Review Report

A practical review of assisted reproductive techniques in cattle. Part 1: Ovarian superstimulation and ovum pick-up

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Abstract

In vitro embryo production is now widely used in many cattle production systems worldwide. Currently, competent personnel are required to perform specialized procedures in the live animal for the harvest and transfer of genetic material. Ovum pick up (OPU) via transvaginal ultrasound-guided follicular aspiration has become the primary means of acquiring oocytes from antral follicles that are subsequently matured, fertilized and cultured in vitro prior to transfer into a recipient female. The number of oocytes recovered during OPU is directly proportional to antral follicle count that is inherent to the individual animal and breed. Superstimulation with exogenous gonadotropins before OPU has increased the quality and developmental competence of oocytes in most embryo production systems. This practical review examines techniques employed for donor selection, ovarian superstimulation, donor preparation, required equipment, the aspiration procedure, and expected recovery rates.

Keywords: Assisted reproductive technology, cattle, ovarian superstimulaton, ovum pick up

Introduction

The substantial increase in bovine embryos produced globally highlights the need for practitioners to be proficient in assisted reproductive techniques. Such proficiency requires a comprehensive understanding of reproductive physiology, endocrinology, and embryology, together with the development and maintenance of technical competency in collection, handling, and transfer of female gametes and embryos. The International Embryo Technology Society (IETS) reported a cumulative total of > 2 million in vitro produced (IVP) bovine embryos worldwide in 2024,[1] a remarkable progression from the pioneering days of embryo transfer in cattle more than half a century ago. Milestones in this field include the first recorded successful transfer of a mammalian embryo in 1890;[2] the birth of the first embryo transfer calf during the 1950s;[3] the first calf born through in vitro fertilization in 1981;[4] the cloning of Dolly the sheep via somatic cell nuclear transfer (SCNT) in 1996;[5] gene editing of drosophila fruit flies in 2002;[6] the advent of CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 technology and reprogramming of mature cells back into a pluripotent state in 2012;[7] and more recently, establishment of pregnancies using induced pluripotent stem cell technologies,[7] and even in vitro gametogenesis.[8] Assisted reproductive technologies have thus evolved from theoretical research to practical tools and to applications on the farm. Despite this remarkable advancement, there remains a considerable need for competent veterinarians, clinicians and technicians to operate as intermediaries between animals and laboratories. This manuscript aims to critically review relevant literature and provide insights into technical procedures involved in using live animals for oocyte collection and transfer of embryos into recipient females.

Ovum pick up and in vitro embryo production

The number of bovine IVP embryos now exceeds those produced by multiple ovulation embryo transfer (MOET)/in vivo derived techniques,[9-11] with > 2 million embryos produced globally as of 2024.[1] In the most recent data reported by the American Embryo Transfer Association (AETA), the total number of OPU procedures performed by registered practitioners was reported at 191,151 compared to 13,835 in vivo collections/flushes.[12] Dairy cattle accounted for a larger proportion of total OPUs whereas beef represented the majority of in vivo collections. The rapid rise in IVP is largely attributed to improved efficiency of

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embryo production, improvements in embryo culture systems, the capacity to generate more embryos per unit of time, the ability to collect oocytes from pre/peripubertal or pregnant animals, and the more efficient use of sexed semen compared to conventional in vivo embryo collection methods.[13,14] Furthermore, widespread adoption of accurate genomic selection has also substantially contributed to this interest and growth.[14] Despite these advances, IVP remains relatively inefficient, as < 20% of aspirated follicles yield an oocyte that develops into a transferrable-quality embryo.[1] Both quantity and quality of oocytes recovered from donor females by OPU are key early determinants of successful embryo development. Therefore, effective donor selection, preparation and refined collection techniques are crucial to maximize successful outcomes.[15]

Donor selection

Typically, oocytes or embryos are collected from genetically superior and/or phenotypically desirable females, based on client discretion. Incorporating selection and dissemination of desirable traits using genomics in young animals enables opportunities to rapidly improve genetics and therefore production, health and efficiency indices.[16,17] Beyond accurate genomic selection, clinicians can employ additional techniques to recover more oocytes from individuals and maximize overall embryo production. For instance, there are clear inherent differences among species, breeds and individual cattle in relation to total antral follicle counts and therefore oocyte numbers and overall oocyte recoveries at OPU.[18-20] Blood concentrations of antimüllerian hormone (AMH), produced by granulosa cells, correlate significantly with antral follicle count (AFC)[21] providing a plausible metric for predicting oocyte harvest in cattle. In addition, AMH is positively associated with oocyte recovery and embryo yield in OPU donors.[22-25] This supports the notion of selecting and managing potential OPU and embryo donors according to their 'ovarian phenotype'.[23] As an example, recent work in Hanwoo cattle has explored embryo development prediction for donors stratified into low, medium or high percentiles of AMH concentrations for their cohort.[26] Similar work has also been performed in *Bos indicus* cattle.[24] This approach may enable targeted donor selection by integrating genotypic and phenotypic data to optimize IVP outcomes and overall efficiency; however, AMH cut-off thresholds for donor selection should be interpreted cautiously due to assay-dependent variability and differences among animal cohorts.[24]

Donor age and physiological state profoundly affect IVP efficiency. For instance, production of embryos from calves, known as juvenile in vitro embryo production (JIVET), is technically possible and physiologically exploitative of a high follicle number before puberty, but has very low production efficiency and commercially unacceptable pregnancy rates after transfer, compared to older heifers.[27] Furthermore, dairy cows early in lactation produce significantly fewer oocytes compared to nonlactating/dry cows and those with greater days in milk.[28,29] Nutrition and metabolic status also clearly influence oocyte quality and subsequent IVP efficiencies, but are beyond the scope of this review. Nonetheless, maintaining donors within a moderate body condition score and feeding adequate maintenance requirements is critical to ensuring consistency and success.[30]

Follicle wave synchronization and ovarian superstimulation

Understanding ovarian physiology and reproductive endocrinology is paramount prior to recommending treatments. Ovarian follicles grow and regress in wave-like patterns, with individual follicles at various stages of growth, stasis and regression/atresia,[31] that affect developmental competency of the oocytes housed within. For instance, oocytes from subordinate/regressing follicles in the presence of a dominant follicle have lower embryo development rates in most bovine IVP systems than those from actively growing follicles.[32] In contrast, equine oocytes with an expanded cumulus recovered from atretic/subordinate follicles are often favoured in IVP systems.[33]

The principles of ovarian superstimulation using follicle stimulating hormone (FSH) prior to OPU are essentially similar to that of donors subjected to conventional in vivo embryo recovery. Namely, initiating emergence of a new follicular wave prior to giving exogenous gonadotropin hormones.[23,34,35] Importantly, AFC is inherent to an individual animal, is heritable, and highly correlated with response to superstimulation.[36] Exogenous FSH does not necessarily increase follicle numbers (despite improving visualization at OPU), but rather rescues subordinate follicles from atresia, a key aspect of superstimulatory physiology.[15,23,35,37] In contrast to some other mammals, it is generally agreed in cattle (despite some variable findings) that oocytes recovered from follicles in advanced atresia have reduced developmental competence compared to those in an active growth phase or those acquiring dominance.[23,32,38] This was supported by findings that in vitro embryo production benefitted from synchronization of follicle wave emergence prior to OPU, even without exogenous FSH.[34,39-41] However, this is not universally accepted, with others reporting conflicting results.[42] Regardless, most agree that giving exogenous gonadotropins concurrent with follicular wave emergence (FWE) optimizes overall blastocyst rates in most donors.[14]

Strategies to implement FWE include mechanical and hormonal approaches (Figure 1). Mechanically, via transvaginal ultrasonographic guided follicular ablation, aspiration of all antral follicles > 5 mm (or the 2 largest antral follicles[43]) is a highly reliable means of stimulating emergence of a new follicular wave within 24-36 hours.[34,35,43,44] Although this technique, known as dominant follicle reduction (DFR) or dominant follicle ablation, is perhaps the most reliable means of achieving follicular turnover, it requires technical competency, largely restricting suitability to donors housed in satellite collection centers.[34,35]

Other methods of synchronizing FWE include hormonal treatment; estradiol (most commonly, estradiol benzoate), gonadotropin releasing hormone (GnRH; e.g. gonadorelin acetate), and progesterone (either injectable or in an intravaginal device).[34,35] Simultaneous treatment of exogenous estradiol and insertion of an intravaginal progesterone devices induces atresia of any dominant follicle within 36 hours and results in FWE in typically 3-5 days.[34,35,45,46] This combination is as effective as physical mechanical ablation, rendering it highly useful for field situations.[43] However, regulatory requirements have restricted the use of estradiol in food producing animals in North America and Europe, and in parts of Australia. GnRH, typically native gonadorelin acetate, is an alternative method of FWE by inducing either ovulation or luteinization of a suitably sized follicle at treatment,[47] enabling emergence of a new follicular wave 26-48 hours after treatment.[35] However, wave emergence depends on having at least 1 follicle that will ovulate (or luteinize) in response to GnRH that historically has been believed to be a diameter of > 10 mm in most *Bos taurus* females.[48] This has been partially addressed by prior insertion of a progesterone device or giving prostaglandin $F_{2\alpha}$, and

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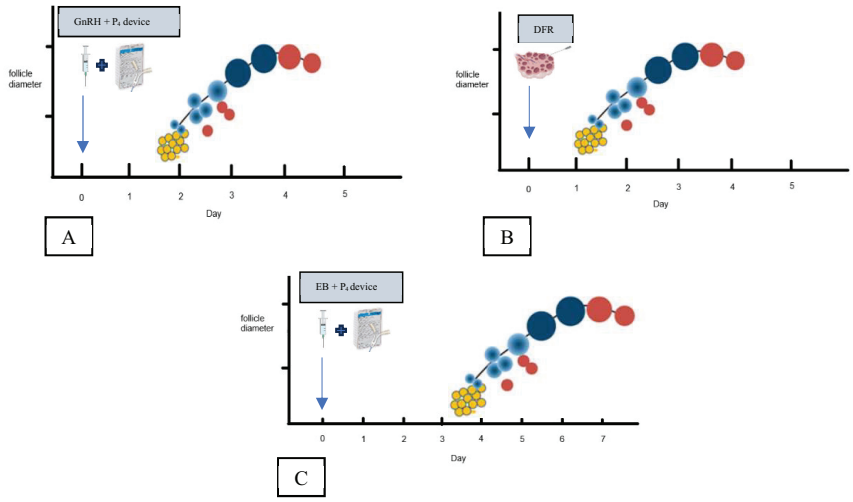

Figure 1. Schematic diagram of pharmaceutical and mechanical techniques for synchronizing follicular wave emergence (FWE) prior to superstimulation with FSH in OPU donors. A. GnRH-based programs have an average interval of 24-48 hours before FWE; B. dominant follicle removal (DFR) via transvaginal ultrasound-guided follicular ablation takes 24-36 hours on average; and C. a combination of estradiol and progesterone takes ∼ 3-5 days before emergence of a new follicular wave

combination protocols with DFR or presynchronization before GnRH.[49,50] Oocytes may be collected after synchronization of few, with or without exogenous FSH; excluding FSH has yielded modest success in situations where FSH is too costly or unavailable, providing another strategy.[23,41] A recent critical appraisal by our laboratory and clinical data indicated mild improvements in embryo production in certain cohorts of animals not stimulated with FSH but presynchronized prior to OPU,[51] despite conflicting results.[42,52]

Collection of oocytes from follicles within the growing or dominance phase appears to optimize both oocyte quality and overall outcomes in IVP systems.[53] Most medium to large follicles (∼ 7-14 mm) appear more likely to harbor viable oocytes despite technical considerations such as reduced oocyte recovery rates in larger follicles.[15,53,54] The relationship between follicle size and developmental competence appears to be related to increasing chromatin condensation within the oocyte nucleus among other maturational changes such as changes to histone methylation, transcription silencing, and redistribution of cytoplasmic organelles.[55,56] However, oocyte developmental competence can be reached at an oocyte diameter of 110 µm (follicular diameter of 3 mm)[57] and some authors have reported equivocal embryo cleavage, embryo development and blastocyst hatch rates from follicles regardless of sizes between ≤ 4 mm or ≥ 4 mm.[56] Therefore, embryos can be produced successfully in nonstimulated programs and could be justified in some situations; there is no 'one size fits all' approach. Readers are directed to other critical reviews on the overall outcomes associated with and without the use of hormonal stimulation in in vitro embryo production.[54,58]

Exogenous FSH prior to OPU increased recovery of high-quality oocytes, improved blastocyst rates and number of overall transferrable embryos.[14,15,23,29,54,59-61] The scale of improved embryo production is partly influenced by animal production systems (intensive versus extensive), breed, animal temperament, practicality and innate ovarian physiology. Despite possible improvements, some commercial IVP companies do not advocate for its use and others actively campaign against it. The rationale could be simply marketing, although some arguments are reasonable (e.g. reduced embryo production costs, reduced laborious hormonal regimens, aspiration frequency, and social perceptions). Nonetheless, there is considerable evidence that FSH increases follicle size and oocyte developmental competency.[15,54,60] Donors are usually superstimulated with purified pituitary-derived porcine FSH (Folltropin, Vetoquinol, USA). Although recombinant versions of FSH have been tried successfully in cattle and often outperform pituitary-derived products,[62-66] limited market availability (and approvals) preclude widespread use. Additionally, there are also reports of batch variations and inconsistencies in early preparations of recombinant products used in cattle. In most superstimulatory regimens, FSH is initiated at synchronized FWE to continue supporting a homogenous growing pool of follicles available for oocyte collection at OPU.[15,23,25,45] There are multiple strategies for FSH treatment, ranging from 1 injection, with or without long-acting delivery vechicles,[29,61,67] to complex, high-dose multiple injection programs similar to that of superovulatory regimens for conventional/in vivo embryo recovery.[14,15,45] Readers are directed to a very comprehensive review on optimization and refinement of superstimulatory protocols for OPU donors.[23]

In short, FSH treatments begin after synchronization of FWE (∼ 24-36 hours after DFR/follicular aspiration; 24-48 hours after GnRH; or 3-5 days after EB + P_4), for a variable number of days, with OPU performed 36-70 hours after the last FSH injection.[68] Examples of superstimulatory protocols after FWE prior to OPU are presented in Figure 2. Although the recommendation for superstimulation in MOET donors is to apply a decreasing FSH dose schedule, it was recently reported that a constant dose schedule in IVP systems was equally efficacious.[25] Furthermore, increasing the total dose of FSH from 160 to 300 mg and increasing from 4 injections once every 12 hours (2-day protocol) to 6 injections once every 12 hours (3-day protocol) also increased blastocyst rates and overall IVP efficiency;[15] however, one could argue the practicality, cost and benefit of this over a conventional/in vivo MOET flush. Not all superstimulatory treatments suit every animal, nor is every regimen practical. Therefore, discussions between practitioners and embryologists on aspects such as follicular diameter at OPU, COC recovery rates, COC quality, embryo cleavage and blastocyst rates are crucial to make adjustments and optimize outcomes in various animal cohorts.

Coasting

'Coasting' is a universal term for the designated gonadotropin withdrawal interval between the last FSH treatment (after superstimulation) and OPU.[15] Theoretically, this mimics the decrease in circulating FSH that occurs naturally during terminal follicle

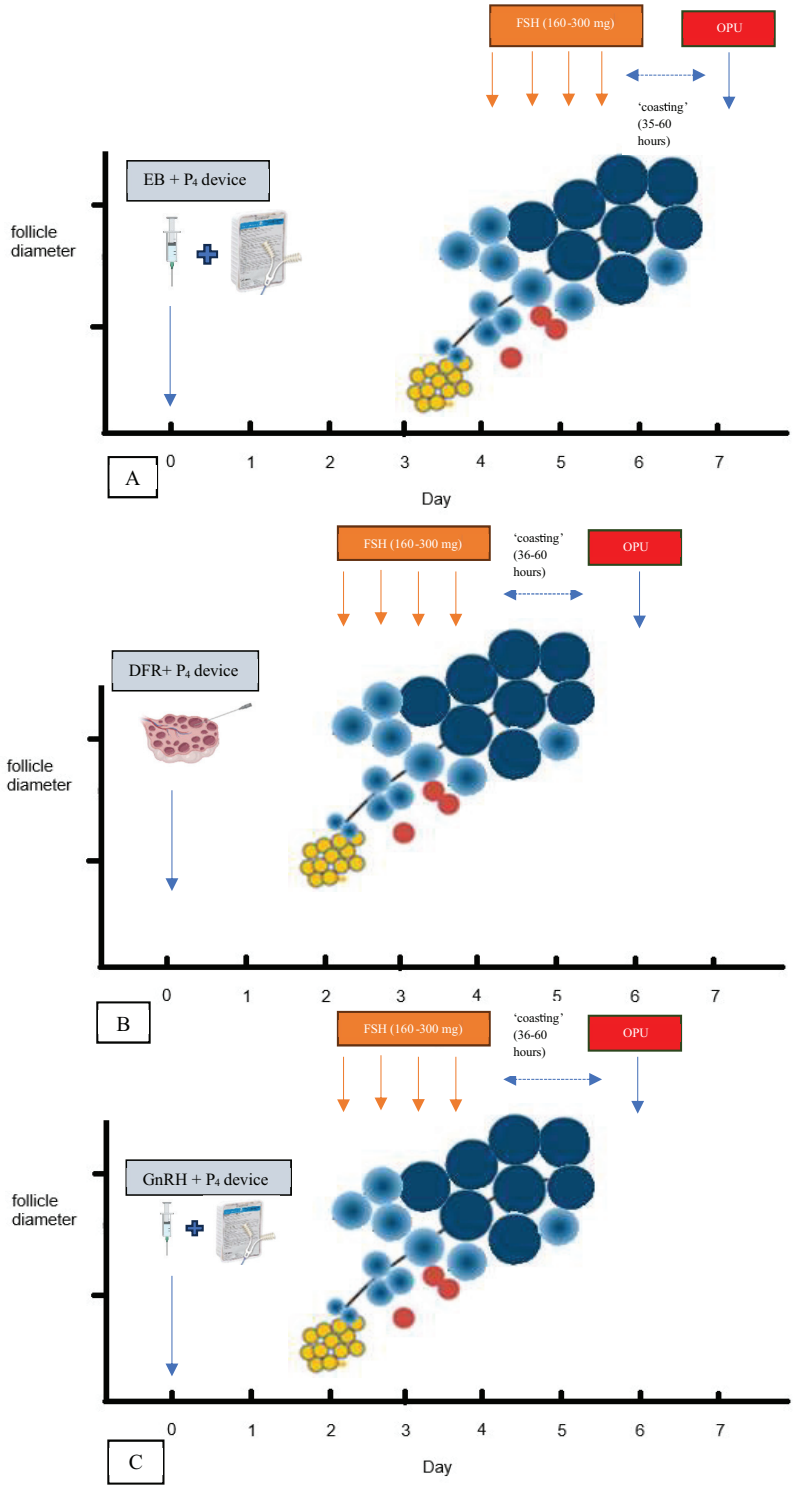

Figure 2. Visual schematic explaining various superstimulation regimens prior to OPU; A. estradiol benzoate and an intravaginal progesterone device; B. dominant follicle removal (DFR); and C. GnRH and intravaginal progesterone device. Note the superstimulation after synchronization of follicular wave emergence, timing of FSH treatment, and coasting periods before OPU. Protocols for FSH treatment vary from 1 injection, 2-day (4 injections), and 3-day (6 injections) regimens

development after selection toward acquisition of dominance.[69,70] Adopting a coasting period after completing ovarian stimulation significantly improves developmental competence of oocytes.[15,69] This is plausibly due to mimicking a preovulatory dominant-like follicle, triggering changes within follicular cellular architecture and the onset of oocyte cytoplasmic maturation before in vitro maturation (IVM) occurs after oocyte recovery in IVP systems.[54,55,70] Other terms include 'follicular capacitation[15,71] or in vivo follicular maturation.[54] In vivo follicular prematuration is achieved between 36-70 hours after the

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Figure 3. First author performing single operator OPU technique in cattle

Figure 4. Equipment necessary to perform OPU in donor cattle. A. Examples of 2 modern ultrasonographic machines/units (on left) capable of attaching to a transvaginal probe (on right)) for imaging ovaries; B. First author's complete setup; C. Example of digital vacuum pumps (Cook medical [above], WTA technologies [below] able to control and select variable vacuum pressures and flow rates; and D. Necessary accessories (e.g. aspiration collection centrifuge tubes [Falcon] commercial flush media [Boviflush, Minitube] supplemented with 5 IU/l heparin, metal needle rod with detachable long-bevel needle (in this case, 3 inch 18 gauge, [SUPRA Minitube Australia]

last FSH injection, with longer coasting intervals producing higher blastocyst rates.[69] Anecdotally, in our group's experience, adult cows perform better with longer coasting periods than peripubertal heifers. As prolonged coasting can significantly increase follicle size and reduce recovery rates, there are efforts to shorten coasting times but still maintain acceptable blastocyst rates.[61]

Antimüllerian hormone/antral follicle count guided superstimulation

Recently, a rather paradoxical relationship between total FSH dose and antral follicle count (AFC)/AMH was reported; donors with low AFC/AMH phenotypes responded poorly to significantly higher pFSH dosages during superstimulation, ultimately leading to reduced IVP efficiency, possibly explained by dysregulated oocyte maturational processes and premature meiotic resumption.[23,72] Regardless, others reported that increasing pFSH doses from 160-300 mg linearly increased visible follicle count, harvested cumulus-oocyte-complexes (COCs) and embryo development, irrespective of circulating AMH concentrations.[15] Despite ambiguity, targeted superstimulation according to measurable proxies (e.g. AFC/AMH) for ovarian phenotype is attractive in a clinical setting.

Aspiration frequency

A common clinical consideration is the optimal frequency at which OPU should occur in repeat donor cattle. This must account for multiple interacting factors, including animal signalment, intrinsic ovarian physiology, donor management (FSH stimulated or nonstimulated cycles), laboratory schedules and logistics, and importantly, animal welfare. Optimal OPU frequency based on total follicle populations available, oocyte recovery and embryo development rates remains somewhat variable between studies.[54] Previous comparisons suggest that twice weekly OPU results in the highest proportion of blastocysts produced from oocytes recovered,[73-75] despite others having documented limited benefit in oocyte recoveries when 3, 4, or 7 day intervals were analysed.[73,76,77] Notably, in the context of SCNT, a longer aspiration interval (14 versus 7 days or less) resulted in highest blastocyst rates.[75] In superstimulated donors, OPU sessions typically occur every 2 weeks to allow enough to permit adequate follicular recruitment, renew superstimulatory treatments and allow coasting before next OPU.[78] Further, emerging evidence suggests that increased OPU frequency (more than once weekly) may elevate some stress-associated biomarkers, e.g. adrenaline concentrations.[79] Collectively, findings indicate that optimal frequency is likely breed and system-specific, requiring alignment between biological response and operational constraints; therefore, OPU every 2 weeks remains widely adopted by many in both commercial and research settings.

Ovum pick up

Donor preparation and anesthesia/analgesia

Animal preparation and restraint are crucial factors contributing to successful collection and good recovery rates. We advise potential clientele of the facilities required to perform OPU in field conditions. This includes a covered chute that ideally keeps the operator and ultrasound machine screen out of direct sunlight and can apply sideward pressure to limit side to side animal movement. An example OPU collection facility is provided (Figure 5). Without appropriate facilities, the use of systemic sedatives/tranquilizers (in addition to essential epidural anaesthesia) may be considered, both for operator and donor safety and to facilitate precise follicular puncture and steady fluid evacuation, improving recoveries. Fractious animals, first-time juvenile donors and poor facilities often justify usage. In most instances, however, sedation is seldom necessary.

Regional anaesthesia and sedation techniques

Effective anesthesia for transvaginal OPU can be achieved with a lidocaine epidural alone, even in heifers undergoing repeated aspirations over several months.[80] Epidural

Figure 5. Ideal facilities to perform OPU (A and B) and aspiration setup to allow 1-operator to collect oocytes without assistance (C and D)

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anesthesia (typically lidocaine hydrochloride or alternatives; e.g. bupivacaine) is a cost effective and clinically reliable method to reduce perineal sensation, rectal tenesmus and provide analgesia to the vagina and vulva.[81] Interchanging the epidural site may also be a helpful option to reduce aversion in repeat donors.[80] Caudal epidural anesthesia is simple, rapid in onset (~ 5 minutes) and hence widely used. A high caudal epidural at the sacrococcygeal space (S5-C1) will typically anesthetize sacral nerves S2-S5 whereas a low caudal epidural as the intercoccygeal space (C1-C2) will typically block S3-S5,[81,82] with cranial spread influenced predominantly by dose volume.[83] Anecdotally, sacrococcygeal (high) caudal epidurals (when appropriately dosed) may provide more consistent efficacy. Most clinicians use lidocaine hydrochloride at a maximum dose of 1 ml/100 kg. Alternative options include 0.6% bupivacaine at 0.6 mg/kg (consider dilution with sterile water for injection to provide a workable volume[84]). Addition of α2-agonists (e.g. xylazine hydrochloride) can extend anesthetic duration from ~ 60-90 minutes to up to 5 hours; that may benefit training procedures, but has limited relevance for experienced operators (who typically complete the procedure in 15-20 minutes).[85] Importantly, there is no significant difference in the onset of analgesia between lidocaine alone and combination protocols, and dosing must balance adequate desensitisation with avoidance of ataxia.

If sedation is indicated, the authors recommend a phenothiazine derivative tranquilizer such as intramuscular acepromazine maleate at the low recommended dose of 0.02 mg/kg given 15-20 minutes before aspiration.[86] Alternatively, low dose intravenous xylazine hydrochloride (0.05 mg/kg) may be given immediately prior to beginning the procedure, or a higher dose intramuscular (double the intravenous dose) 15-20 minutes prior. Dose selection is crucial to avoid ataxia and/or risk of recumbency. Although most collections can be completed without sedation, it may be beneficial in fractious animals, particularly those that are superstimulated (with larger follicles) or inadequately restrained. In rare cases where epidural anaesthesia is ineffective (e.g. obesity, fibrosis from repeated use), alternative regional techniques (including sacral paravertebral anesthesia, local splash blocks [intrarectal or intravaginal], or a pudendal nerve block) may be considered to reduce rectal tenesmus and provide perineal analgesiae.[82] Should these techniques fail to prevent rectal tenesmus and provide sufficient analgesia it may be prudent to withdraw from the procedure on the grounds of animal welfare.

OPU technique

Oocyte collection by transvaginal ultrasonography guided OPU technique was initially intended for problem donors that underperformed in conventional superstimulation and in vivo embryo recovery programs.[87] It has since evolved considerably from the first described nonsurgical technique for cattle in 1987.[88] The procedure involves the operator aspirating all antral follicles within ovaries that are manipulated transrectally and guided by a transvaginal ultrasonographic probe and needle.[73] As opposed to other species, most bovine clinicians would opt for a 1-operator technique, with their nonpalpating arm holding both the transvaginal probe and manipulating the aspiration needle. Sometimes an assistant is helpful (particularly when learning), but most experienced clinicians can aspirate donor cattle without assistance.

The main components involved in aspiration include a microconvex ultrasonographic transducer fitted within a moulded plastic casing that is inserted intravaginally. Usually, a sanitary sheath or sterilized rectal sleeve is placed over the transvaginal probe before insertion and replaced between donors. The moulded casing also houses a needle channel that can fit either a complete system with a long needle or a stainless-steel rod/guide that can fit a detachable disposable needle of variable size and length (18-21 gauge and 1.5-3 inches long). This is attached to plastic tubing (1.1 mm diameter and ~ 55 inches long) that transports fluid and collected COCs to a collection vessel (typically a 50 ml conical centrifuge tube). Some clinicians mount the collection tube on the transvaginal probe, facilitating a shorter transport tube, whereas others mount it on themselves. Negative aspiration pressure is achieved with an aspiration pump (Cook medical; WTA [College Station, TX, USA], or other) connected to the collection vessel with rubber tubing, and with an in-line water trap/filter to avoid aspiration of fluid into the pump.

In summary, the procedure/protocol of oocyte recovery in a live animal is as follows. Typically, the donor animal is restrained in a cattle chute and an epidural anesthesia (~ 20 mg/100 kg lidocaine hydrochloride [i.e. 3-5 ml of lidocaine 20 mg/ml solution] is given). Sedation and/or tranquilization is generally unnecessary but may be beneficial should donor restraint be suboptimal. Following this, the operator evacuates the rectum and the vulva is wiped to remove debris or feces and then cleaned with disinfectant. Some clinicians advocate a vaginal 'washout' with sterile saline (with or without diluted antiseptic such as Virkon S or iodine) in donors that had an intravaginal device.[89] A plastic chemise/covering is placed over the probe casing with a small amount of sterile ultrasonographic gel applied to the transducer head. The transvaginal probe is then introduced into the vulva, past the vestibule at a 45° angle and then into the vaginal fornix immediately adjacent to the external cervical os ipsilateral to the ovary to be aspirated. The ovary is grasped firmly toward the transducer end and any rectal folds or tissue between probe, vaginal fornix and ovarian stroma are pushed away. While manipulating the ovary via transrectal palpation, the needle guide is aligned to the follicle of choice and entered by advancing the needle; that entry should be visualised on the ultrasonographic viewing screen. Once the follicle contents have been evacuated, the ovary should then be again manipulated transrectally to realign the next follicle and the needle subsequently readvanced. It is imperative during nonstimulated collections to avoid penetrating any deeper than 5-10 mm into the ovarian stroma and reducing retraction and repenetration of the vaginal wall. After a follicle is ablated, the authors gently retract the needle (not completely remove it) back through the ovarian stroma, realign and then penetrate another follicle. Complete needle retraction back from the peritoneal space into the vaginal cavity only occurs if the animal makes dangerous movements or when shifting between ovaries. Larger follicles are aspirated last to assist ovarian manipulation and 'wash' the aspiration line after completing an ovary. In FSH-stimulated donors with larger follicles (> 6 mm), the authors seek to envision a route whereby the needle is moved from follicle to follicle without needing to make major realignments. Following collapse of larger follicles needle scraping is employed (twisting 90-120°) to ensure that all fluid is recovered from the follicle. The ovary should be manipulated transrectally over the transducer, not by moving the ultrasonographic transducer; a skill that is important to learn. It is important not to apply excessive pressure to the ovary during collection, particularly when working with stimulated donors, as this can lead to the bursting of follicles prior to aspiration, reducing recovery rates due to follicle leakage.

This is repeated for every follicle on both ovaries; depending on the number of available antral follicles the procedure may take 10-20 minutes.

Equipment and preparation

As there is a plethora of equipment available for aspirating oocytes from donor cattle, the choice is typically dependent on clinician preference, availability, cost and compatibility with existing systems. Firstly, the use of an ultrasonographic machine that has exceptional image quality (able to image antral follicles < 5 mm) is paramount for unstimulated collections. The first author prefers a 5-10 MHz broadband array microconvex OPU transducer probe (eC9OPU; E.I. Medical Imaging) coupled with a portable B-mode ultrasonographic scanner (Ibex EVO III; E.I. Medical Imaging). Other crucial factors include maintaining adequate flow rates and vacuum pressures to support oocytes after extraction from follicles. The first author also prefers to use a human medical-grade aspiration pump (Cook vacuum pump; Cook Medical, Australia) and 1.1 mm aspiration tubing that is ~ 55 inches long (WTA Technologies or AgTech Inc [Manhattan, KS, USA]) connected to a disposable IVF threaded centrifuge cap (MAI Animal Health, Elmwood, WI) or reusable aluminium stopper (WTA Technologies) fitted on a sterile 50-ml propylene conical Falcon centrifuge tube (available from laboratory suppliers or Falcon). The Falcon/centrifuge tube is kept warm using a tube warmer device (WTA Technologies or AgTech Inc) that is held around the operator's neck or by an assistant. For each donor, the sterile 50-ml Falcon tube is prefilled with ~ 7-10 ml of commercial buffered pH stable bovine embryo flushing medium (EmCare, ICPbio, Auckland NZ; or Bovifush, Minitube) or commercial bovine aspiration medium (IVFBioscience, Farmouth, UK) supplemented with 5-10 IU/ml heparin prior to beginning the aspiration and kept warm in a tube heater or digital dry bath/block heater (WTA Technologies or Thermo Scientific). The first author now generally uses only 5 IU/ml heparin (despite higher doses during training). Although marginally higher doses do not appear to impair embryo production, experience in other species (e.g. horses) suggests caution, and a higher dose is only used if blood clots begin to impair flow during OPU (Figure 6) or interfere with searching after recovery.

Imaging and visualization

Advances in transvaginal ultrasonography are partially responsible for increases in numbers of follicles punctured and oocytes recovered.[77] Convex sector transducers provide superior visualization and are favoured over linear-array transducers,[90] particularly when operating at higher frequencies (up to 9-10 MHz) with adjustable settings for contrast, gain and color flow Doppler. Image quality can be enhanced with a small drop of ultrasonographic gel on the probe/transducer (with a sanitary sleeve), although some operators prefer a small volume of collection media (as gel may trap oocytes). Improved imaging facilitates precise needle alignment, minimises inadvertent puncture of ovarian stroma and reduces hemorrhage, blood clot formation and potential ovarian damage.[91]

Needle size and aspiration pressures

No single preset needle size, aspiration pressure or flow-rate suits every animal or every system. Adjustments are necessary based on donor preparation (stimulated versus nonstimulated), follicle size, historical recoveries, per hoc recoveries, oocyte morphology and ultimately blastocyst rates. The relationship between flow-rates (i.e. amount of fluid aspirated per unit time) and pressures (mm Hg) according to needle size/gauge have been reported.[92] Recommended flow rates vary, although ~ 15 ml/minute is likely best, as higher rates only slightly improve recoveries, but start to reduce cumulus layer preservation.[93] Flow rates should be checked regularly; our laboratory checks routinely before every OPU session. This is achieved by aspirating and recording a volume of media drawn through the line over a set period at animal working height; for instance, 7-8 ml of fluid aspirated within 30 seconds would indicate this recommended flow rate. Flow rates are related to vacuum pressures that are dependent on needle size. Overall recovery rates are also dependent on this complex interaction among flow-rates, vacuum pressures, needle diameter, and needle bevel.[54]

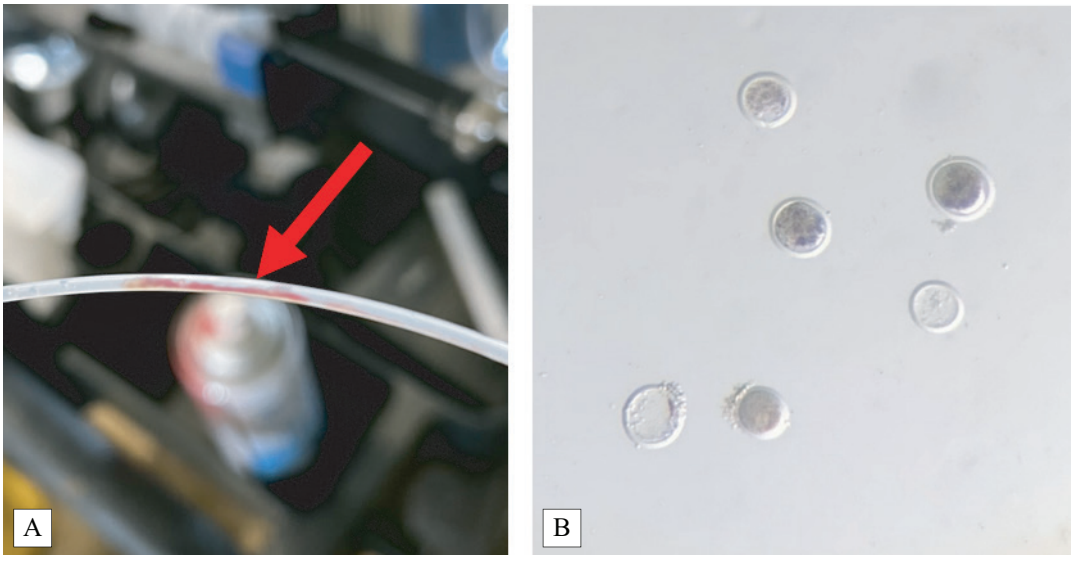

Figure 6. Note a large clot in the aspiration line (A) that can alter intraluminal pressure and consistent flow (possibility of oocyte cumulus layers stripping [B]); note also oocyte cytoplasm (poor oocyte quality) not related to the procedure

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In summary, flow rates increase with needle size at a given pressure;[92] blastocyst rates are inversely proportional to vacuum pressure (ideally 70-130 mmHg);[92] and that although thinner (~ 20 gauge) needles preserve compact cumulus layers,[91,92] larger gauge, longer bevel needles achieve superior overall oocyte recovery despite reduced cumulus retention.[94,95] Notably, these findings, generated largely with abattoir-derived ovaries, may not fully reflect in vivo OPU conditions, with limited live-animal studies reporting different optimal needle sizes and vacuum pressures.[96] This discrepancy warrants further investigation, consistent with recent findings in mares where increasing aspiration pressure alone does not necessarily improve recovery.[97] In practice, our laboratory uses a 3-inch 18-gauge long-bevel needle with vacuum pressures of 40-110 mm Hg, adjusted according to donor characteristics, embryologist feedback, and historical performance data, aiming to recover oocytes with > 3 cumulus layers in stimulated donors while maintaining overall yield. We attempt to use vacuum pressures that promote recovery of > 3 layers of cumulus in stimulated donors, while still optimizing overall recoveries. Careful attention to maintaining consistent flow and minimizing clot formation during OPU (Figure 6) also essential to reduce pressure fluctuations within the collection system.

Follicular curettage

Twisting the needle after collapse of larger follicles can improve recovery rates of oocytes;[98] accordingly, gentle 90-180 degree twisting of the needle is recommended after aspiration of all follicles in stimulated collections and in follicles > 6-7 mm in unstimulated cycles in order to ensure that all pockets of fluid (that may be outside the plane of the ultrasonographic image) are recovered.

Follicular flushing

Follicular flushing has been explored to enhance recovery from larger stimulated follicles, as done in other species (e.g. women and mares).[93] A radial-delivery aspiration system ('OxIVF') to deliver flushing fluid in a radial spread from the needle and was compared this to follicular flushing and aspiration with a double-lumen needle.[93] An intrafollicular vortex was created, and recovery rates improved (particularly in follicles ≥ 7 mm) when this technique was applied.[93] However, follicular flushing and use of conventional follicular flushing with double-lumen needles is not cost-effective and does not consistently improve recovery rates at scale.[98]

Maintaining temperature control

Maintaining consistent oocyte temperature from the follicle to the laboratory, including in the collection line, is critical to maximizing embryo development rates and potentially pregnancy rates.[99] In field settings, battery-powered 'tube-warmer' devices (WTA Technologies) are often used to warm collected aspiration fluid. Aspirating donors in an insulated room maintained at 26-30°C also reduces temperature changes. Nevertheless, some temperature variation during transit through the collection line is unavoidable, highlighting the need for improved strategies to stabilise thermal conditions.

Oocyte handling and grading

Once aspiration has been completed and follicular contents are in the collection vessel, the embryologist begins processing the aspirate. In short, while maintaining temperature control (i.e. ideally working on a heated stage or plate set between 37.5 and 38.5°C) the fluid contents are passed through a 70-100 µm cell strainer/filter (either a Greiner BIO-ONE 100 µm EASYstrainer™ or 75 µm IVF Oocyte filter (Pacific Vet, Australia) and then washed with gentle fluid pressure (using needle and syringe) onto a searching dish. Searching for COCs is done using a stereomicroscope at 30 x magnification. Following location, COCs are then assessed and graded morphologically and then typically washed 2 or 3 times with a bovine serum albumin (BSA) + antimicrobial (kanamycin sulphate) supplemented Dulbecco's phosphate buffered saline solution, then placed in IVM transport medium. Again, this will vary among commercial laboratory protocols.

Grading is partially subjective and most often performed according to guidelines established by the IETS.[73,100] However, various IVP systems may use different nomenclature for grading scales. Morphological evaluation is often performed using a combination of number of cumulus layers as well as general appearance of the oocyte cytoplasm on a 1-4 (or A-D) scale. Furthermore, there are effects of breed and metabolic status (i.e. lactating dairy cows) on morphological features, particularly ooplasm appearance. Heat stress can also have substantial impacts on ooplasm appearance with subsequent effects on blastocyst rates. Interestingly, as a means to reduce some of the variability associated with subjective analysis of oocyte grade, authors have recently described artificial intelligence-assisted selection for bovine oocytes, similar to what is conducted in the embryo grading space.[101]

Obtaining high quality oocytes is important, as oocyte grade does not only have a relationship with embryo development and blastocyst rates[29,102-105] but is also related to postthaw embryo viability[102] and potentially pregnancy rates. Also, others are seeking to explore other noninvasive markers that may assist in predicting developmental competency beyond rudimentary morphological evaluation.[106] Preserving cumulus layers is important in oocyte maturation and can promote meiotic resumption when group cultured with oocytes without sufficient cumulus[107] suggesting that preservation of cumulus layers during oocyte recovery is important for success in IVP (Figure 7D). Beyond maturational support, cumulus cells also provide metabolic and antioxidative substrates that promote fertilization.[108] Collectively, these findings strongly indicate the opportunity for clinicians to optimize donor management (i.e. nutrition), superstimulation regimens and oocyte recovery techniques to enhance the overall IVP process.

Expected recovery rates and technical competence in OPU

There are limited published data that document when a practitioner can achieve acceptable technical competence. Expectedly, reported recovery rates vary widely among commercial laboratories,[54] among technicians/practitioners,[38] stage of estrous cycle and follicular dynamics,[38] stimulation strategies and follicle size,[23,45] and animal demographics/ovarian phenotype.[109] Published recovery rates (i.e. proportion of oocytes recovered from number of follicles punctured) in studies vary widely ~ 35-90%, with most agreeing that recoveries from stimulated cycles average ~ 50-70%[15,45,110] and in nonstimulated sessions, recoveries are often higher, ~ 65-85% (or more). Follicle size and stimulation regimen may have no influence on recoveries,[23] and in fact may be even be higher[111]

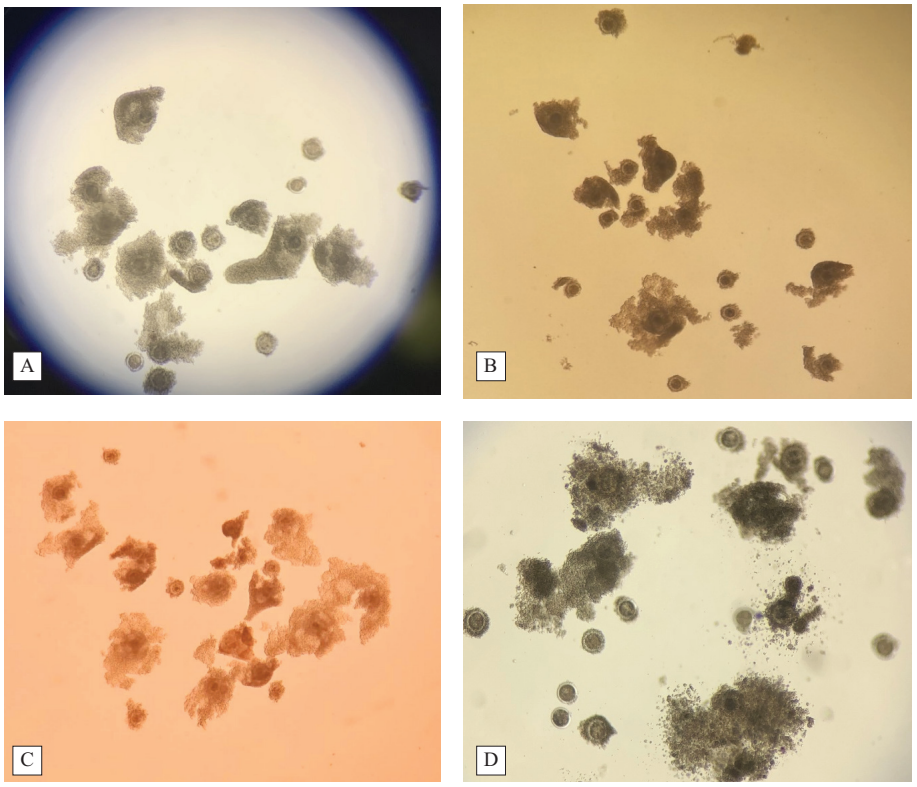

Figure 7. Oocytes collected from donor cattle via transvaginal ultrasound-guided OPU; A. From 13-month heifer (collected 36 hours after 1 FSH injection [175 IU/10 mg]); B. From an adult cow (collected with higher vacuum pressure [110 mm Hg] after stimulated cycle with 4 x injections of total dose [200 mg/350 IU] of FSH]); C. another donor under similar stimulation protocol; however, with low vacuum pressure (40 mm Hg), note the multiple cumulus layers; and D. example of an unstimulated collection with varying oocyte quality and stages of cumulus compaction and expansion

implying many confounding factors and countering the colloquial notion that increased follicular size reduces recovery rates. Reporting of recovery rates is therefore probably obsolete, and rather focusing on quality (and quantity) of oocytes recovered and overall embryo development rates per cycle/OPU is perhaps more applicable. An important consideration is getting accurate follicle counts before OPU during an ovarian survey, as underestimating/miscounting follicle numbers will bias overall recovery rates. After > 25-30 follicular punctures, counts and therefore recovery rate percentages, become highly unreliable.

Overall published total oocyte recoveries per donor vary among technicians, breeds, species, animal age, physiological stage, superstimulation regimens and animal management practices.[29,54,61] For instance, breed average recovery rates for Holstein-Friesian heifers are ~ 15-16 total usable oocytes per OPU (< 10 months) to 12 total oocytes per OPU (> 10 months), lactating cows 15 total oocytes, and nonlactating cows 20-21 oocytes/OPU.[29] Higher recovery rates are typically reported for beef cattle, with *Bos taurus* (Angus) donors reporting 18-25 oocytes/OPU[54] and *Bos indicus* (Brahman and Nelore) donors averaging ~ 30 or more oocytes/OPU.[112] For accurate, larger-scale reported data, the reader is directed to the IETS Annual Data Committee Retrieval reports[9] and AETA reports.[12]

As a rough guide, it is recommended that 50 individual OPUs are performed under supervision to ensure safety; 500 are performed to develop proficiency; and 1,000 are performed to master the procedure. However, this will very among individuals, prior reproductive experience, and regularity and consistency of repeat OPU sessions. Nearly 1 year of data collected for a farm in which regular OPU sessions were performed fortnightly exemplifies the expected acquisition of technical competency (Figure 8) (there was some prior experience and basic training in OPU prior to beginning these sessions). Overall recoveries and recovery rates improve substantially during the first 300-400 donors/OPUs and then plateaued. Recovery rate percentages recorded by practitioners should be interpreted with caution (and requires scepticism) as it is easily biased. Reassuringly, overall recoveries were in-line with those reported for Holstein-Friesian heifers by other large-scale OPU/IVP programs.[29,54,61]

Conclusion

Despite advancements in laboratory protocols, embryo culture conditions and cryopreservation techniques, there are still opportunities to improve the overall process of in vitro embryo production, particularly when attention is paid to procedures relevant to the individual donor. The overall IVP process is still considered relatively inefficient with only 18% (on average) of aspirated follicles resulting in development of a transferrable embryo.[14] Optimizing collection of high-quality oocytes with acceptable developmental competence can be pursued based on individually targeted superstimulation regimens, donor selection in IVP programs according to ovarian phenotype/AFC, and refinement of the OPU procedure itself.

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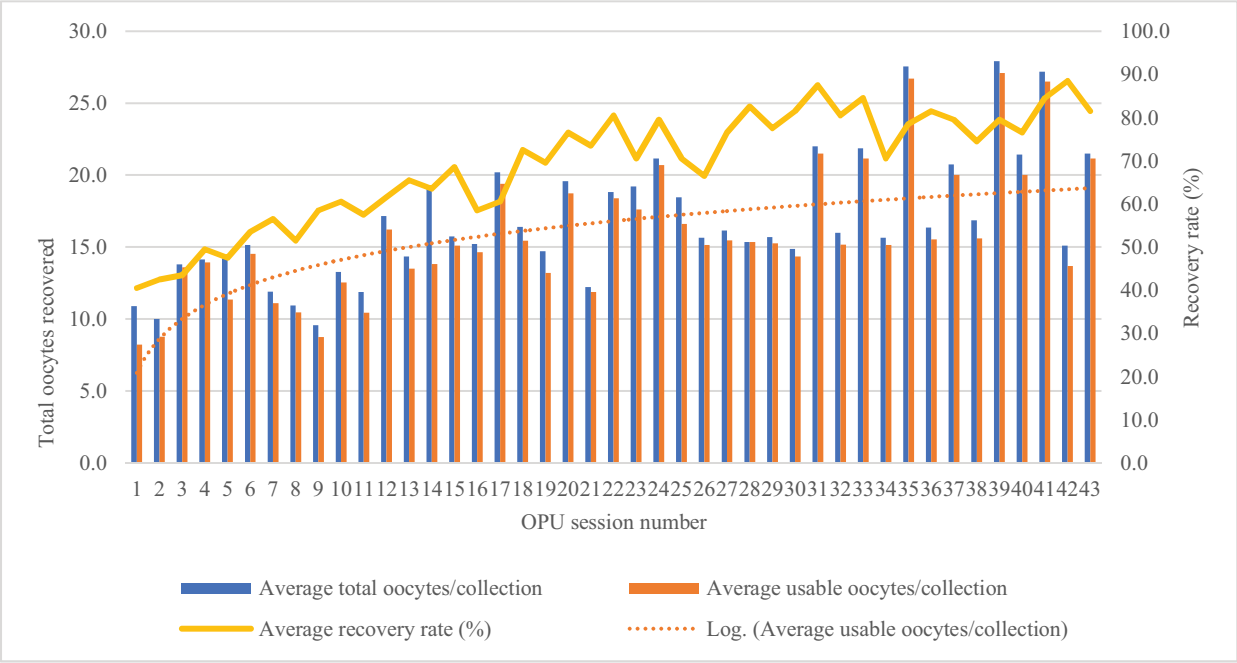

Figure 8. Figure demonstrating acquisition of technical competence. Oocytes recovered by first author from 723 individual OPUs out of 43 sessions performed fortnightly for a farm over an 11-month interval on non-stimulated Holstein-Friesian dairy heifers [9-14 months]. Total oocytes recovered, and total oocytes were set aside for IVM (left Y-axis). Recovery rates from a rough estimate by the author at OPU (right Y-axis)

Making improvements in oocyte harvest techniques should be a valid target for researchers and practitioners alike. An important message to reinforce, however, is that performing these procedures at an industry competitive level requires countless hours of practice, dedication and consistent refinement to maintain proficiency.

Authors' contribution statement and agreement

RN: conceptualization and preparation of original manuscript, image collection and preparation, manuscript editing and revision and **RS:** manuscript conceptualization and period after review. Authors have read and approved final submission.

Conflict of interest

None to report.

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