU within 2 h of slaughter, so the tissue breakdown that facilitates oocyte dislodgment would have been minimal at that time [26].

The higher incidence of smaller follicles (<10 mm) observed in the *postmortem* group could have also contributed to the increased oocyte recovery rates. Previous research has shown that smaller follicles can yield higher recovery rates [12,28]. Recovery rates are also reported to be higher for small immature follicles than for large immature follicles [29,30].

Some other known factors that influence oocyte recovery include mare age [12] and breed [21]. It has been shown that increasing mare age is correlated to a lower oocyte recovery rate [11,12]. In this study, age data were not available but might have contributed to the outcome. A study of 473 mares engaged in a commercial OPU program showed an average of 12 years of age [11], and the most common age for slaughtering of meat horses is < 2.5 years in Europe [31]. So, it is likely that the mean age of the mares from the *postmortem* group was significantly younger than the mares from the *in vivo* group which belonged to a commercial OPU program.

Regarding the *in vivo* recovery rates, a higher standard deviation was seen in this group (SD = 21.5 %; min 22.5 % and max. of 88.2 %) compared to the *postmortem* group (SD = 12.3 %; min. 44.4 % and max. 84.6 %), due to the strong variability between individual mares and possible differences in the intrinsic difficulty of aspirating certain live mares and oocyte searching due to the amount of blood clots during some OPUs. Nevertheless, the recovery rate aligns with recent reports which indicate that when using a 12-gauge double lumen needle and repeated flushing of the follicle, an average oocyte recovery rate from immature follicles of around 50–55 % can be expected (50.1 % [32], 54 % [33], and 57.6 % [12]).

Notably, a higher number of expanded oocytes were recovered in the *postmortem* group. During *in vivo* OPU procedures, more denuded oocytes are usually obtained than when the oocytes are obtained by follicle scraping of individual follicles [13], suggesting that the aspiration pressure, flushing of the follicle, or needle scraping of the follicle wall might affect the morphology of the oocytes obtained; although in this study, these factors were standardized for both groups. On the other hand, recovery of expanded oocytes has been associated with the aspiration of follicles undergoing atresia, while oocytes classified as compact originate from viable follicles [34,35]. Therefore, a higher proportion of atretic follicles in the *postmortem* ovaries compared with those from live mares might explain the differences in the percentage of expanded oocytes.

Another observation was the occasional clogging of the bottle cork and needle, which occurred in both groups, suggesting that this common clogging issue likely stems from the ovarian tissue itself rather than from blood or blood clots, since no blood was apparently present in the *postmortem* aspirated fluid.

Additionally, the discovery of oocytes in the leaked fluid during *postmortem* OPU highlights an often-overlooked aspect of the procedure.

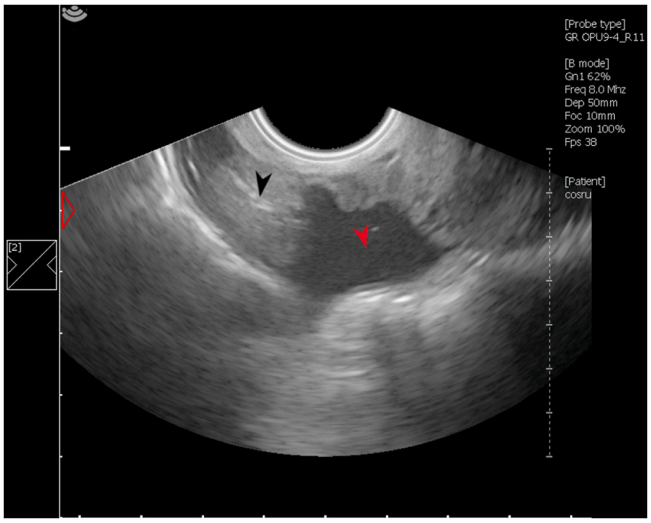

Fig. 5. Representative ultrasonogram of a mare from the *in vivo* group, just after the aspiration of the last follicle during OPU. The black arrowhead (left) shows part of the ovarian stroma; The red arrowhead (right) shows the slightly echoic free fluid accumulated around the ovary. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 5
Specifics of the 3 replicates (6 ovaries) in the *postmortem* group in which overflowed fluid was analyzed.

Replicate	Number of ovaries	Number of follicles	Oocytes recovered in effluent OPU bottle	Oocytes recovered in overflowed (leaked) fluid	ORR effluent OPU bottle	ORR overflowed fluid
1	2	14	8	1	8/14 57.1 %	1/14 7.1 %
2	1	25	19	1	19/25 76.0 %	1/25 4.0 %
3	3	42	25	3	25/42 59.5 %	3/42 7.1 %
Overall	6	81	52	5	52/81 64.2 %	5/81 6.2 %

ORR: oocyte recovery rate.

Commonly after an OPU procedure, clinicians anecdotally report observing free fluid around the ovary and in the abdomen of the mare. This was confirmed during the OPU in the *in vivo* group with live mares, as free fluid was identified around the ovary in every mare after OPU. This fluid was likely to originate from a mixture of flushing media overflowing out of the ovary mixed with blood from follicle scraping, which may contain extravasated oocytes too, as shown in the *postmortem* group. The loss of oocytes within the ovarian stroma or even outside of the ovary may be attributed to the injection pressures and total volume of media used during follicle flushing, or potentially to the mechanical disruption caused by follicle puncturing. A considerable percentage of the oocytes recovered in the *postmortem* group was recovered in the leaked fluid, which is an interesting finding requiring further research. The use of excised ovaries for OPU as experimental model, could be used to determine the effect of volume of media, the number of flushes per follicle and the number of holes made in each ovary on the percentage of oocytes lost in the leaked fluid, and so understand the factors involved in the oocyte recovery of live mares. Unfortunately, the number of holes made in each ovary with the needle during follicle aspiration was not recorded. This could have played a role in the likelihood of an oocyte being lost in the overflowed fluid through the hole made by the needle.

The functionality and developmental potential of oocytes retrieved from excised ovaries was not pursued in this study, although embryos developed from abattoir derived oocytes have been obtained and reported before [22–25]. The proposed OPU system described in the current study of ultrasound-guided follicle puncture and aspiration of excised ovaries, although time-consuming and using a high volume of flushing media, yielded a considerably high number of recovered oocytes per ovary (mean of 8.8 oocytes), which is greater than previously reported following scarping of excised ovaries [24,25].

In conclusion, oocyte recovery from *postmortem* excised ovaries by ultrasound-guided OPU proved to be viable. The main differences observed in the *postmortem* group were: 1) lack of blood into the recovered fluid; 2) 10 % higher oocyte recovery rate compared to live mares, and 3) higher percentage of expanded COC. The higher recovery rate obtained in the *postmortem* group and the finding of oocytes in the leaked fluid during OPU, suggests that there is still room for improvement in the *in vivo* OPU technique. Utilizing excised ovaries could offer an alternative for further research into factors affecting oocyte recovery and oocyte leakage during OPU procedures.

CRediT authorship contribution statement

Laura Sala-Ayala: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Aleksandra T. Pytel:** Writing – review & editing, Investigation. **Karolina Stychno:** Writing – review & editing, Resources, Investigation. **Juan Cuervo-Arango:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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