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Ovarian follicular flushing as a means of increasing oocyte yield and *in vitro* embryo production in cattle

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Abstract

Context: The number of developmentally competent cumulus-oocyte complexes (COCs) retrieved during Ovum Pick-Up (OPU) determines success in both bovine and human assisted reproduction. Follicular flushing for COC retrieval is practiced widely in humans but not in cattle. **Aims:** To determine the benefits of follicular flushing in cattle and assess the merits of a novel 16G double-lumen needle ('OxIVF') that flushes laterally to the needle shaft. **Methods and key results:** Experiment 1 flushed 655 antral follicles (≥7 mm) from 255 abattoir-derived cattle ovaries. COC recovery was greater (P = 0.034) for the OxIVF vs Standard needle (mean ± SE; 74.1 ± 2.10% vs 67.0 ± 2.23%); yield of Grade 1 COCs was also greater (20.1 ± 1.97% vs 8.2±1.38%; P < 0.001). In Expt. 2, twelve Holstein heifers underwent two cycles of OPU in a cross-over design comparing both needle types. Recovery of COCs was greater (P = 0.045) for the OxIVF vs Standard needle (89.1±2.98% vs 79.6±3.47%). Day 6 embryo yield was also greater (P = 0.017) for the OxIVF vs Standard needle (87.2±4.38% vs 67.6±6.73%). In Expt. 3, eleven Holstein heifers underwent two cycles of OPU using the OxIVF needle in a cross-over design: flushing (≥7 mm follicles) vs a 'Hybrid' approach of flushing (≥7 mm follicles) and aspiration (5-7 mm follicles); followed by two cycles of standard follicle aspiration (> 5 mm follicles). Recovery of COCs was greater (P = 0.033) for 'Flush' vs 'Aspirate' groups (82.1±5.06% vs 66.2±3.48%). However, number of Day 8 blastocysts for the 'Hybrid' vs 'Flush' approach (9.2±1.39 vs 6.5±1.05 per cycle) did not reach statistical significance. **Implications:** Follicle flushing using the OxIVF needle, embracing the 'Hybrid' approach, has the potential to increase oocyte retrieval and blastocyst number per donor cycle but requires further validation.

Introduction

A key indicator of success for embryo-based platforms used in assisted reproduction (ART) relates to the number of live births generated per donor cycle. This can be influenced by several factors including methods employed for *in vitro* culture (IVC), cryopreservation and transfer (ET) of embryos in both livestock and humans (Hansen, 2023; Niederberger et al., 2018). An additional factor limiting the success of IVC-ET in cattle relates to the *in vitro* maturation (IVM) of germinal-vesicle (GV) intact oocytes prior to *in vitro* fertilization (IVF) (Lonergan and Fair, 2016). Although recognised as a key step determining ET success in this species, IVM is not currently practised widely in human ART (Smeenk et al., 2023), aside from cases of fertility preservation or where ovarian stimulation is contraindicated (Walls and Hart, 2018; Ahmad et al., 2023). Regardless of species, however, ART cycles commence with sessions of transvaginal follicular aspiration (Ovum Pick-Up; OPU) for the collection of GV (cattle) or predominantly metaphase II (MII) oocytes (humans). It follows that the number of developmentally competent oocytes retrieved during such sessions is also a major determinant of ART success in both species (Hansen, 2020; Vermey et al., 2019).

Oocyte retrieval in cattle can be undertaken from donors stimulated with exogenous follicle stimulating hormone (FSH), or from non-stimulated donors; the latter scenario is more common among *Bos indicus* genotypes which have larger numbers of antral follicles compared to *Bos taurus* genotypes (Baruselli et al., 2021). These structures are generally smaller in this sub species of cattle where aspiration is typically undertaken in follicles ≥ 3 mm in diameter. In contrast, controlled ovarian stimulation prior to oocyte retrieval is more commonly practiced in *Bos taurus* breeds (Fry, 2020; Sarwar et al., 2020). Whilst various protocols for ovarian stimulation exist (Galli et al., 2001; Seneda et al., 2020), those described in the current report are based on exogenous FSH treatment followed by a short period (~42 h) of gonadotrophin withdrawal (termed 'coasting') prior to OPU (Blondin et al., 2002; Nivet et al., 2012). In our hands these protocols typically result in between 17 to 30 follicles ≥ 5 mm in diameter for sexually mature donors, and up to 40 follicles ≥ 5 mm for peri-pubertal donors (Tutt et al., 2021; Simmons et al, 2023; Tutt et al., 2023). Furthermore, the distribution of follicle diameters recorded at OPU in these stimulated cycles are typically 36% (5-7 mm), 55% (>7-10 mm), and 9% (>10 mm); and the percentage oocytes recovered following standard aspiration has averaged 66.6 ± 1.03%. These results are generally consistent with other studies adopting similar approaches to oocyte retrieval in taurine cattle (Gimenes et al., 2015; Sarwar et al., 2020).

The situation in human ART is somewhat different, although there are important parallels. Controlled ovarian stimulation is standard practice for the most part, with gonadotrophin administration tailored to ensure a high percentage (>70%) of MII oocytes (from typically 16-22 mm follicles) on day of retrieval (Abbara et al., 2018); with a further 10 to 20% of oocytes transitioning towards MII at this time (Shu et al., 2007; Braga et al., 2020). This is important because cumulus expansion dissociates (to an extent at least) the cumulus-oocyte complex (COC) from the follicular wall rendering this

structure more amenable to retrieval during OPU (Lainas et al., 2023a). Retrieval is mostly undertaken by follicular aspiration (using single-lumen needles) but also by combined aspiration and flushing (using double-lumen needles) (D'Angelo et al., 2019). Whilst early nonrandomized studies indicated that follicular flushing could increase oocyte yield over aspiration, more recent randomized controlled trials found no improvement. Accepted COC recovery rates range between 60 to 80% (e.g., Haydardedeoglu et al., 2017; von Horn et al., 2017; Calabre et al., 2020; Malhotra et al., 2020; de Souza et al., 2021). The consensus at present suggests no overall benefit of follicular flushing in human ART (Georgiou et al., 2022).

Follicular flushing is also widely practiced in equine ART, aspects of which overlap with both bovine and human OPU. Whilst retrieval of COCs is easier from large dominant follicles (typically 20 to 35 mm), ovarian stimulation in the mare is not practiced widely. This is due, in part, to lack of commercial availability of appropriate gonadotrophins but it also relates to complexities in cycle regulation and monitoring. Consequently, retrieval of GV oocytes from non-stimulated follicles (typically 8 to 25 mm) is favoured (Hinrichs, 2018; Stout, 2020). However, the problem with this approach is that attachment of GV oocytes to the follicle wall is particularly strong in this species, requiring repeated flushing with double-lumen needles (typically 12G) combined with scraping of the granulosa to achieve clinically acceptable (50 to 70%) recovery rates.

In contrast to human and equine ART, follicular flushing is not generally practiced in cattle. Consistent with experience in humans, early studies in cattle (working mostly with abattoir derived ovaries) found no advantage, in terms of COC yield or quality, of follicular flushing over conventional aspiration (Fry et al., 1997; Sasamoto et al., 2003). Consequently, interests turned to other factors that can affect yield and quality of COCs when using single-lumen needles, including needle diameter and bevel length, vacuum pressure, and frequency of aspiration (Ferré et al., 2023). However, retrieval rates following standard follicular aspiration are modest and highly variable both between donors, in a given cycle, and within donors between cycles (our experience (Tutt et al., 2021; Simmons et al, 2023; Tutt et al., 2023) and those of others (e.g., Sarwar et al., 2020; Ferré et al., 2023)).

Consequently, and with the foregoing discussion in mind, the current series of experiments sought to re-evaluate follicular flushing as a means of oocyte retrieval in cattle. Our motivation was encouraged, in part, by the recent creation of a novel double-lumen needle (referred to as the 'OxIVF' needle) by members of the current consortium. This needle differs from other double-lumen needles in that it flushes laterally to the needle shaft through 12 radial perforations approximately 7 mm from the needle point (**Fig. 1**). Mathematical modelling, and computational fluid dynamics-based evaluation of flow behaviour, indicated that the new design elements generate a rich laterally induced full-volume rather than frontal flow field within the ovarian follicle (Cimpeanu et al., 2023).

Consequently, model predictions are that the intrafollicular vortical structure generated during flushing will (i) negate the importance of COC location within the follicle relative to proximity of the needle tip at the time of aspiration, and (ii) achieve enhanced beneficial COC movement with no additional shear stress experienced during flushing; and with theoretically measured velocities inside the needle matching those quantified during aspiration. The predicted outcomes, therefore, are that oocyte yield following OPU would be enhanced and trauma to the COC (potentially leading to loss of cumulus cells and/or damage/stress to the oocyte itself) reduced in view of comparatively more favourable directionally distributed flow characteristics. This latter aspect could have implications for post-fertilisation development and pregnancy outcomes following embryo transfer. In addition to its potential use in bovine OPU, the outcome of these experiments could also inform on the efficacy of this new needle design ahead of future clinical trials in humans.

The three experiments reported in this article began with a pilot study which compared the new 16G OxIIVF double-lumen needle (prototype by Vitrolife, Sweden) to a standard 16G double-lumen needle (also manufactured by Vitrolife, Sweden) used widely for human OPU. In addition to providing preliminary data on the relative merits of the OxIVF needle, this experiment helped establish the flushing/aspiration parameters and instrument settings required for the two subsequent *in vivo* studies. The first of these *in vivo* studies directly compared the two double lumen needles in a cross-over design comprising two OPU cycles and 12 virgin heifers, where only follicles ≥ 7 mm in diameter were punctured. The second of these *in vivo* studies extended the first by comparing follicle flushing only (as described) to a hybrid approach of flushing follicles ≥ 7mm followed by aspirating follicles between 5 and 7 mm in diameter (follicles < 7 mm were deemed to be too small to flush). This was also a cross over study and involved 11 virgin heifers. At completion, all animals from this study underwent two cycles of follicular aspiration following a standard protocol reported previously by our group (Tutt et al., 2021; Simmons et al, 2023; Tutt et al., 2023).

Materials and Methods

All procedures were approved by the Animal Welfare and Ethical Review Board (AWERB) of the University of Nottingham. In addition, procedures undertaken on living donors were performed under the auspices of the Animal Scientific Procedures Act (1986). Associated protocols complied with the ARRIVE guidelines with project licensed authority (PDBF3E539; 29/05/2019). All chemicals and reagents were sourced through Sigma-Aldrich Company Ltd (Dorset, UK) unless otherwise specified.

Design features of needles and related equipment

The conventional needle (Vitrolife; outer diameter 1.65 mm/16G, 455 mm length) had a standard double-lumen design aspirating through the inner lumen and flushing through the outer lumen. The experimental OxIVF double-lumen needle was identical in dimension (outer diameter of 1.65

mm/16G, 455 mm length) with an inner lumen for aspiration and an outer lumen for flushing. However, it had 12 radial perforations approximately 7 mm from the needle point, with the flushing lumen sealed at the distal end (**Fig. 1**), enabling flush media to be delivered through the perforations rather than the needle tip. These perforations were 0.1 mm in diameter and tilted at 30° to the distal needle end to facilitate 'backwards flushing' (Cimpeanu et al., 2023) to initiate and maintain a rich flow field inside the entire intra-follicular volume that would facilitate oocyte extraction from previously inaccessible locations.

Aspiration was achieved using a digital vacuum pump (K-MAR-5200, Cook Medical, USA) and flushing controlled with a prototype peristaltic pump, assembled by Labman Automation Ltd (Middlesborough, UK). Aspiration and flushing tubing (both 2.5 mm OD, 1.9 ID, and 900 mm in length) were supplied attached to the needles supplied by the manufacturer. We used a thicker-walled silicone tube (supplied with the pump) attached to the flush line by a Luer fitting which was run through the peristaltic pump from the reservoir of flush media (held at around 37˚C).

Experiment 1. Oocyte retrieveal from abattoir derived ovaries (pilot study)

Ovaries were obtained from local commercial abattoirs. They were placed into pre-warmed Thermos-type flasks (~35°C) immediately following recovery and transferred to the laboratory for oocyte retrieval. Ovaries were washed with a commercial bovine oocyte aspiration medium (IVF Bioscience®, Falmouth UK) prior to aspiration. They were then allocated randomly to each of the two treatment groups. A ruler was used to measure follicle diameter, and follicles aspirated or flushed according to the following size categories: Small, 7-10 mm; Medium, 11-14 mm; and Large >15 mm. Both needle types were attached to a 14 mL collection tube and the aforementioned aspiration and peristaltic flushing pump, which was set at a flow rate of 15 mL/min for both aspiration and flushing (Ward et al., 2000).

Each follicle was aspirated to the point of collapse before aspiration ceased and a commercial bovine aspiration medium (IVF Bioscience, Falmouth, Cornwall) flushed into the follicle to around 80% capacity. Follicle contents were then aspirated for a second and final occasion. The needle was maintained in position within the follicle throughout. The next follicle of appropriate size was then selected, and the process repeated. For each needle, aspirants from small (7-10 mm) follicles were collected initially followed by medium and then large follicles. Following oocyte retrieval from each follicle size class, the aspiration tubing was rinsed to ensure no recovered oocytes remained within the needles or tubing. Recovered aspirants were filtered using a 230 mL embryo filter (Em conTM, Wisconsin, US). Collection tubes were rinsed with aspiration medium to recover any oocytes that may have adhered to the wall of the tube. The filter was then rinsed three to four times and aspirants dispensed into 30-mm petri dishes for grading. Fresh needles were used for each session.

Cumulus-oocyte complexes (COCs) were graded on a four-point scale and described previously (Goodhand et al., 1999; Sinclair et al., 2008). Briefly, Grade 1 COCs had >5 layers of compact cumulus cells with a clear, even ooplasm; Grade 2 COCs had <5 layers of compact cumulus cells with a clear, even ooplasm; Grade 3 COCs had <5 layers of cumulus cells which were slightly expanded, and the ooplasm was slightly uneven; Grade 4 COCs had <5 layers of expanding cumulus and uneven ooplasm or were denuded or fully expanded.

Experiment 2. Transvaginal 'follicular flushing' (OxIVF vs standard double lumen needle)

Twelve 13-14-month-old sexually mature Holstein-Friesian heifers underwent two stimulated cycles of transvaginal ovarian-follicular flushing/aspiration (Ovum Pick-Up; OPU) which, with respect to estrous synchrony and ovarian stimulation, followed previously published protocols (Tutt et al., 2021; Simmons et al., 2023; Tutt et al., 2023). However, at OPU, oocyte collection was limited to follicles ≥7 mm in diameter. Oocyte retrieval was achieved using a combination of follicle aspiration and flushing (i.e., aspirate-flush-aspirate) with one of two needles: (i) a conventional double lumen needle (Vitrolife; Control) or (ii) the OxIVF double lumen needle. Retrievals took place from comparatively large (i.e., ≥7 mm) follicles in this experiment for the following reasons: (i) Expt. 1 established that it wasn't feasible to flush follicles < 7 mm in diameter due to the radial perforations being approximately 7 mm from the needle point; (ii) larger antral follicles also more closely resemble those flushed in human assisted reproduction (an important translational consideration); and (iii) oocytes from larger antral follicles are intrinsically more developmentally competent (Sirard, 2019; Aguila et al., 2020). Six donors were allocated to each of these two needle-treatment groups during Cycle 1 and then swapped over, using the other needle type, for Cycle 2. All donor animals underwent these two cycles with intra-vaginal progesterone (P4) releasing implants in place between interventions as described previously (Simmons et al., 2023). The P4 devices used were Progesterone Releasing Intravaginal Devices (PRID® Delta, CEVA, Santé Animale, Libourne, France; impregnated with 1.55g P4). Each cycle commenced with aspirating all follicl8es ≥ 5 mm in diameter (dominant follicle removal; DFR). A PRID® Delta was inserted at DFR (Day 0) and ovarian stimulation proceeded 48 h later. This consisted of six injections (i.m.) of follicle stimulating hormone (FSH; Folltropin, 70 IU dose per dose, Vetoquinol UK Ltd, Towcester, UK) at 12 h intervals. OPU was undertaken on Day 6, approximately 38-42 h following final FSH injection. A replacement progesterone implant was inserted following OPU, and the process repeated with DFR on Day 14 and OPU on Day 20.

Oocyte collection was undertaken in a dedicated procedures room with ambient temperature maintained at ~33ºC. Briefly, COCs were flushed using one of the two needle types. These were connected to 900 mm flush and aspiration tubing. Flushing media comprised HEPES buffered TCM199 media with added (injectable) heparin (heparin sodium, Wockhardt UK Ltd, Wrexham, UK). Flow rates for both aspiration and flushing were set to 15 mL/min, and aspiration pressure at a constant -70 mm Hg. The procedure entailed aspirating each follicle until it visibly collapsed (viewed

by ultrasound), followed by flushing (until the follicle inflated to its original size) and finally aspiration for the second time. Visualisation was undertaken using a 7.5 MHz ultrasound scanner (Expad, IMV Imaging, Glasgow, UK). Aspirants were collected into 50 mL culture flasks, hand-held to maintain temperature. The time taken to undertake these procedures was recorded as the interval between initial restraint of each donor once they had entered theatre.

Collections were then passed through a heated (~37°C) filter and rinsed repeatedly with pre-warmed media (~50 mL) to remove excess cell debris and blood. The filtrate was then rinsed into a 100 mm petri dish on a warm stage (~38°C) and COCs retrieved. These were graded according to appearance and density of attached cumulus cells, and homogeneity of cytoplasm, as described for Experiment 1. Oocytes with sparse, expanded or absent cumulus or with fragmented, pale or irregular cytoplasm (i.e., Grade 4 COCs), were rejected. Oocyte maturation was as previously described (Tutt et al., 2021; Simmons et al., 2023), with oocytes cultured in 1.8 mL of HEPES buffered TCM 199 based maturation media (Sigma Aldrich, Poole, Dorset, UK), in a screw top cryovial (Nunc, ThermoFisher Scientific, Loughborough, UK) at atmospheric CO_2 and 38.5°C, for 23-24 h. Whilst it was not possible to blind OPU operators in this study, outcome assessors in the culture lab were blinded to type of needle used.

Frozen/thawed semen from a single bull was used for IVF. Sperm preparation was by centrifugation through a 45%/90% BoviPure (Nidacon International AB, Mölndal, Sweden) gradient. Fertilization occurred in 50 µL drops of modified TL fertilisation media as previously described (Tutt et al., 2021; Simmons et al., 2023) under oil. Oocytes were washed in fertilization media then placed in drops at a maximum of 5 per drop. 2 µL of sperm preparation media was added to each drop to give a final concentration of 70,000 sperm per drop. Oocytes and sperm were co-incubated for 18-21 h in a humidified environment of 5% CO_2 in air at 38.5°C.

Embryos were cultured in SOF based sequential culture media as described previously (Tutt et al., 2021; Simmons et al., 2023), in a humidified environment under oil at 6.9% CO_2, 5% O_2 and 38.5°C. Briefly, 21 h post fertilisation, (a.m. of Day 1), presumptive zygotes were denuded by repeated pipetting, and transferred at no more than 11 per drop to 10 µL drops of the first culture media. Cleavage was assessed 30 h later (p.m. of Day 2) and oocytes classified according to cell number (i.e., 1, 2-3, 4-5 and >6 cells). Zygotes were transferred approximately 42 h later (Day 4) to 10 µL drops of the second culture media. Progression to morula was assessed 48 h later (Day 6), and embryos transferred to 20 µL drops of the third culture media. Embryos were assessed again 48 h later (Day 8), for stage and quality in accordance to the International Embryo Technologies Society (IETS) guidelines for bovine embryo assessment (Stringfellow and Givens, 2010).

Experiment 3. Hybrid approach: 'follicular flushing' and aspiration (OxIVF needle only)

Eleven Holstein heifers underwent two stimulated cycles of OPU-IVP in a similar cross-over design to that in Expt. 2, however both treatments used the OxIVF needle. One treatment flushed ≥7mm follicles only (as in Expt. 2), the other flushed follicles ≥7 mm followed by aspiration of 5-6 mm follicles using the same needles. Retrieved oocytes from these cycles underwent standard IVM-IVF-IVC (blinded to treatment) as described for Expt. 2. There then followed two cycles of conventional (18G single-lumen needle) follicle aspiration as undertaken in previous studies at this centre (Tutt et al., 2021; Simmons et al., 2023; Tutt et al., 2023). This was for the sole purpose of directly comparing oocyte recovery between treatments (i.e., proportion of punctured follicles that led to oocyte recovery). Oocytes from these two standard OPU cycles did not undergo conventional IVP but were used for other purposes unrelated to this project. The time taken to undertake procedures was recorded as the interval between initial restraint of each donor once they had entered theatre.

Statistical analyses

Analyses were performed using the GenStat statistical package (21st Edition, VSN International, 2022; https://www.vsni.co.uk/). For Experiment 1, which concerned COC recovery from abattoir derived ovaries, proportion data were analysed using REML generalized linear models that assumed binomial errors and used logit-link functions. Terms fitted to these models were 'Replicate', 'Needle type', and 'Follicle size category' or 'COC grade category', plus interactions between 'Needle type' and these latter two twems. For Expeiments 2 and 3, whch concerned OPU from living donors, proportion data were analysed using REML generalized linear mixed models that assumed binomial errors and used logit-link functions. In these models 'Donor' formed the random effect, and 'Cycle' and 'Needle type' were fixed effects. Analyses of the number of follicles aspirated and oocytes retrieved assumed Poison errors and used log-link functions. Data are presented as means ± SEM, and considered signifcant at $P < 0.05$.

Results

Experiment 1. Oocyte collection from abattoir derived ovaries (pilot study)

In all, 463 COCs were retrieved from 655 antral follicles (≥ 7 mm) present on 255 ovaries spanning eight occasions (i.e., representing eight experimental replicates). The average number of follicles flushed for small, medium and large categories was approximately 20, 14 and 6 respectively for each of the two needle types on each occasion. Overall, percentage COC retrieval was greater for the OxIVF than the Control needle (74.1 ± 2.10% vs 67.0 ± 2.23%; $P = 0.034$). Although retrieval from larger antral follicles appeared greater for the OxIVF than the Control needle (**Fig. 2A**), there was no statistically significant interaction ($P = 0.101$) between these terms to confirm that this was indeed the case. However, there was a significant ($P < 0.001$) interaction between needle type and COC grade which indicated that morphologically better quality COCs were retrieved with the OxIVF than the Control needle (**Fig. 2B**). Specifically, the percentage Grade I COCs was greater ($P < 0.001$) for

the OxIVF than Control needle (20.1 ± 1.97% vs 8.2 ± 1.38%; **Fig. 2C**). These differences in morphological grade were based mostly on the extent of cumulus investment following collection.

Experiment 2. Transvaginal 'follicular flushing' (OxIVF vs standard double lumen needle)

The number and distribution of antral follicles flushed were similar for the 12 cycles of collection undertaken by both needle types (Table 1). Each cycle typically lasted around 20 minutes (from when the donor entered to when they left the procedures room), and this interval did not differ between the two needle types. Recovery of COCs was greater (P = 0.045) for the OxIVF compared to the Control needle (89.1 ± 2.98% vs. 79.6 ± 3.47%; Table 1), however, morphological grade of cumulus-oocyte-complexes at the point of recovery was unaffected by flushing-needle type. The percentage oocytes fertilised following IVF did not differ statistically (P = 0.06) between the OxIVF needle (91.0 ± 4.67%) and the Control needle (77.9 ± 7.00%) (Table 1). By Day 6, the percentage morulae and blastocysts were greater (P = 0.017) for the OxIVF than the Control needle; the latter needle type led to a higher (P = 0.017) percentage of developmentally retarded (>12 cells) embryos. The percentage Day 8 blastocysts of oocytes inseminated for the OxIVF needle (64.7 ± 5.59%) did not differ statistically (P = 0.075) from the Control needle (52.4 ± 5.99%).

Experiment 3. Hybrid approach: 'follicular flushing' and aspiration (OxIVF needle only)

The time required to undertake each cycle (determined from when the donor entered to when they left the procedures room) was less (P < 0.001) for conventional aspiration (~18 min) compared to either follicular flushing (~20 min) or the hybrid approach (~22 min) (Table 2). The number of follicles punctured was less (P < 0.001) for the 'Flush' treatment group compared to the other two retrieval groups (i.e., 'Hybrid' and 'Aspirate') as only follicles ≥ 7 mm in diameter were considered (Table 2). This led to fewer (P = 0.057) COCs recovered per donor cycle; although it should be noted that numerically more follicles were punctured during standard aspiration. However, the percentage 9-10 mm follicles punctured was greater (P < 0.001) for the 'Fush' than the two other retrieval groups, and the percentage 11-12 mm follicles punctured was greater (P = 0.035) for 'Flush' and 'Hybrid' retrieval treatments compared to the standard 'Aspirate' treatment. This was also the case numerically for the ≥13 mm follicle size category, although it didn't reach statistical significance. The consequence was that the percentage COCs retrieved was greater (P = 0.033) for the 'Flush' than the 'Aspirate' retrieval groups (82.1 ± 5.06% vs 66.2 ± 3.48%), with the 'Hybrid' group being intermediate (74.2 ± 4.91%). In the end, a greater percentage (80.0 ± 4.72% vs 64.1 ± 3.15%; P = 0.036) of COCs were matured. Morphological grade of COCs going into maturation did not differ between retrieval groups.

Only oocytes matured from the 'Flush' and 'Hybrid' retrieval groups were inseminated and resultant zygotes cultured to Day 8 (Table 3). The number of oocytes inseminated did not differ statistically (P

= 0.066) between the 'Hybrid' (16.3 ± 1.60) and the 'Flush' (11.9 ± 1.28) retrieval groups. However, the number of oocytes that subsequently cleaved was greater (P = 0.038) for the 'Hybrid' than the 'Flush' retrieval groups (Table 3). This was due to the number of COCs retrieved being greater for the 'Hybrid' than the 'Flush' group (Table 2). However, all other developmental parameters were similar for these two retrieval groups. The yield of Day 6 and 8 blastocysts were both numerically greater for the 'Hybrid' than 'Flush' retrieval groups (11.3 ± 1.79 vs 8.0 ± 1.37, Day 6; 9.2 ± 1.39 vs 6.5 ± 1.05, Day 8), although these differences did not reach statistical significance.

Discussion

The individual scale of each of the three experiments reported in this article is such that some degree of caution should be exercised when interpreting findings and extrapolating outcomes to alternative systems of OPU in cattle or to human ART. With that cautionary note in mind, however, several important findings emerge from this study which are of great potential value to those undertaking OPU in these species. Firstly, regarding follicular flushing, there is clear evidence from Experiments 1 and 2 that oocyte yield and quality are both greater for the OxIVF compared to the standard double lumen needle under test. This is reflected by improved morphological grades of COCs from non-stimulated abattoir derived ovaries (Expt. 1; Fig. 2) and by enhanced post-fertilisation development following stimulated cycles of OPU (Expt. 2; Table 1). Secondly, in Expt. 3 (Table 2), follicular flushing with the OxIVF needle on its own led to a sixteen-percentage point increase (~82 vs 66%) in the yield of oocytes recovered over conventional follicular aspiration. The recovery rate of 66.2 ± 3.48% reported for follicular aspiration in Table 2 agrees well with a mean ± SE of 66.6 ± 1.03% observed in similar cycles of follicular aspiration reported previously by our group (Tutt et al., 2021; Simmons et al, 2023; Tutt et al., 2023). This agreement enhances our confidence that the sixteen-percentage point increase in oocyte recovery is real. However, the technical limitation of only being able to flush follicles ≥ 7 mm in diameter meant that the total number of oocytes retrieved per donor cycle was numerically (P = 0.057) less for follicular flushing compared to aspiration. This was the motivation behind the 'hybrid' approach which involves flushing follicles ≥ 7 mm in diameter combined with aspirating follicles between 5 and 7 mm in diameter. This 'hybrid' approach negates differences in the number of oocytes retrieved (Table 2). The failure to demonstrate an absolute increase in oocyte yield over standard aspiration could be attributed to two factors: (i) small scale of the study undertaken and, related to this, (ii) the numerically greater number of follicles punctured during aspiration, which we consider to be a chance difference. Consequently, the 'hybrid' approach could represent a way forward for cattle undergoing similar stimulated ('coasting') cycles of OPU, although this awaits confirmation requiring further experimentation to bolster numbers represented in the current study.

Follicular flushing vs standard aspiration

Donor preparation ahead of OPU in the two *in vivo* experiments reported in this article used a protocol developed for taurine cattle which involves a short period of FSH stimulation ahead of gonadotrophin withdrawal (termed 'coasting'). This protocol results in high yields of Day 7 blastocysts (Blondin et al., 2002; Nivet et al., 2012), and was used in a recent series of studies at our centre which investigated the benefits of progesterone support during ovarian stimulation (Simmons et al., 2023), consequences of removing complex proteins and adding melatonin during IVM (Tutt et al., 2023), and establishing the nature and extent of chromosomal abnormalities that arise with such interventions (Tutt et al., 2021). Here we demonstrate a clear benefit, in terms of percentage COC recovery, of follicular flushing using the OxIVF needle over conventional aspiration with this 'coasting' protocol (Table 2). This is even more significant given that the improvement in oocyte yield arose following a single flush. That is, each follicle underwent a cycle of aspiration-flush-aspiration. In contrast, it is typical for equine OPU to flush and aspirate each follicle several times (Hinrichs, 2018; Stout et al., 2020; Heida et al., 2024).

In human OPU, 'closed flushing' (i.e., each follicle is flushed 3 to 4 times before tubes are returned to the laboratory) has been proposed for patients with > 6 follicles, and 'open flushing' (i.e., each follicle is flushed until an oocyte is detected or no cellular material detected) recommended for those with ≤ 6 follicles (D'Angelo et al., 2019). Such practices were not possible in the current study where we typically flushed between 15 and 25 follicles per donor, and up to 12 donors per session. Although the conduct of our experiments differs from that of human and equine OPU, it closely resembles commercial practice in cattle (Ferré et al., 2023). However, future studies could consider working with fewer donors per session in order to flush each follicle more than once. It is uncertain at this juncture if this could increase the yield of COCs further, but it would increase the time required to complete procedures.

In the current study, improvements in percentage oocyte recovery can be attributed to improved efficiency of COC retrieval from antral follicles (particularly from larger follicles as observed in Expt. 1) and/or improved retrieval from the collection flask, as flushing during OPU resulted in clearer collections with fewer blood clots (our observations and those of others (Daya et al., 1990; Rose and Laky, 2013)). The relative contribution of each of these factors awaits further experimentation. However, it is evident that the advantage of the OxIVF needle over the standard double-lumen needle (Fig. 1A, Table 1) extends beyond generating clearer collections (as these were similar for both needle types), indicating improved intrafollicular retrieval of oocytes when flushing/aspirating with this needle.

Regrettably, oocytes retrieved by conventional follicular aspiration in Expt. 3 were required for alternative purposes and so, whilst it was possible to gain an insight into comparative retrieval rates at OPU (Table 2), it was not possible to compare directly post-fertilisation development to the

blastocyst stage. However, we can with caution compare embryo development to that obtained in recent studies employing conventional follicular aspiration at our centre (Tutt et al., 2021; Simmons et al, 2023; Tutt et al., 2023). These studies used donors of a similar age from the same herd, together with similar protocols for ovarian stimulation and *in vitro* production of embryos. Importantly, it was the same team of operators who undertook these procedures, and semen from the same sire was used for IVF in all studies. Percentage Day 6 and Day 8 blastocysts of oocytes inseminated averaged 75% and 61% respectively across those studies, which is comparable to mean values (72% and 57% respectively) obtained for Experiments 2 and 3 in the current study. Consequently, whilst ovarian follicular flushing (particularly using the OxIVF needle) can improve the percentage oocytes retrieved, further direct comparisons concerning embryo development during IVC are required to determine if this parameter can also be enhanced.

OxIVF vs standard double lumen needles

Based on model predictions during needle design (Cimpeanu et al., 2023), and picking up on a point raised in the preceding section, improved yields of COCs in Experiments 1 and 2 using the OxIVF needle likely arose due to the favourable intrafollicular vortex structure generated during flushing which enhanced retrieval during aspiration. Indeed, model predictions indicated that this would negate the positional effect of the COC, relative to proximity of the needle tip, at the time of aspiration. Given the novelty of this needle design there are no other studies that could serve as a direct comparator. The fact that flushing with double-lumen needles in cattle failed to increase oocyte retrieval relative to aspiration with single-lumen needles in the past (Fry et al., 1997; Sasamoto et al., 2003) may simply be due to needle design and frontal directionality of flow during flushing/aspiration.

The percentage Grade 1 COCs retrieved in the current study was greater for the OxIVF compared to the standard double lumen needle for all follicle size categories in non-stimulated abattoir ovaries (Expt. 1; Fig. 2). However, no discernible improvement in COC morphological grade was evident for the OxIVF needle over the standard double lumen needle following OPU in Expt. 2 (Table 1); which was comparable to aspirated COC grades following OPU in Expt. 3 (Table 2). Although follicular-size categories were comparable across all three experiments, the cumulative percentage of Grade 1 and 2 COCs retrieved was considerably greater (~83%) for stimulated ovaries of Experiments 2 and 3 compared to non-stimulated ovaries of Expt. 1 (~52%). The high percentage of morphological good quality COCs, which is a characteristic feature of stimulated compared to non-stimulated cycles (Sarwar et al., 2020; Tutt et al., 2021), probably contributed to the lack of an effect of flushing needle design on this parameter in the two OPU experiments.

The marginal improvement in embryo development for the OxIVF relative to the standard double-lumen needle in Expt. 2 (Table 1) may have arisen in part due to a greater proportion of oocytes

recovered from larger (≥ 10 mm) antral follicles (Sirard, 2019; Aguila et al., 2020, as witnessed in Expt. 1; although recovery by follicle-size category couldn't be discerned in our OPU cycles in Expt. 2. It follows that the inclusion of oocytes aspirated from 5-7 mm follicles in our 'Hybrid' approach may have negated mean differences in embryo development between retrieval strategies (i.e., 'Hybrid' vs Flush) in Expt. 3. Nevertheless, compared to follicular flushing alone, the 'Hybrid' approach led to a mean increase of 5.0 cleaved zygotes and 2.7 Day 8 blastocysts per donor cycle (Table 3).

Translational value of findings for human and equine ART

Follicular flushing is practiced widely in both human (D'Angelo et al., 2019) and equine (Hinrichs, 2018; Stout, 2020) assisted reproduction, although the consensus at present is that it offers little advantage over standard aspiration in humans (Georgiou et al., 2022). The working hypothesis of the current series of experiments, however, was that the intrafollicular vortex generated by the OxIVF needle, resulting from a lateral rather than a frontal flow field (Cimpeanu et al., 2023; also **Fig. 1**), would enhance oocyte retrieval; and the evidence we present generally supports this proposition. Consequently, as a means of oocyte retrieval, this novel needle design may enhance success with follicular flushing in human ART and offer an alternative approach for recoveries in equine ART. However, a translational limitation of the current study relates to the size distribution of bovine antral follicles selected for aspiration/flushing. There were only a small number of large (14 to 18 mm) follicles punctured in the current study. This represents the lower range of follicles commonly aspirated/punctured in stimulated cycles of human OPU (Abbara et al., 2018). Indeed, it's generally recommended that smaller (< 10 mm) follicles, which made up the largest cohort in the current study, are left un-punctured in such cycles to avoid collection of non-responsive GV oocytes (D'Angelo et al., 2019). The situation concerning follicle size in equine practice is more variable, with both smaller (5 to 8 mm; from non-stimulated cycles) and larger (typically 20 to 35 mm) follicles aspirated/flushed (Hinrichs, 2018; Stout, 2020). However, a further translational limitation of the current study relates to needle size. Needles of different sizes are commonly used for human OPU (typically 17-19G single-lumen needles for aspiration and 16-17G double lumen needles for flushing (Lainas et al., 2023b)). In contrast, common practice in equine OPU is to aspirate/flush using 12G in preference to 15-16G double-lumen needles (Velez et al., 2012; Hedia et al., 2024). Nevertheless, the current report provides the first proof of concept that ovarian follicular flushing using the OxIVF needle can improve percentage oocyte recovery and thus serves as an impetus for follow-up studies in human and equine ART.

Final reflections and conclusions

The 'hybrid' approach of flushing follicles ≥ 7 mm in diameter, combined with aspirating follicles between 5 and 7 mm in diameter, leads to high yields of developmentally competent oocytes from stimulated cycles of OPU in cattle using the OxIVF needle, which was found to be superior to the standard double lumen needle assessed. We would advocate the 'hybrid' approach of flushing and

aspirating when using this needle in similar stimulated cycles in cattle. The design of the OxIVF needle (which features 12 radial perforations 7 mm from the needle point), however, prohibits the flushing of follicles <7 mm in diameter. It should be noted that this is not a limitation with standard double lumen needles of similar gauge. The flushing of all antral follicles ≥ 5 mm in diameter using such a needle was not assessed in the current series of experiments but is an alternative approach in cattle worthy of consideration. In contrast, follicle size is not a concern in stimulated cycles of OPU in humans and so wouldn't limit the use of the OxIVF needle for flushing in this species. Finally, for the protocols described in the current series of experiments in cattle, follicular flushing extended OPU by between 3 to 5 min per donor cycle. The procedure itself required little additional effort on the part of the operators and there were no adverse effects for the donors. Whilst we can conclude that ovarian follicular flushing, particularly using the OxIVF needle, is a suitable means of achieving high yields of oocytes during OPU in cattle, larger scale follow-up studies are required to establish the advantage of this approach over standard follicular aspiration.

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Data availability: The data that support this study will be shared upon reasonable request to the corresponding authors.

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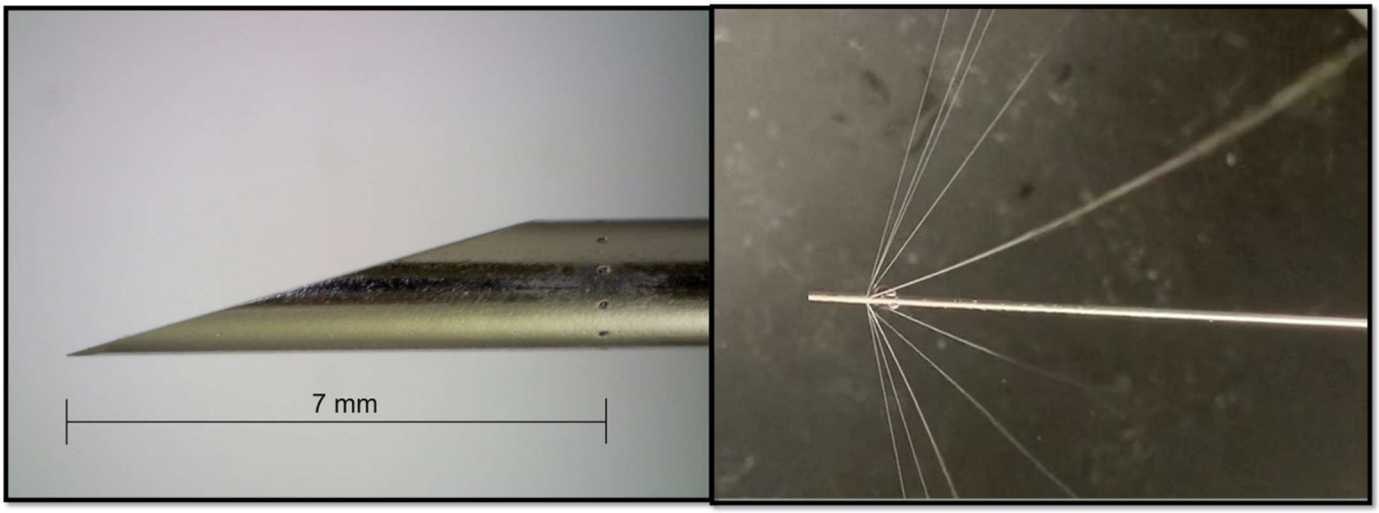
Figure 1. Design features of OxIVF double-lumen needle. It has an outer diameter of 1.65 mm/16G and is 455 mm in length. The needle tip is modified with the outer lumen sealed to the inner lumen distally and flushing perpendicular to the needle shaft through 12 radial perforations (0.1 mm in diameter tilted at 30° to the distal needle end) approximately 7 mm from the needle point. The image on the right depicts the backwards flow of flushing media released from each of the 12 perforations.

Figure 2. Retrieval of cumulus-oocyte complexes (COCs) from abattoir derived ovaries in Experiment 1 (pilot study) using either Control or OxIVF double-lumen needles to aspirate, flush and then aspirate for a second time. **A.** COC retrieval (proportion) per follicle-size category; **B.** COC morphological grade (Goodhand et al., 1999; Sinclair et al., 2008) distribution across all follicles; **C.** Proportion of morphological Grade I COCs by follicle-size category. Analyses based on eight biological replicates (i.e., collections on eight separate occasions). Data presented as means ± SEM based on eight replicated experiments.



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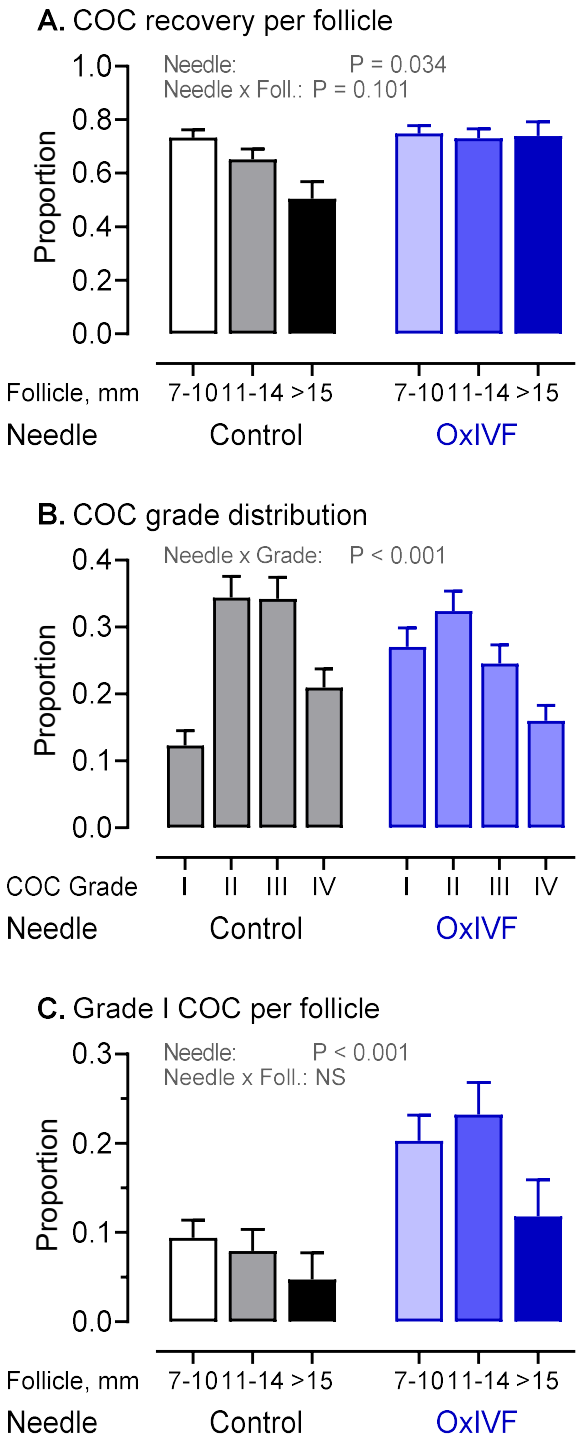

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Table 1. Expt. 2. Ovarian follicle status at the point of OPU, oocytes retrieved and *in vitro* embryo development following IVF for each of the two double-lumen needle types. Data presented as means ± SEM.

Needle	Control	OxIVF	Significance (P)
Donor cycles	12	12	
Donor turnaround†, min	20.3 ± 1.89	19.4 ± 1.90	-
A. Ovarian response to stimulation			
Follicles flushed, n	18.8 ± 2.19	15.3 ± 2.00	-
7-8 mm of flushed, %	51.8 ± 6.48	54.1 ± 7.18	-
9-10 mm of flushed, %	32.3 ± 3.69	29.5 ± 4.00	-
>10 mm of flushed, %	15.9 ± 4.44	14.2 ± 4.71	-
B. Oocyte recovery			
Oocytes recovered, n	15.0 ± 1.99	13.6 ± 1.90	-
Recovered of flushed, %	79.6 ± 3.47	89.1 ± 2.98	0.045
Grade 1, %	63.3 ± 4.39	56.5 ± 4.70	-
Grade 2, %	23.3 ± 3.25	25.2 ± 3.50	-
Grade 3, %	13.3 ± 3.74	12.9 ± 3.87	-
C. Day 2 embryos#			
Oocytes inseminated, n	14.8 ± 1.99	14.2 ± 1.95	-
Cleaved of inseminated, %	77.9 ± 7.00	91.0 ± 4.67	0.06
2 cells of cleaved, %	5.2 ± 2.60	5.6 ± 2.45	-
3-4 cells of cleaved, %	22.4 ± 6.22	21.1 ± 5.51	-
5-6 cells of cleaved, %	25.0 ± 4.95	26.1 ± 4.53	-
>4 cells of cleaved, %	72.4 ± 8.06	73.2 ± 7.22	-
>6 cells of cleaved, %	47.4 ± 8.00	47.2 ± 7.21	-
D. Day 6 embryos			
Day 6 embryos, n	10.2 ± 1.51	11.3 ± 1.46	-
Day 6 of inseminated, %	70.5 ± 7.17	80.1 ± 6.12	-
>12 cells of D6, %	32.4 ± 6.73	12.8 ± 4.40	0.017
Morula of D6, %	60.0 ± 7.17	79.2 ± 5.45	0.031
Morula & Blasts of D6, %	67.6 ± 6.73	87.2 ± 4.38	0.017
Blastocysts of D6, %	7.6 ± 3.66	8.0 ± 3.43	-
E. Day 8 blastocysts			
Day 8 blastocysts, n	7.8 ± 1.14	9.2 ± 1.18	-
D8 of inseminated, %	52.4 ± 5.99	64.7 ± 5.59	0.075
D8 of cleaved, %	67.2 ± 4.21	71.1 ± 3.67	-
D8 of D6, %	74.3 ± 4.28	80.8 ± 3.53	-
D8 (IETS Stage 7 to 9)‡ of D8, %	70.5 ± 8.95	70.3 ± 7.88	-
D8 (IETS Stage 9)‡ of D8, %	23.1 ± 6.97	32.7 ± 6.76	0.127

Donor turnaround† - Interval between successive donors entering the procedures room. ‡ All IETS morphological Grade 1 (Excellent)

Table 2. Expt. 3. Ovarian follicle size distribution and COC retrieval following 'follicle flushing' (all follicles ≥7mm), aspiration (all follicles ≥ 5 mm) or a hybrid approach (i.e., combining 'flushing' of all follicles ≥7mm with aspiration only for 5-6 mm follicles). The 'flushing' and 'hybrid' approaches used the OxIVF needle. Follicle aspiration alone employed standard 18-gauge needles reported previously (Tutt et al., 2021; Simmons et al., 2023). Data presented as means ± SEM.

	Method of oocyte retrieval			Significance (P)
	Flush	**Hybrid**	**Aspirate**	
Donor cycles, n	11	11	22	
Follicles punctured per donor, n	15.3 ± 1.16[a]	21.7 ± 1.39[b]	25.1 ± 1.05[b]	<0.001
Donor turnaround[†], min	20.4 ± 1.19[a]	22.5 ± 1.19[a]	17.6 ± 0.92[b]	<0.001
A. Follicle size category (mm)	**Percentage of total follicles punctured**			
5 - 6	0.0[a]	32.7 ± 3.00[b]	35.6 ± 2.02[b]	<0.001
7 - 8	37.9 ± 3.72	28.0 ± 2.85	32.4 ± 1.96	-
9 - 10	41.0 ± 3.80[a]	20.5 ± 2.63[b]	21.8 ± 1.75[b]	<0.001
11 - 12	16.9 ± 2.80[a]	15.6 ± 2.32[a]	8.6 ± 1.17[b]	0.035
≥ 13	3.7 ± 1.37	3.4 ± 1.24	1.6 ± 0.53	-
B. Oocyte recovery per donor				
Recovered, n	12.6 ± 1.20	16.2 ± 1.36	16.6 ± 0.97	0.057
Recovered of punctured, %	82.1 ± 5.06[a]	74.2 ± 4.91[ab]	66.2 ± 3.48[b]	0.033
Matured, n	12.3 ± 1.17	15.6 ± 1.32	16.1 ± 0.95	0.064
Matured of punctured, %	80.0 ± 4.72[a]	71.3 ± 4.52[ab]	64.1 ± 3.15[b]	0.036
Grade 1 COCs, n	7.6 ± 0.85	9.6 ± 0.95	10.4 ± 0.70	0.075
Grade 2 COCs, n	2.1 ± 0.59	2.4 ± 0.63	2.9 ± 0.49	-
Grade 3 COCs, n	2.5 ±0.62	3.5 ± 0.73	2.7 ± 0.45	-
Grade 1 COC, %	62.7 ± 3.87	59.0 ± 3.50	62.3 ± 2.41	-
Grade 2 COC, %	15.4 ± 3.90	14.3 ± 3.39	18.3 ± 2.67	-
Grade 3 COC, %	19.4 ± 4.34	22.9 ± 4.18	16.3 ± 2.53	-

Donor turnaround[†] - Interval between successive donors entering the procedures room. Within each row, means with a different superscript are different at P<0.05.

Table 3. Expt. 3 *In vitro* embryo production from oocytes retrieved by 'follicle flushing' (all follicles ≥7mm) or using a hybrid approach of flushing all follicles ≥7mm and aspirating 5-6 mm follicles. Both approaches used the OxIVF needle. Data presented as means ± SEM.

Oocyte retrieval	Flush	Hybrid	Significance (P)
Donor cycles, n	10	10	
A. Day 2 embryos			
Oocytes inseminated, n	11.9 ± 1.28	16.3 ± 1.60	0.066
Cleaved, n	9.7 ± 1.20	14.7 ± 1.62	0.038
Cleaved of inseminated, %	80.7 ± 7.26	89.7 ± 4.74	-
2 cells of cleaved, %	5.4 ± 2.67	4.6 ± 1.72	-
3-4 cells of cleaved, %	8.1 ± 3.74	10.0 ± 3.54	-
5-6 cells of cleaved, %	17.5 ± 3.16	21.6 ± 3.07	-
>6 cells of cleaved, %	69.3 ± 4.44	64.0 ± 3.93	-
B. Day 6 embryos			
Day 6 embryos, n	8.0 ± 1.37	11.3 ± 1.79	-
Day 6 of inseminated, %	67.0 ± 8.79	69.3 ± 7.63	-
C. Day 8 blastocysts			
Day 8 blastocysts, n	6.5 ± 1.05	9.2 ± 1.39	-
Day 8 of inseminated, %	54.2 ± 7.49	56.5 ± 6.56	-
Day 8 of cleaved, %	67.9 ± 5.08	61.9 ± 4.48	-
Day 8 of Day 6 embryos, %	82.1 ± 7.70	80.6 ± 6.65	-
Ci. Advanced Day 8 blastocysts			
Stage 7(1‡) to 9†, n	4.3 ± 0.93	6.3 ± 1.26	-
Stage 7(1‡) to 9† of inseminated, %	34.7 ± 6.69	39.7 ± 6.42	-
Stage 7(1‡) to 9† of cleaved, %	43.7 ± 5.63	43.2 ± 4.97	-
Stage 7(1‡) to 9† of Day 6, %	52.8 ± 7.46	56.3 ± 6.73	-
Stage 7(1‡) to 9† of Day 8, %	63.1 ± 6.42	70.9 ± 5.55	-
Stage 8 to 9†, n	3.5 ± 0.79	3.9 ± 0.92	-
Stage 8 to 9† of inseminated, %	28.8 ± 5.39	24.3 ± 4.82	-
Stage 8 to 9† of cleaved, %	34.7 ± 5.30	27.3 ± 4.55	-
Stage 8 to 9† of Day 6, %	41.1 ± 5.82	36.3 ± 5.47	-
Stage 8 to 9† of Day 8, %	49.2 ± 7.56	46.1 ± 7.39	-

†IETS developmental Stage (7, 8 and 9 refers to Expanded, Hatching and Hatched blastocysts respectively)
‡Numbers within parentheses refer to IETS morphological grade (1 = Excellent)