Theriogenology 229 (2024) 47–52

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Original Research Article

Successful ultrasound-guided ovum pick-up (OPU) and subsequent in vitro embryo production in a domestic cat

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ARTICLE INFO

Keywords:
Ovum pick up (OPU)
Oocyte retrieval
Assisted reproduction
Domestic cat
In vitro production

ABSTRACT

Ovum Pick Up (OPU) is a minimally invasive technique widely used in cattle and mares for oocyte retrieval, involving ultrasound-guided puncture of ovarian follicles. It has been demonstrated that this technique is safe for its repeated use in the same female without affecting her reproductive health, allowing for the retrieval of oocytes in individuals regardless of their reproductive status. The oocytes obtained through OPU can subsequently be used for *in vitro* embryo production (IVP) using assisted reproductive techniques (ARTs) or be cryopreserved in biobanks for their future use. Traditionally, the minimally invasive technique of choice performed *in vivo* in domestic and wild felines was LOPU (laparoscopic-guided ovum pick up). The present study was designed to explore if ultrasound-guided OPU in the domestic cat is safe and effective. In an initial series of *ex vivo* experiments (n = 92 ovaries, n = 434 oocytes), the effect of different aspiration pressures for oocyte collection was explored. These experiments identified 43 mmHg as the optimal aspiration pressure, resulting in the highest recovery rate and a favorable maturation and blastocyst rate. Subsequently, 16 grade I and II oocytes were retrieved by OPU and 101 oocytes were retrieved following ovariectomy and slicing. Sixteen oocytes obtained with each technique were subjected to *in vitro* maturation (IVM) and *in vitro* fertilization (IVF). A total of 14 presumptive zygotes were selected for *in vitro* culture (IVC) from each group (OPU and slicing), obtaining a cleavage rate of 57.1 % and 64.2 %, a morula rate of 28.5 % in both groups, and a blastocyst rate of 7.14 % and 14.2 % respectively. The hormonal stimulation protocol was well-tolerated, with no adverse effects observed. Moreover, no complications arose during the ovariectomy performed post-OPU. The use of this technique in domestic cats represents a significant step forward in terms of safety, replicability, and invasiveness, serving as a valuable model for its application in wild felids species. Additional research involving a greater number of animals is required to validate these encouraging findings.

1. Introduction

Ovum Pick Up (OPU) is a minimally invasive technique employed to retrieve oocytes, involving the ultrasound-guided transvaginal puncture of ovarian follicles. Initially developed for use in bovine livestock, this methodology has subsequently been adapted to other species such as horses or buffalo due to its inherent advantages over alternative techniques (such as superovulation or *post-mortem* retrieval) and its minimal contraindications [1].

Studies have shown that repeated OPUs can be carried out in the same animal without posing risks to health or reproductive function, being extremely useful for the development of *in vitro* assisted

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https://doi.org/10.1016/j.theriogenology.2024.08.017
Received 5 June 2024; Received in revised form 1 August 2024; Accepted 13 August 2024
Available online 13 August 2024

reproduction techniques (ARTs) [1,2]. OPU has emerged as an useful tool which can be performed with or without superovulation, and can be used regardless of the reproductive status of the donor [1,3].

In recent years, this technique has increasingly been adapted for its use in wild animals, as it can play a significant role in conservation programs employing assisted reproductive techniques. In felines, so far, this procedure has exclusively been performed laparoscopically (LOPU) in domestic cat [4] or wild individuals, such as jaguar, puma and cheetah [5,6]. In other species of wild mammals, such as rhinoceroses [7,8] and owl monkeys [9], the implementation of transrectal or transabdominal OPU protocols has enabled the successful execution of advanced assisted reproduction procedures, providing substantial advantages in terms of invasiveness, safety, and feasibility.

Infertility across domestic species, wild animals and humans poses challenges that are widely addressed with well-established ARTs such as *in vitro* maturation (IVM), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) or somatic cell nuclear transfer (SCNT) to produce *in vitro* embryos that can be subsequently transferred or cryopreserved [10–12]. Furthermore, the cryopreservation of gametes is a crucial tool for biodiversity conservation, aiding in narrowing the gap between *ex situ* and *in situ* populations [13,14]. Therefore, the retrieval of oocytes through minimally invasive procedures, followed by their cryopreservation or utilization for ARTs, emerges as an invaluable approach for safeguarding genetic resources from endangered felines in cryobanks [15].

Additionally, the domestic cat has proven to be a promising model for the development of ARTs that could be applicable to conservation programs for wild felids [16,17]. Therefore, the protocol we present for performing transabdominal OPU in domestic cats has been designed with the aim of implementing its use in clinical and research settings for domestic species, as well as for its subsequent adaptation in wild felid conservation initiatives.

2. Material and methods

2.1. Ethics statement

The present study was approved by the Animal Experimentation Ethics Committee (CEEA) of the Veterinary Clinical Hospital of the Faculty of Veterinary Medicine at the Complutense University of Madrid (UCM) under no. 05/2024, following the regulations outlined in Royal Decree 53/2013.

2.2. Conditions for oocyte aspiration (in vitro maturation, fertilization and culture from ex vivo OPU-retrieved oocytes)

Several *ex vivo* studies were conducted to compare different puncture procedures and pump pressure settings. For each study, a different pool of ovaries was used, as the experiments were designed and performed independently. These studies aimed to evaluate the recovery rate, the morphology of the retrieved oocytes, the maturation rates, and *in vitro* embryo production, including cleavage, morula, and blastocyst rates. The main objective was to identify the pressure that provided the best balance between recovery rate and oocyte maturation, ultimately selecting the pressure that caused the least damage to the oocytes during the retrieval process and thereby achieving the highest blastocyst production rate. For these studies, ovaries were collected following elective gonadectomies at various associated centers.

In the first study, the oocyte recovery rate relative to the number of punctured follicles was evaluated using three different vacuum pump (Cook® KMAR-5100, Eight Mile Plains, Australia) pressures (43 mmHg, 65 mmHg, and 90 mmHg) [18–21]. This pump was connected to a 120 cm long polyvinyl chloride (PVC) extension line (Bexenmedical, Guipúzcoa, Spain) attached to a 20G needle. A total of 36 ovaries from 18 cats undergoing elective sterilization procedures were used in the study (n = 6 replicates). All visible follicles (0.5–4.0 mm in diameter) were punctured, totalling 230 follicles, from which 111 oocytes were recovered. The ratio of recovered oocytes to punctured follicles was recorded.

In a second study, based on the results of recovery rate, two pressures were selected. All visible follicles (0.5–4.0 mm in diameter) from 42 ovaries of 21 cats were punctured, resulting in a total of 612 follicles punctured and 329 oocytes retrieved (n = 4 replicates). Among these, 227 grade I and II selected oocytes underwent IVM for 28 h in a laboratory-made medium [TCM-199 based, hepes-buffered, supplemented with L-glutamine, $NaHCO_3$, cysteine, lactate, pyruvate, bovine serum albumin, and 25 ng/mL epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA)], at 38.5 °C, 20 % O_2, and 5 % CO_2. After 28 h, the oocytes were denuded using hyaluronidase, fixed in 4 % paraformaldehyde (PFA) at 20°C-22 °C for 30 min, and then washed in phosphate buffered saline (PBS) for 15 min. Finally, the oocytes were mounted on slides with Hoechst 33,342 (H342) and observed under fluorescence microscopy to evaluate the extrusion of the first polar body as a sign of maturation.

Selection of the oocytes was performed following the morphological classification established by Wood and Wildt in 1997 [22], which is based on the assessment of cytoplasmic appearance and the number of cumulus cell layers. According to these criteria, oocytes were classified as follows: grade I included those with opaque and uniform cytoplasm along with 5 or more layers of cumulus cells. Grade II consisted of oocytes with uniform cytoplasm but 2–4 layers of cumulus cells. Grade III encompassed oocytes displaying cytoplasmic fragmentation, a complete corona radiata, and partial cumulus cells. Grade IV included oocytes showing cytoplasmic fragmentation combined with total-to-partial denudation.

Finally, based on all previously obtained data, a third experiment was designed to compare *in vitro* embryo production (IVP) of aspirated oocytes recovered using two different pressures (43 mmHg and 65 mmHg). To reduce the bias arising from the selection of ovaries, one ovary from each female was chosen for aspiration with each of the pressures. A total of 14 ovaries from 7 queens, which were electively gonadectomized at feline colony management centers, were used in the study (n = 3 replicates). All visible follicles were punctured, and 96 grade I and II oocytes were selected and subsequently matured for 28 h in the same laboratory-made IVM medium as the previous study (n = 52 using 43 mmHg and n = 44 using 65 mmHg respectively). The oocytes were then transferred to a laboratory-made IVF medium (supplemented FERT-TALP medium) and co-incubated with frozen-thawed epididymal spermatozoa for 18–20 h at a final concentration of 1×10^6 spermatozoa/mL. Subsequently, culture was performed in laboratory-made *in vitro* culture (IVC) medium [Synthetic oviductal fluid supplemented with 3 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA)] and renewed on day 4 post-insemination (dpi) to synthetic oviductal fluid supplemented with 10 % fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) until 10 dpi. Cleavage and morula rates were evaluated at 4 dpi and blastocyst rates at 6–8 dpi.

2.3. Queen preparation, superstimulation and anesthesia

The OPU was performed in a one-year-old female cat that came to the Complutense Veterinary Clinical Hospital (CVCH) for an open ovariectomy. The owner of the animal provided informed consent for the procedure. A comprehensive examination, complete blood work and an electrocardiogram were performed to ensure the optimal health status of the animal before the procedure.

The queen was hormonally stimulated following a protocol with pregnant mare serum gonadotropin (PMSG) (Folligon®, MSD, Salamanca, Spain) administering a total dose of 200 IU, 84 h prior to OPU [23,24]. The PMSG was prepared by dissolving the lyophilized tablet in approximately 5 mL of solvent, gently agitating the solution, and injecting it into the solvent vial to mix thoroughly. The solution was used immediately after preparation. The use of human chorionic gonadotropin (hCG) (Gestron1500®, Kyoritu Seiyaku, Tokyo, Japan)

was dismissed as a study by Yoshimura et al., in 2021 described a decrease in the mean numbers of ovarian follicles after hCG injection because of its influence on ovulation [25].

The presence of ovarian follicles was monitored by abdominal ultrasound using a 22 MHz ultra-high frequency transducer (Canon Aplio i800, Canon Medical Systems Corporation, Japan), one week after the onset of estrous and 84 h after the administration of PMSG.

The cat was subjected to sedation, during which 2 μg/kg of dexmedetomidine IM (Sedadex®, Dechra Pharmaceuticals, Barcelona, Spain) and 0.3 mg/kg of methadone IM (Semfortan®, Dechra Pharmaceuticals, Barcelona, Spain) were administered. Once the sedative effect was achieved, the cephalic vein was catheterized. Heart rate, respiratory rate, non-invasive arterial blood pressure, electrocardiogram, and temperature were continuously monitored. The abdominal area was clipped and aseptically prepared.

Before the procedure, preparation of the medium was carried out. This involved commercial TCM-199 medium (M7528, Sigma Aldrich, St. Louis, MO, USA) to which 10 mcg/mL of sodium salt heparin was added (H3393, Sigma Aldrich, St. Louis, MO, USA), and then warmed at 38.5 °C.

2.4. 2.4. ovum pick up

For the aspiration of antral follicles (defined as those larger than 2 mm), a vacuum pump (Cook® KMAR-5100, Eight Mile Plains, Australia) connected to a system comprising of a 120 cm long PVC extension line (Bexenmedical, Guipúzcoa, Spain) attached to a 23G needle was used. The needle was inserted into a vacutainer along with the end of the pump. The pump was set to have a suction pressure of 3 mL/min (60 drops/min approximately, equivalent to 43 mmHg) and the entire system was subsequently purged with pre-warmed TCM-199 medium.

For the aspiration, the cat was positioned in lateral recumbency, and the ovaries were located ultrasonographically (Fig. 1). All follicles larger than 2 mm were punctured using a 23G needle (Fig. 2). After puncturing the first ovary, the whole system was flushed three times with pre-warmed TCM-199 medium to ensure that any oocytes remained in the system before starting the procedure on the second ovary. To aspirate the other ovary, the cat was repositioned from one side to the other.

After puncturing the follicles, a final flushing of the system with TCM-199 medium was performed, and the tube was quickly transferred to the laboratory for the evaluation and selection of the recovered oocytes. Lastly, the queen was transferred to the operating room, where anesthetic induction was achieved with 1 mg/kg propofol (Lipuro®, Braun Medical SA, Barcelona, Spain). An ovariectomy was then performed, and the ovaries were promptly taken to the Reproduction Laboratory to be processed for collecting the remaining oocytes via slicing, retrieving oocytes form follicles of various sizes. Open surgery allowed for verification that no internal damage had occurred during the OPU procedure.

2.5. In vitro embryo production

All retrieved oocytes from OPU, and selected grade I and II oocytes from slicing were subjected to IVM for 28 h in IVM medium, at 38.5 °C, 20 % O_2, 5 % CO_2. Matured oocytes (Fig. 3) were transferred to a laboratory-made IVF medium (supplemented FERT-TALP medium) and co-incubated with frozen-thawed epididymal spermatozoa for 18–20 h, at a final concentration of 1×10^6 spermatozoa/mL. Degenerated presumptive zygotes were discarded prior to IVC, and thus, fourteen selected presumptive zygotes per group were cultured under mineral oil, in 25 μL microdroplets of IVC medium (Synthetic oviductal fluid supplemented with 3 mg/mL BSA) for 4 dpi at 38.5 °C, 5 % O_2, 5 % CO_2, 90 % N_2. The IVC medium was changed on 4 dpi [26] to synthetic oviductal fluid supplemented with 10 % FBS until 8 dpi. Cleavage and morula rate were recorded on 4 dpi, and blastocyst rate on 6–8 dpi (Fig. 3).

2.6. Statistics

Data were analyzed with SPSS Statistics 29 software (IBM, USA). One-way ANOVA was used to assess recovery rates differences at 43, 65 and 90 mmHg. Then, one-way ANOVA was used to assess recovery rates and maturation rates differences at 43 and 65 mmHg. Lastly, the differences between IVP values were analyzed using Mann-Whitney. Differences were considered significant with a P value lower than 0.05 ($p < 0.05$).

3. Results

3.1. Oocyte aspiration

First, follicle aspiration was tested at various pressures to compare recovery rates. This was done by counting the number of punctured follicles and the oocytes recovered from each ovary. A total of 36 ovaries were punctured, resulting in the aspiration of 230 follicles and the recovery of 111 oocytes. Aspiration using pressures of 43, 65 or 90 mmHg resulted, respectively, in the recovery of 41/64 (65.8 %), 51/105 (51.6 %) and 19/61 (27.8 %) oocytes from follicles (Table 1). There were significant differences in recovery rates between the three pressure groups, with the highest recovery rate observed in the 43 mmHg group. These results indicate that a pressure of 90 mmHg led to a lower recovery rate and significantly damaged the retrieved oocytes.

Subsequently, based on the recovery rate results above, two pressures were selected to test oocyte maturation post-aspiration to assess potential damage during the procedure. A total of 612 follicles from 42 ovaries were punctured, resulting in the recovery of 329 oocytes. The results revealed no statistical differences between the groups in terms of recovery and maturation rates, ($P > 0.05$) (Table 2). Furthermore, morphological analysis following Wood & Wilt's criteria [22] was carried out. Even though the homogeneity of the cytoplasm was similar between the groups, the surrounding layers of cumulus cells were

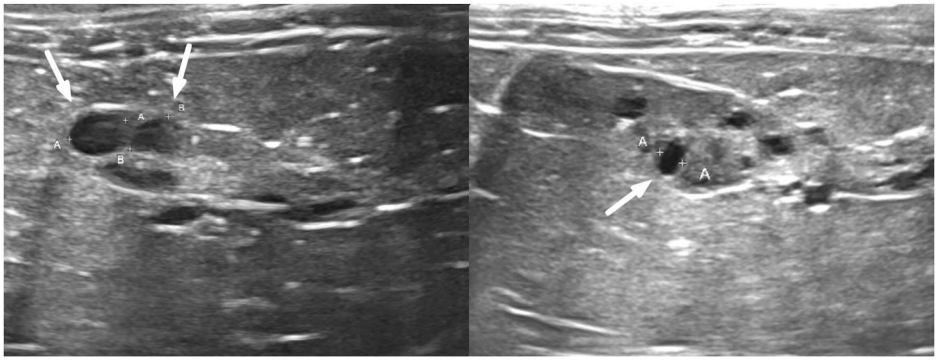

Fig. 1. Ultrasound images of the ovaries prior to performing the Ovum Pick Up (OPU). White arrows show follicles larger than 2 mm.

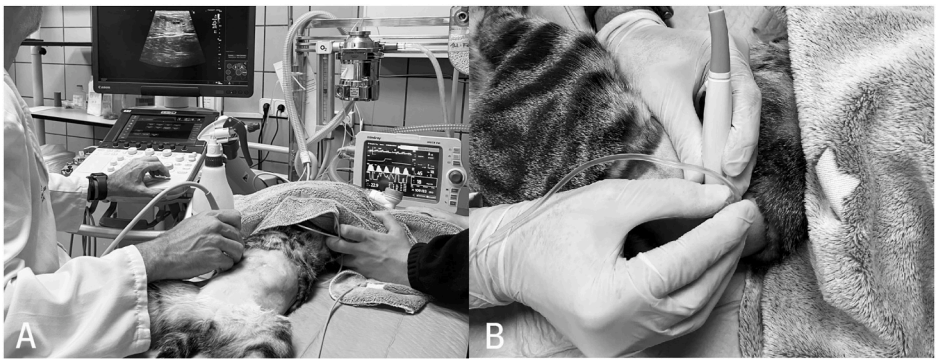

Fig. 2. Ultrasound localization of the ovaries in supine position (A). Ultrasound probe position for OPU performance in the left ovary (B).

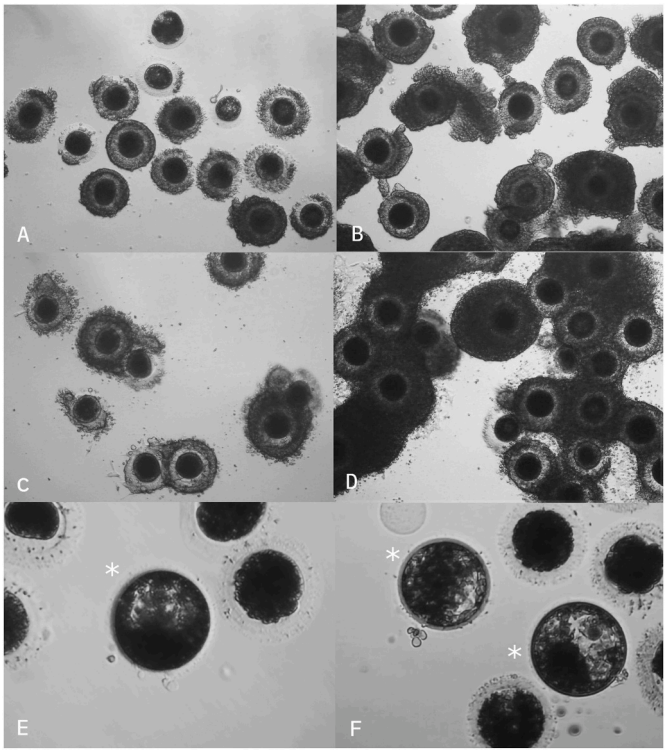

Fig. 3. Images of the oocytes obtained through OPU (A) and slicing (B), prior to IVM. Images of the same oocytes, OPU (C) and slicing (D), after IVM. Morulae and blastocysts (indicated with an asterisk) obtained in each group, OPU (E) and slicing (F).

Table 1
Effect of aspiration pressure on oocyte recovery rates in cat ovaries. Values are presented as mean ± Standard Deviation.

Pressures (mmHg)	Nº ovaries	Nº punctured follicles	Nº retrieved oocytes	Recovery rate (M% ± SD)
43 mmHg (3 mL/min)	12	64	41	65.8 ± 10.9[a]
65 mmHg (4 mL/min)	14	105	51	51.6 ± 12.4 [b]
90 mmHg (5.5 mL/min)	10	61	19	27.8 ± 12.0 [b]
Total	36	230	111	

Mean values with different superscripts (*a* and *b*) show differences between rows (experimental groups). Significance level is defined as $P < 0.05$.

noticeably lower in the group of oocytes aspirated using 65 mmHg. As a result, the proportion of grade I oocytes was higher in the group recovered using 43 mmHg (Fig. 4A) than in the group aspirated using 65 mmHg (Fig. 4B) (Table 2).

Finally, in the last experimental series, 14 ovaries were punctured, retrieving 96 oocytes. The results obtained from the IVP experiment showed no significant differences between groups ($P > 0.05$). Cleavage rates were 42.6 % in the 43 mmHg group and 38.4 % in the 65 mmHg group. Additionally, morula rates were 22.7 % and 21.4 %, respectively; while blastocyst rates were 9.9 % and 7.9 %. Finally, blastocyst over cleavage rate were 24.4 % and 17.7 % in the 43 and 65 mmHg groups respectively (Table 3). Although no significant differences were revealed, the values of all IVP rates were slightly higher in the 43 mmHg group. Thus, considering the results from all experiments, 43 mmHg (3 mL/min) was identified as the optimal pressure for performing OPU, minimizing damage to the oocytes while maintaining a good recovery rate, and thus enhancing the opportunities to obtain adequate IVM and IVP rates.

3.2. Oocyte collection using OPU

The fluid recovered during the OPU procedure was evaluated under the microscope (Ti-Eclipse, Nikon, Japan), revealing 12 grade I oocytes and 4 grade II oocytes (Fig. 3). In comparison, the slicing procedure performed on the same ovaries following ovariectomy yielded a total of 101 oocytes, with 56 of them being grade I and II (Fig. 3).

All retrieved oocytes (n = 16) from OPU, and 16 grade I and II oocytes from the slicing procedure were subjected to IVM and 14 presumptive zygotes from each group were selected for IVC. The OPU-obtained oocyte group showed a cleavage rate of 57.1 %, 28.5 % of morula were produced and a blastocyst rate of 7.14 %, with a blastocyst rate over cleaved embryos of 12.5 %. In contrast, the slicing-obtained oocyte group exhibited cleavage, morula, blastocyst rates, and a blastocyst rate over cleaved embryos of 64.2 %, 28.5 %, 14.2 %, and 22.2 %, respectively (Table 4).

4. Discussion

To the best of our knowledge, this is the first reported case of transabdominal ultrasound-guided OPU in the domestic cat, subsequently achieving successful IVP. This technique has been developed by adapting existing protocols from LOPU in domestic cats [20] and small ruminants [21], and ultrasound-guided OPU in cattle [2], considering that due to the average size of the domestic queen, transvaginal technique was not feasible.

Thus, the OPU technique can be useful in retrieving oocytes from both healthy animals and those with reproductive disorders that cause infertility [1]. Additionally, this technique may be used in young prepubertal animals as well as in geriatric animals that are no longer in their reproductive prime, as has been demonstrated in both cattle and wild mammals such as rhinoceroses and large felines [6,7,18]. Taking all of this into account, OPU could be an extremely useful tool for the conservation of endangered species, as it allows a safe retrieval and

Table 2
Effect of aspiration conditions on oocyte recovery rates and oocyte maturation rates. Values are presented as mean ± Standard Deviation.

Pressures (mmHg)	Nº ovaries	Nº punctured follicles	Nº retrieved oocytes	Grade I oocytes	Recovery rate (M% ± SD)	Maturation rate (M% ± SD)
43 mmHg (3 mL/min)	26	383	215	134	55.6 ± 6.2	45.4 ± 4.8
65 mmHg (4 mL/min)	16	229	114	93	48 ± 11.3	43 ± 15.3
Total	42	612		329	227	

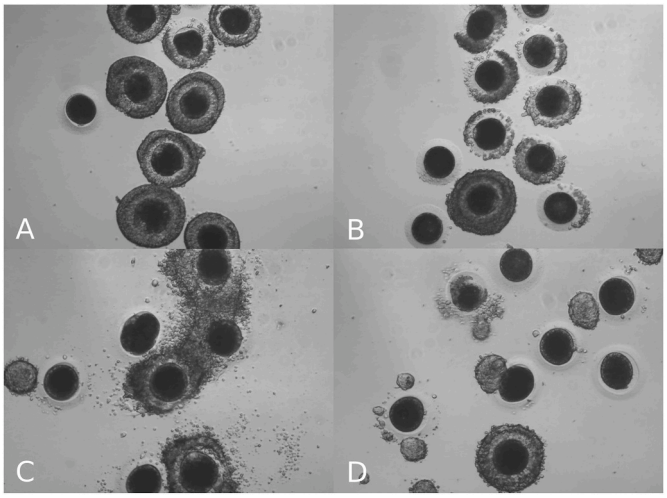

Fig. 4. Images of the oocytes obtained through *ex vivo* follicle aspiration at 43 mmHg (A) and 65 mmHg (B), before IVM. Images of the same oocytes obtained at 43 mmHg (C) and 65 mmHg (D), after IVM.

cryopreservation of highly valuable genetic material from individuals that are not suitable for reproduction.

The development of OPU protocols represents significant improvements over the existing LOPU technique, as it allows a reduction of the time under anesthesia, avoids intraoperative complications by being a non-invasive technique, and reduces postoperative pain [9]. This technique has proven to be effective and reliable for oocyte retrieval that can subsequently be used in multiple applications, such as IVP through IVF or ICSI, or SCNT [4]. Furthermore, previous studies have not been able to compare *in vitro* procedure success rates between OPU and slicing post-surgery in the same individual.

While further studies are necessary to improve the OPU technique in domestic cats, initial studies have determined an optimal pump speed and pressure for oocyte retrieval in this species, resulting in minimal damage and thus allowing their use in IVC with successful development of blastocysts. The pressure applied in these experiments aligns with the parameters proposed by Baldassarre et al. (1994) for sheep [21], and is slightly lower than those utilized in large felines and cattle [5], as well as other LOPU protocols described for domestic cats [19], which could be justified by the size differences among these species. The pressure of 43 mmHg demonstrated the highest recovery rate in these experiments. Although no differences were observed in IVP when compared to the 65 mmHg pressure, the oocytes obtained with 43 mmHg had more layers of cumulus cells, resulting in a higher percentage of grade I oocytes.

Throughout this study, the safety of the approach was continuously evaluated. During the implementation of this technique, it was verified that the hormonal stimulation protocol is safe, not causing any undesired side effects in the queen. Additionally, no complications occurred during the OPU and the recovery after the procedure was rapid and painless. The laparotomy performed for the ovariectomy surgery, which took place 1 h after the OPU procedure, served to thoroughly confirm that there were no internal abdominal injuries to the ovaries or other structures.

While the blastocyst rate obtained was satisfactory (12.5 % over cleaved embryos), the results were lower than those obtained using the slicing technique. However, IVP rates of the present study were comparable to the ones obtained in the initial IVP studies performed throughout this research It must be taken into account that this procedure was performed *in vivo* once in a single animal; thus, further studies involving a substantially larger number of animals are necessary to estimate differences between groups and to identify critical aspects of the protocol to enhance outcomes. Nevertheless, at this point, we can indicate that the damage during the process is limited, making it suitable for IVP using IVM and IVF techniques.

Considering the positive outcomes achieved following the implementation of this procedure, we consider this a safe technique that offers significant advantages over existing protocols and that is easily reproducible. Lastly, further research is warranted to develop a protocol and refine the technique for use in both domestic and wild felids.

5. Conclusions

Transabdominal ultrasound-guided OPU emerges as a safe, effective, and rapid technique for minimally invasive oocyte retrieval in domestic cats, yielding high quality oocytes suitable for IVP. This technique has potential applicability in wild felid species, offering advantages over LOPU, such as minimized anesthesia duration and reduced postoperative complications. Thus, OPU stablishes as a versatile tool in assisted reproduction in this specie, offering substantial potential to

Table 3
Effect of aspiration conditions on oocyte cleavage rates, morula and blastocyst production. Values are expressed as mean ± standard deviation.

Pressures (mmHg)	Nº ovaries	Nº oocytes	No. zygotes IVC	Cleaved/zygotes (% ± SD)	Morulae (% ± SD)	Blastocysts (% ± SD)	Blastocysts/cleaved (% ± SD)
43 mmHg (3 mL/min)	7	52	47	21/47 (42.6 ± 6.8)	11/47 (22.7 ± 3.9)	4/47 (9.9 ± 6.2)	4/21 (24.4 ± 15.8)
65 mmHg (4 mL/min)	7	44	40	16/40 (38.4 ± 11.9)	8/40 (21.4 ± 6.2)	3/40 (7.9 ± 7.2)	3/16 (17.7 ± 16.7)
Total	14	96	87	37	19	7	

Table 4
Effect of method of oocyte collection on oocyte cleavage rates, morula and blastocyst production.

Retrieval method	No. Oocytes	No. zygotes IVC	Cleaved/zygotes (%)	Morulae (%)	Blastocyst (%)	Blastocysts/cleaved (%)
OPU	16	14	8 (57.1 %)	4 (28.5 %)	1 (7.14 %)	1 (12.5 %)
Slicing	16	14	9 (64.2 %)	4 (28.5 %)	2 (14.2 %)	2 (22.2 %)

enhance conservation programs involving endangered felines.

Funding

This work was supported by the Community of Madrid, as part of the grants aimed at carrying out industrial doctorates awarded to Andrea Priego-González and Ana Muñoz-Maceda (IND2023/BIO-27134 and IND2022/BIO-23496).

CRediT authorship contribution statement

Andrea Priego-González: Writing – original draft, Visualization, Methodology, Conceptualization. **Ana Munoz-Maceda:** Writing – original draft, Methodology, Formal analysis. **Joaquín Cerdeira-Lozano:** Writing – review & editing, Investigation. **Hernán Fominaya:** Writing – review & editing, Methodology. **Manuel Fuertes-Recuero:** Writing – review & editing, Methodology. **Gustavo Ortiz-Díez:** Writing – review & editing, Validation, Methodology, Formal analysis. **Manuel Gardoqui Arias:** Writing – review & editing, Methodology, Funding acquisition. **Eduardo R.S. Roldan:** Writing – review & editing, Validation, Supervision, Conceptualization. **María Jesús Sánchez-Calabuig:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Acknowledgements

The authors are deeply grateful to the Municipal Feral Cat Colony Management Center of the Madrid City Council and its dedicated staff for their indispensable help in obtaining ovaries and testicles after routine gonadectomy procedures carried out at their facilities.

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