



Original Research Article

Sequential refinement of ovum pick-up technical parameters improves oocyte recovery and developmental competence in Holstein cows

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ABSTRACT

Ovum pick-up (OPU) technical variability limits the efficiency and consistency of bovine in vitro embryo production (IVP). Here, we established a sequential refinement framework for OPU by systematically evaluating key technical parameters across seven cumulative experiments in Holstein cows. An 18 G aspiration needle achieved significantly higher cumulus-oocyte complex (COCs) recovery than 20 G and 22 G needles (53.05% vs. 40.03–45.79%; $p < 0.05$). 90° improved recovery compared with non-vertical insertion (55.64% vs. 48.86%), while insertion to one-third to one-half follicle depth and bevel-down orientation further enhanced recovery efficiency (56.52% and 57.97%, respectively; $p < 0.05$). Polyvinyl chloride (PVC) tubing provided superior COCs recovery (63.29%) and morphological quality, with a tubing length of 1.2–1.5 m. Vacuum pressure of 50 mmHg resulted in higher COCs recovery (70.27%) and blastocyst rate (40.65%), significantly outperforming the previously used 70 mmHg setting. In contrast, excessive aspiration pressure (80 mmHg) was associated with mitochondrial dysfunction, disrupted cortical granule distribution, and increased apoptosis ($p < 0.05$), indicating impaired oocyte nuclear and cytoplasmic maturation under excessive mechanical stress. Notably, recovery efficiency progressively increased from 53.05% to 70.27% throughout the sequential optimization process throughout the sequential refinement process. Collectively, this study provides a field-based refinement approach for OPU procedures and demonstrates the association between aspiration parameters and bovine oocyte developmental competence under the tested conditions.

1. Introduction

Ovum pick-up coupled with in vitro embryo production (OPU-IVP) has become an indispensable reproductive biotechnology for accelerating genetic improvement in dairy and beef cattle [1,2]. By enabling repeated, non-surgical collection of oocytes from genetically superior donors, OPU-IVP substantially shortens generation intervals and accelerates the dissemination of desirable genetic traits [3]. Despite its widespread commercial application, large inter-operator and inter-session variability remains a major limitation affecting the efficiency, reproducibility, and scalability of bovine IVP systems. Although donor-associated factors such as body condition score, age, parity, and

lactation status are known to influence oocyte competence [4,5], inconsistencies in OPU technical procedures represent an additional and often underappreciated source of variability that directly constrains downstream embryo production efficiency [6]. The success of OPU depends on multiple interrelated technical parameters, including aspiration needle gauge, insertion angle and depth, bevel orientation, tubing material and length, and vacuum pressure. These parameters collectively determine aspiration hydrodynamics, mechanical shear stress, and cumulus-oocyte complex (COCs) transit stability during follicular evacuation, thereby influencing not only the number of COCs recovered but also their structural integrity and developmental competence [7,8]. For example, needle diameter influences aspiration flow dynamics, with

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larger-gauge needles potentially improving recovery while reducing shear-induced damage to COCs [9,10]. Vacuum pressure must also be carefully calibrated: insufficient pressure may result in incomplete follicular evacuation, whereas excessive pressure can strip cumulus cells and induce subcellular damage [11]. Similarly, tubing characteristics and needle manipulation technique may substantially affect oocyte quality, however, these factors have rarely been evaluated in a systematic and integrated manner under field conditions [2]. Previous studies have primarily investigated individual OPU parameters in isolation, frequently using abattoir-derived ovaries rather than live donor animals, thereby limiting their translational relevance to commercial OPU-IVP programs [10,12]. Moreover, most studies have focused mainly on oocyte recovery rate and gross morphological quality, with limited attention to the downstream developmental and subcellular consequences of subrefined aspiration conditions. To our knowledge, no previous study has systematically integrated sequential parameter optimization with developmental and ultrastructural evaluation under live commercial OPU conditions. Consequently, there remains a need for a comprehensive, field-based optimization that systematically refines multiple OPU parameters in a cumulative manner while maintaining operational feasibility in commercial settings.

Therefore, the objectives of this study were: (1) to systematically evaluate, through a sequential experimental design, the effects of aspiration needle gauge, insertion angle, insertion depth, bevel orientation, tubing material and length, and vacuum pressure on COCs recovery, morphological quality, and developmental competence in Holstein cows; (2) to characterize the ultrastructural damage induced by excessive aspiration pressure; and (3) to establish a field-validated OPU framework for improving the efficiency and reproducibility of commercial bovine IVP systems.

2. Materials and methods

All experimental procedures were approved by the Animal Care and Use Committee of Northwest A&F University and conducted in accordance with national animal welfare regulations.

2.1. Experimental farms and animals

This study was conducted from March 2022 to December 2024 at three large-scale commercial dairy farms located in Ningxia, Gansu, and Shaanxi provinces, China. Briefly, all farms employed total mixed ration feeding three times daily, milking three times daily at 8-h intervals, and digital herd management using the "Yimuyun" system. Throughout the study period, herd structure, diet formulation, and management routines remained stable, and no outbreaks of infectious or metabolic diseases occurred.

Multiparous Holstein cows in the voluntary waiting period (50–70 days postpartum) were enrolled. Across the seven experiments, a total of 1665 unique multiparous Holstein cows were enrolled. Each experiment used a separate cohort of donor cows, and no cow was used in more than one experiment. Within each experiment, each donor underwent a single OPU session. Furthermore, we clarified that all recovered COCs and embryo developmental outcomes were summarized at the donor level to avoid pseudoreplication caused by multiple oocytes or embryos originating from the same donor. To isolate the effect of each technical parameter, a controlled variable design was employed: when evaluating a given parameter, donor groups were matched for body condition score, age, parity, days in milk, and milk yield to minimize confounding. Sample sizes for each experiment (30–55 donors per group) were determined based on practical constraints of commercial farm operations, the availability of eligible multiparous Holstein cows within the voluntary waiting period, and effect sizes anticipated from previous OPU studies.

2.2. General OPU procedure

Cows were restrained in a chute, and caudal epidural anesthesia was administered using 5 mL of 2% lidocaine hydrochloride. The perineal region was cleaned and disinfected. A portable ultrasound system (BCF Easi-Scan, IMV Technologies, France) equipped with a 7.5 MHz convex transducer was used. Donors received an ovarian superstimulation protocol tailored to the presence or absence of a corpus luteum, involving dominant follicle aspiration, prostaglandin $F_{2\alpha}$ (25 mg, i.m.; Ningbo Sansheng, China), and FSH (100 IU, i.m., every 12 h for five injections; Ningbo Sansheng), as previously described [13]. OPU was conducted 36 h after the final FSH injection. Follicles ≥ 2 mm were aspirated, and follicular aspirates were collected into 50-mL conical tubes containing pre-warmed (37 °C) PBS supplemented with 3% (v/v) fetal bovine serum (FBS; Gibco, USA).

The specific OPU parameters evaluated in each experiment (needle gauge, insertion angle, insertion depth, bevel orientation, tubing material, tubing length, and vacuum pressure) were varied according to the experimental design described in Section 2.3. For each experiment, the specific settings of the parameter under investigation and the fixed settings of all other parameters are detailed in Sections 2.3.1–2.3.7.

2.3. Sequential experimental design for parameter optimization

The optimization of OPU parameters was conducted through seven sequential experiments. In each experiment, a single target parameter was varied while all other parameters were held constant at values derived from previously published OPU protocol, routine clinical practice at the time of the study, or the refined values identified in preceding experiments within this study. The experimental sequence was: (1) needle gauge, (2) needle insertion angle, (3) needle insertion depth, (4) needle bevel orientation, (5) tubing material, (6) tubing length, and (7) vacuum pressure. This sequential approach allowed the cumulative refinement of the OPU protocol, with each successive experiment benefiting from the refined parameter settings established in prior experiments.

2.3.1. Experiment 1: Aspiration needle gauge

Six types of single-lumen aspiration needles were compared: 22 G-S (0.9×70 mm), 22 G-L (0.9×80 mm), 20 G-S (0.9×70 mm), 20 G-L (0.9×80 mm), 18 G-S (1.2×70 mm), and 18 G-L (1.2×80 mm), with 50 donors per group. For this initial experiment, parameters that had been explicitly specified in previously published OPU protocol were set accordingly: needle insertion to approximately one-half of the follicular diameter, silicone capillary tubing of 1.5 m length, and a vacuum pressure of 70 mmHg (flow rate: 20–30 mL/min). For parameters not explicitly specified in that protocol, the routine clinical practice at the time of the study was followed: non-vertical insertion and bevel oriented upward.

2.3.2. Experiment 2: Needle insertion angle

Based on the results of Experiment 1, which identified the 18 G needle as refined, the 18 G-L needle was adopted for all subsequent experiments. Two insertion angles were compared—vertical (90°) and non-vertical—in 30 donors per group. All other parameters were maintained as in Experiment 1 (one-half follicle depth, bevel upward, silicone tubing of 1.5 m, 70 mmHg).

2.3.3. Experiment 3: Needle insertion depth

Four insertion depth ranges, expressed as the fraction of follicular diameter penetrated by the needle tip, were compared: $0 < D < 1/3$, $1/3 \leq D \leq 1/2$, $1/2 < D \leq 2/3$, and $D > 2/3$, with 40 donors per group. Based on the results of Experiments 1 and 2, the 18 G-L needle and 90° were used. All other parameters followed Experiment 1 settings (bevel upward, silicone tubing of 1.5 m, 70 mmHg).

2.3.4. Experiment 4: Needle bevel orientation

Four bevel orientations were compared—upward, downward, leftward, and rightward—in 55 donors per group. Based on cumulative optimizations, the 18 G-L needle, 90°, and one-third to one-half follicle depth were adopted. Remaining parameters followed Experiment 1 settings (silicone tubing of 1.5 m, 70 mmHg).

2.3.5. Experiment 5: Tubing material

Three tubing materials (inner diameter 1.2 mm, outer diameter 2.4 mm) were compared: silicone capillary, PVC capillary, and PTFE (Teflon) tubing, with 40 donors per group. Based on the preceding optimization, the 18 G-L needle, 90°, one-third to one-half follicle depth, and bevel downward were used. Tubing length was maintained at 1.5 m and vacuum pressure at 70 mmHg, consistent with settings from Experiments 1–4.

2.3.6. Experiment 6: Tubing length

Five lengths of PVC capillary tubing were compared: 1.0 m, 1.2 m, 1.5 m, 1.7 m, and 2.0 m, with 30 donors per group. Based on the preceding optimizations, the 18 G-L needle, 90°, one-third to one-half follicle depth, bevel downward, and PVC tubing were used. Vacuum pressure was maintained at 70 mmHg.

2.3.7. Experiment 7: Vacuum pressure

Six vacuum pressure levels were compared: 20, 35, 50, 65, 70, and 80 mmHg, with 32 donors per group. Based on all preceding optimizations, the 18 G-L needle, 90°, one-third to one-half follicle depth, bevel downward, and PVC tubing were used. Tubing length was set at 1.5 m.

2.4. In vitro embryo production

2.4.1. COCs grading and in vitro maturation (IVM)

COCs were isolated from follicular aspirates using a 75- μ m filter and graded morphologically according to the Chinese national standard (GB/T 45202): Grade A, ≥ 5 compact cumulus cell layers and homogeneous cytoplasm; Grade B, 3–5 cumulus layers and homogeneous cytoplasm; Grade C, 1–3 cumulus layers or heterogeneous cytoplasm; Grade D, expanded cumulus or denuded oocytes. Only Grade A and B COCs were selected for IVM.

IVM was performed in 100- μ L microdrops of oocyte maturation medium consisting of TCM-199 (Gibco) supplemented with 10% (v/v) FBS, 1 μ g/mL estradiol, 0.075 IU/mL hMG, 20 ng/mL bFGF, 20 ng/mL EGF, and 1% (v/v) ITS, overlaid with mineral oil, and cultured at 38.5 °C in 5% CO₂ for 22–24 h.

2.4.2. In vitro fertilization (IVF)

Frozen-thawed semen from a single proven Holstein sire was used throughout the study. Motile spermatozoa were isolated by Percoll density gradient centrifugation (45%/90%). Briefly, 2 mL of 90% Percoll solution was placed at the bottom of a 15-mL centrifuge tube, and 2 mL of 45% Percoll solution was carefully layered on top to form a discontinuous gradient. The gradient was equilibrated at 38.5 °C in 5% CO₂ for at least 2 h. Frozen-thawed semen was layered onto the gradient and centrifuged at 1470 rpm (approximately 350 $\times$ g) for 10 min at room temperature. The supernatant was discarded, and the sperm pellet was resuspended in 1 mL of TL-Hepes medium and centrifuged at 1380 rpm (approximately 300 $\times$ g) for 5 min. The final sperm pellet was resuspended in approximately 100 μ L of TL-Hepes medium for subsequent use. Matured COCs were partially denuded by gentle pipetting and washed in TL-Hepes medium. Groups of 20–25 COCs were transferred to 100- μ L microdrops of IVF medium (BO medium supplemented with 6 mg/mL BSA and 10 μ g/mL heparin). Spermatozoa were added at a final concentration of 1.5–2.0 $\times 10^6$ /mL, and gametes were co-incubated for 10–12 h at 38.5 °C in 5% CO₂.

2.4.3. In vitro culture

Following IVF, presumptive zygotes were denuded of remaining cumulus cells and spermatozoa by vortexing in hyaluronidase solution, washed in synthetic oviductal fluid (SOF) medium, and cultured in 100- μ L microdrops of SOF supplemented with 8 mg/mL BSA, 2% (v/v) essential amino acids, and 1% (v/v) non-essential amino acids. Culture was performed at 38.5 °C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂. Cleavage rate was assessed at 48 h post-insemination, and blastocyst rate was evaluated on Day 7.

2.5. Immunofluorescence staining

To assess the effects of vacuum pressure on oocyte nuclear and cytoplasmic maturation, oocytes collected under 50 mmHg and 80 mmHg in Experiment 7 were subjected to IVM for 22 h. Only oocytes that had extruded the first polar body were selected for subsequent analyses.

2.5.1. Mitochondrial membrane potential assessment

Mitochondrial membrane potential was assessed using a JC-1 kit (C2006; Beyotime, China). According to the manufacturer's instructions, a JC-1 staining working solution was prepared by diluting the JC-1 stock solution (200 $\times$) with ultrapure water and JC-1 staining buffer. Matured oocytes were incubated in the JC-1 working solution at 37 °C in a humidified atmosphere of 5% CO₂ for 20 min in the dark. After staining, oocytes were washed three times with ice-cold JC-1 staining buffer, mounted on glass slides with an antifade mounting medium, and immediately imaged using a laser scanning confocal microscope (Nikon A1+/A1R+, Japan). JC-1 monomers (green fluorescence) and J-aggregates (red fluorescence) were detected at excitation/emission wavelengths of 488/530 nm and 543/590 nm, respectively. The ratio of red to green fluorescence intensity was calculated to quantify mitochondrial membrane potential.

2.5.2. Mitochondrial distribution assessment

Mitochondrial distribution was examined using Mito-Tracker Deep Red FM (C1032; Beyotime, China). A Mito-Tracker working solution was prepared by diluting the stock solution to a final concentration of 100 nM in pre-warmed PBS. Matured oocytes were incubated in the working solution at 38.5 °C in 5% CO₂ for 30 min in the dark. After incubation, oocytes were washed three times with PBS, fixed in 4% paraformaldehyde for 30 min at room temperature, washed again, counterstained with DAPI (C1006; Beyotime) for 8–10 min, mounted on glass slides, and imaged using the confocal microscope at an excitation/emission wavelength of 644/665 nm.

2.5.3. Cortical granules (CGs) detection

CGs distribution was evaluated by FITC-conjugated peanut agglutinin (PNA-FITC) staining. Matured oocytes were fixed in 4% paraformaldehyde at 4 °C overnight or at room temperature for 30 min. After fixation, oocytes were washed three times in PBS containing 0.1% polyvinyl alcohol (PBS-PVA) on a shaker for 5 min each. Oocytes were then permeabilized with 0.1% Triton X-100 (P0097; Beyotime) at 4 °C for 30 min, followed by three washes in PBS-PVA. Permeabilized oocytes were blocked in immunofluorescence blocking solution (P0102; Beyotime) at room temperature for 2 h and washed three times. In the dark, oocytes were incubated in PNA-FITC solution (Y269614; Beyotime) at a dilution of 1:300 at room temperature for 1 h. After three washes in PBS-PVA, oocytes were mounted on glass slides with a small drop of antifade mounting medium (P0126; Beyotime), gently covered with a coverslip sealed with nail polish, and immediately imaged using the confocal microscope at an excitation/emission wavelength of 488/530 nm.

2.5.4. Early apoptosis detection

Early apoptosis was detected by Annexin V staining using an Annexin V-FITC Apoptosis Detection Kit (C1062S; Beyotime, China). Matured

oocytes were fixed in 4% paraformaldehyde at 4 °C overnight. After fixation, oocytes were washed three times in PBS-PVA on a shaker for 5 min each. Oocytes were then permeabilized with 0.1% Triton X-100 at room temperature for 30 min, followed by three washes. A working solution was prepared by diluting 5 µL of Annexin V-FITC in 195 µL of Annexin V binding buffer. Oocytes were incubated in the working solution at room temperature for 15 min in the dark. After staining, oocytes were washed three times in PBS-PVA, counterstained with DAPI for 8–10 min, washed again, mounted on glass slides with antifade mounting medium, and imaged using the confocal microscope at an excitation/emission wavelength of 488/530 nm.

2.6. Embryo transfer and pregnancy diagnosis

To evaluate the *in vivo* developmental competence of embryos produced under different OPU conditions, Grade A blastocysts derived from the pre-optimization baseline protocol and the post-optimization protocol were transferred into synchronized Holstein heifers. Recipients were synchronized using a standard protocol (Day 0: CIDR insertion + GnRH; Day 7: CIDR removal + PGF2α; Day 9: GnRH; Day 10: estrus detection). Fresh blastocysts were non-surgically transferred into the uterine horn ipsilateral to the corpus luteum on Day 7 post-estrus (one blastocyst per recipient). Pregnancy was diagnosed by transrectal ultrasonography on Day 35 post-transfer.

2.7. Outcome measures

The primary outcome measures were: (1) COCs recovery rate = (number of COCs recovered/number of follicles aspirated) × 100%; (2) proportion of grade A/B COCs = (number of grade A and grade B COCs/total number of COCs recovered) × 100%; (3) maturation rate = (number of oocytes extruding the first polar body/number of COCs cultured) × 100%; (4) fertilization rate = (number of zygotes with two polar body/number of matured oocytes) × 100%; (5) cleavage rate = (number of cleaved embryos/number of fertilized oocytes) × 100%; and (6) blastocyst rate (number of blastocysts/number of cleaved embryos) × 100%.

2.8. Statistical analysis

Statistical analyses were performed using SPSS Statistics 25 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 10.1.3 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard error of the mean (SEM). Prior to analysis, data distribution and homogeneity of variance were evaluated to confirm suitability for parametric testing. For percentage outcomes (COCs recovery, grade A/B COCs, maturation, fertilization, cleavage, and blastocyst rates), linear regression models were fitted. Estimated marginal means (LS-means ± SEM) were derived from the models, and pairwise comparisons were performed using Tukey's HSD test. For non-percentage continuous outcomes, comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Comparisons between two groups were conducted using Student's *t*-test. For comparisons of pregnancy rates, the Chi-square test was used. Donor allocation was balanced among treatment groups to minimize donor-associated variation during repeated OPU procedures. Therefore, donor identity was maintained as the unit of analysis to avoid pseudoreplication. Complementary generalized linear mixed model (GLMM) analyses were additionally performed for the major proportional outcomes using a binomial error distribution and logit link function. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Aspiration needle gauge and COCs recovery (Experiment 1)

To optimize aspiration needle configuration during OPU, six types of single-lumen needles (22 G, 20 G, and 18 G in short and long variants) were compared using 50 donors per group. The results showed that the COCs recovery rate increased progressively with increasing needle diameter. The 18 G-S and 18 G-L needles achieved the highest recovery rates (53.05% and 52.05%, respectively), significantly exceeding those of the 20 G groups (45.59–45.79%) and the 22 G groups (40.03–40.16%) ($p < 0.05$; Table 1). The 18 G needles achieved a relative improvement of approximately 30% over the poorest-performing 22 G needles. The proportion of grade A/B COCs was comparable between the 18 G (54.92–55.27%) and 20 G (51.76–53.17%), both of which were significantly higher than those observed in the 22 G groups (33.81–34.99%) ($p < 0.05$). Maturation rates followed a similar pattern, with 18 G and 20 G groups (76.69–79.69%) significantly outperforming the 22 G group (64.90–65.55%) ($p < 0.05$). No significant differences were observed between short and long variants within the same gauge for any evaluated parameter ($p > 0.05$). The 18 G-L needle was selected based on superior performance under the tested condition.

3.2. Needle insertion angle (Experiment 2)

Using the 18 G-L needle identified as refined in Experiment 1, 90° was compared with non-vertical insertion using 30 donors per group. All remaining parameters were maintained as in Experiment 1. The results showed that 90° significantly improved COCs recovery rate compared with non-vertical insertion (55.64% vs. 48.86%; $p < 0.05$; Table 2). In contrast, no significant differences were observed in the proportion of grade A/B COCs (50.92% vs. 47.58%; $p > 0.05$) or maturation rate (79.70% vs. 76.74%; $p > 0.05$) between groups. These findings suggest that insertion angle primarily influences aspiration efficiency rather than subsequent oocyte quality or maturation competence. Therefore, 90° was selected based on superior performance under the tested condition.

3.3. Needle insertion depth (Experiment 3)

Following identification of the 18 G-L needle and 90° as refined parameters in Experiments 1 and 2, four needle insertion depth ranges were evaluated using 40 donors per group. All remaining parameters were maintained as in previous experiments. The results showed that the highest COCs recovery rate (56.52%) was achieved when the needle tip was positioned at one-third to one-half of the follicular diameter, significantly exceeding those obtained at shallower insertion depths (50.44%; $0 < D < 1/3$), moderately deep insertions (45.66%; $1/2 < D \leq 2/3$), and deep insertions (34.67%; $D > 2/3$) ($p < 0.05$; Table 3). In contrast, no significant differences were observed in the proportion of grade A/B COCs or maturation rate among groups ($p > 0.05$). Collectively, these findings indicate that refined intrafollicular needle positioning is critical for maximizing COCs recovery efficiency. Therefore, insertion to one-third to one-half of the follicular diameter was selected based on superior performance under the tested condition.

3.4. Needle bevel orientation (Experiment 4)

Building on the cumulative optimizations results from Experiments 1–3, four bevel orientations were evaluated using 55 donors per group. All remaining parameters were maintained unchanged. The results showed that bevel orientation significantly influenced COCs recovery, with the downward orientation achieving the highest recovery rate (57.97%), significantly exceeding the upward (52.28%), leftward (51.38%), and rightward (53.00%) orientations ($p < 0.05$; Table 4). In contrast, no significant differences were observed among groups in the

Table 1

Effect of aspiration needle gauge on COCs recovery and maturation in Holstein cows.

Needle(G)	Donors (n)	Follicles (n)	COCs (n)	COCs recovery rate (%)	Grade A/B COCs (%)	Maturation rate (%)
22 G-S	50	1065	426	40.03 ± 1.11 ^a	34.99 ± 0.99 ^a	65.55 ± 1.90 ^a
22 G-L	50	1516	608	40.16 ± 1.06 ^a	33.81 ± 1.20 ^a	64.90 ± 2.08 ^a
20 G-S	50	1567	714	45.59 ± 0.69 ^b	51.76 ± 1.38 ^b	76.69 ± 2.36 ^b
20 G-L	50	1189	544	45.79 ± 0.81 ^b	53.17 ± 0.81 ^b	77.27 ± 1.49 ^b
18 G-S	50	1189	631	53.05 ± 1.64 ^c	55.27 ± 1.17 ^b	77.97 ± 1.57 ^b
18 G-L	50	1257	654	52.05 ± 1.90 ^c	54.92 ± 0.64 ^b	79.69 ± 1.68 ^b

Data were presented as LS-means ± SEM. Different letters within the same column indicate significant differences ($p < 0.05$); identical letters indicate no significant difference ($p > 0.05$, linear regression models followed by pairwise comparisons using Tukey's HSD test). In this experiment, the parameters used were: one-half follicle depth, non-vertical insertion, bevel upward, silicone tubing of 1.5 m, 70 mmHg vacuum pressure.

Table 2

Effect of needle insertion angle on COCs recovery and maturation in Holstein cows.

Insertion angle	Donors (n)	Follicles (n)	COCs (n)	COCs recovery rate (%)	Grade A/B COCs (%)	Maturation rate (%)
Non-vertical	30	635	299	48.86 ± 0.65 ^a	47.58 ± 1.22 ^a	76.74 ± 1.39 ^a
Vertical (90°)	30	754	420	55.64 ± 0.66 ^b	50.92 ± 1.18 ^a	79.70 ± 0.70 ^a

Data were presented as LS-means ± SEM. Different letters within the same column indicate significant differences ($p < 0.05$); identical letters indicate no significant difference ($p > 0.05$, linear regression models). The 18 G-L needle was used; all other parameters were as in Experiment 1.

Table 3

Effect of needle insertion depth on COCs recovery and maturation in Holstein cows.

Insertion depth	Donors (n)	Follicles (n)	COCs (n)	COCs recovery rate (%)	Grade A/B COCs (%)	Maturation rate (%)
0 < D < 1/3	40	1361	686	50.44 ± 1.54 ^a	48.29 ± 1.65 ^a	76.84 ± 1.60 ^a
1/3 ≤ D ≤ 1/2	40	1305	738	56.52 ± 0.96 ^b	52.22 ± 1.56 ^a	80.09 ± 1.49 ^a
1/2 < D ≤ 2/3	40	1164	531	45.66 ± 0.78 ^c	50.78 ± 1.20 ^a	70.45 ± 8.00 ^a
D > 2/3	40	1258	436	34.67 ± 0.98 ^d	51.34 ± 1.33 ^a	76.06 ± 5.59 ^a

Data were presented as LS-means ± SEM. Different letters within the same column indicate significant differences ($p < 0.05$); identical letters indicate no significant difference ($p > 0.05$, linear regression models followed by pairwise comparisons using Tukey's HSD test). D, insertion depth as a fraction of follicular diameter. The 18 G-L needle and 90° were used; all other parameters were as in Experiment 1.

Table 4

Effect of the orientation of the oocyte aspiration needle tip on COCs recovery and maturation in Holstein cows.

Bevel orientation	Donors (n)	Follicles (n)	COCs (n)	COCs recovery rate (%)	Grade A/B COCs (%)	Maturation rate (%)
Upward	55	1562	817	52.28 ± 0.79 ^a	48.36 ± 1.31 ^a	76.28 ± 1.67 ^a
Downward	55	1936	1122	57.97 ± 0.95 ^b	49.85 ± 1.10 ^a	75.52 ± 2.93 ^a
Leftward	55	1984	1019	51.38 ± 0.53 ^a	52.11 ± 0.94 ^a	77.39 ± 1.97 ^a
Rightward	55	1595	845	53.00 ± 0.48 ^a	51.07 ± 1.04 ^a	74.63 ± 2.49 ^a

Data were presented as LS-means ± SEM. Different letters within the same column indicate significant differences ($p < 0.05$); identical letters indicate no significant difference ($p > 0.05$, linear regression models followed by pairwise comparisons using Tukey's HSD test). The 18 G-L needle, 90°, and one-third to one-half follicle depth were used; remaining parameters were as in Experiment 1.

proportion of grade A/B COCs (48.36–52.11%) or maturation rate (74.63–77.39%) ($p > 0.05$), suggesting that bevel orientation primarily affects recovery efficiency without substantially altering oocyte quality. Based on these findings, the downward bevel orientation. Therefore, insertion to one-third to one-half of the follicular diameter was selected based on superior performance under the tested condition.

3.5. Tubing material (Experiment 5)

Using the cumulatively refined needle parameters established in Experiments 1–4, three tubing materials—silicone, PVC, and PTFE—were compared using 40 donors per group. The results showed that PVC capillary tubing achieved the highest COCs recovery rate (63.29%), significantly exceeding those obtained with silicone (58.53%) and PTFE tubing (58.61%) ($p < 0.05$; Table 5). The proportion of grade A/B COCs was significantly higher in the PVC (52.18%) and silicone

Table 5

Effect of tubing material on COCs recovery and maturation in Holstein cows.

Tubing material	Donors (n)	Follicles (n)	COCs (n)	COCs recovery rate (%)	Grade A/B COCs (%)	Maturation rate (%)
Silicone	40	1402	819	58.53 ± 1.33 ^a	49.61 ± 1.03 ^a	73.39 ± 1.89 ^a
PVC	40	1150	728	63.29 ± 0.67 ^b	52.18 ± 1.35 ^a	75.72 ± 2.66 ^a
PTFE	40	1350	791	58.61 ± 1.23 ^a	36.06 ± 1.23 ^b	51.31 ± 1.91 ^b

Data were presented as LS-means ± SEM. Different letters within the same column indicate significant differences ($p < 0.05$); identical letters indicate no significant difference ($p > 0.05$, linear regression models followed by pairwise comparisons using Tukey's HSD test). Cumulative refined needle parameters from Experiments 1–4 were used; tubing length was 1.5 m, vacuum pressure 70 mmHg.

groups (49.61%) than in the PTFE group (36.06%; $p < 0.05$). Similarly, maturation rate was lowest in the PTFE group (51.31%; $p < 0.05$), whereas PVC and silicone tubing maintained comparatively higher maturation outcomes. Collectively, these findings suggest that tubing material influences aspiration stability and oocyte preservation during OPU. Therefore, PVC tubing insertion to one-third to one-half of the follicular diameter was selected based on superior performance under the tested condition.

3.6. Tubing length (Experiment 6)

Following identification of PVC as the refined tubing material in Experiment 5, five tubing lengths (1.0, 1.2, 1.5, 1.7, and 2.0 m) were evaluated using 30 donors per group. The results showed, tubing lengths of 1.0, 1.2, and 1.5 m yielded comparably high COCs recovery rates (64.71–64.96%), proportions of grade A/B COCs (51.67–54.53%), and maturation rates (78.76–81.61%), with no significant differences observed among these groups ($p > 0.05$; Table 6). In contrast, extending the tubing length to 1.7 m significantly reduced all evaluated parameters, and further deterioration was observed at 2.0 m, where recovery rate, grade A/B proportion, and maturation rate declined to 49.57%, 33.89%, and 53.45%, respectively ($p < 0.05$). Collectively, these findings indicate that tubing length influences both aspiration efficiency and operational stability during OPU. Therefore, a tubing length range of 1.2–1.5 m was selected based on superior performance under the tested condition.

3.7. Vacuum pressure and oocyte developmental competence (Experiment 7)

Following cumulative optimization of all preceding OPU parameters, six vacuum pressure levels (20, 35, 50, 65, 70, and 80 mmHg) were evaluated using 32 donors per group. The COCs recovery rate exhibited a clear pressure-dependent response, increasing progressively from 37.28% at 20 mmHg to a maximum of 70.27% at 50 mmHg, before declining to 63.15% at 70 mmHg and 57.99% at 80 mmHg ($p < 0.05$; Table 7). Recovery efficiency at 50 mmHg was significantly higher than that observed at 70 mmHg ($p < 0.05$), demonstrating superior performance of the refined pressure setting. Similarly, the proportion of grade A/B COCs was highest at 50 mmHg (62.72%), significantly exceeding all other pressure groups, including 70 mmHg (56.80%) ($p < 0.05$). In contrast, the proportion of high-quality COCs declined sharply to 32.99% at 80 mmHg, suggesting substantial disruption of cumulus cell integrity under excessive aspiration pressure.

To evaluate the downstream developmental consequences of aspiration pressure, COCs recovered under each condition were subjected to IVF and IVC across seven independent replicates. Developmental competence was maximized at 50 mmHg, which yielded the highest maturation rate (82.16%), fertilization rate (84.56%), and blastocyst rate (40.65%) (Table 8). These parameters were significantly higher than those obtained at 65, 70, and 80 mmHg ($p < 0.05$). At 70 mmHg, blastocyst rate declined to 28.14%, whereas further deterioration was observed at 80 mmHg, where blastocyst formation decreased to 23.57%, representing a reduction of more than 40% relative to the 50 mmHg

Table 7

Effect of vacuum pressure on COC recovery and quality in Holstein cows.

Vacuum pressure (mmHg)	Donors (n)	Follicles (n)	COCs (n)	COCs recovery rate (%)	Grade A/B COCs (%)
20	32	1015	378	37.28 ± 1.79 ^a	54.44 ± 1.58 ^a
35	32	1120	602	53.77 ± 0.88 ^b	57.39 ± 1.31 ^a
50	32	1254	881	70.27 ± 1.03 ^c	62.72 ± 1.08 ^b
65	32	1352	869	64.29 ± 0.78 ^d	51.64 ± 0.51 ^{ad}
70	32	1287	864	63.15 ± 1.10 ^d	50.86 ± 0.95 ^{ad}
80	32	1103	640	57.99 ± 2.06 ^{be}	32.99 ± 1.01 ^c

Data were presented as LS-means ± SEM. Different letters within the same column indicate significant differences ($p < 0.05$); identical letters indicate no significant difference ($p > 0.05$, linear regression models followed by pairwise comparisons using Tukey's HSD test). All other parameters were set to their refined values as determined in Experiments 1–6.

group. In contrast, low-pressure conditions (20 and 35 mmHg) primarily reduced the quantity of recovered COCs without significantly impairing subsequent developmental competence. Collectively, these findings demonstrate that aspiration pressure critically influences both COCs recovery efficiency and oocyte developmental competence, with 50 mmHg representing the refined balance between recovery yield and preservation of developmental potential.

3.8. Ultrastructural effects of excessive vacuum pressure

To investigate the potential cellular alterations associated with reduced developmental competence under high aspiration pressure, oocytes collected under the refined pressure condition (50 mmHg) and the highest tested pressure (80 mmHg) in Experiment 7 were subjected to IVF, followed by assessment of nuclear and cytoplasmic maturation-related characteristics. Compared with the 50 mmHg group, oocytes collected at 80 mmHg exhibited significantly reduced cumulus cell expansion scores ($p < 0.05$; Fig. 1A and B). Similarly, the first polar body extrusion rate was significantly decreased in the 80 mmHg group ($p < 0.05$; Fig. 1C and D). Assessment of cytoplasmic maturation-related characteristics showed that mitochondrial membrane potential, measured by the JC-1 red-to-green fluorescence ratio, was significantly lower in oocytes collected at 80 mmHg compared with those collected at 50 mmHg ($p < 0.05$; Fig. 1E and F). Cortical granule distribution evaluated by FITC-PNA staining was also altered, with reduced fluorescence intensity observed in the 80 mmHg group ($p < 0.05$; Fig. 1G and H). Furthermore, Annexin V staining revealed a significantly higher proportion of early apoptotic oocytes in the 80 mmHg group ($p < 0.05$; Fig. 1I and J). Mitochondrial distribution analysis using Mito-Tracker Red staining showed differences in mitochondrial localization patterns between groups, with more diffuse cytoplasmic distribution observed in oocytes collected at 80 mmHg compared with the 50 mmHg group ($p < 0.05$; Fig. 1K and L). Collectively, these findings indicate that excessive aspiration pressure is associated with alterations in multiple nuclear and cytoplasmic maturation-related characteristics, which may contribute to reduced oocyte developmental competence.

Table 6

Effect of PVC tubing length on COCs recovery and maturation in Holstein cows.

Tubing length (m)	Donors (n)	Follicles (n)	COCs (n)	COCs recovery rate (%)	Grade A/B COCs (%)	Maturation rate (%)
1.0	30	780	507	64.96 ± 1.56 ^a	51.67 ± 0.85 ^a	80.78 ± 2.31 ^a
1.2	30	1170	757	64.71 ± 1.54 ^a	54.33 ± 0.61 ^a	81.61 ± 1.86 ^a
1.5	30	936	600	64.96 ± 1.58 ^a	54.53 ± 1.19 ^a	78.76 ± 1.02 ^a
1.7	30	1230	692	56.24 ± 0.94 ^b	45.69 ± 0.49 ^b	64.44 ± 1.26 ^b
2.0	30	1115	550	49.57 ± 1.09 ^c	33.89 ± 0.84 ^c	53.45 ± 1.02 ^c

Data were presented as LS-means ± SEM. Different letters within the same column indicate significant differences ($p < 0.05$); identical letters indicate no significant difference ($p > 0.05$, linear regression models followed by pairwise comparisons using Tukey's HSD test). Cumulative refined parameters from Experiments 1–5 were used; vacuum pressure was 70 mmHg.

Table 8

Effect of vacuum pressure on oocyte developmental competence in Holstein cows.

Vacuum pressure (mmHg)	replicates (n)	COCs (n)	Maturation rate (%)	Fertilization rate (%)	Cleavage rate (%)	Blastocyst rate (%)
20	7	206	76.77 ± 0.66 ^a	80.83 ± 1.42 ^a	82.6 ± 0.57 ^a	38.76 ± 0.91 ^a
35	7	346	76.77 ± 1.91 ^a	80.15 ± 1.11 ^a	82.11 ± 0.95 ^a	43.23 ± 3.98 ^a
50	7	553	82.16 ± 0.94 ^b	84.56 ± 0.63 ^b	82.76 ± 1.57 ^a	40.65 ± 1.26 ^a
65	7	449	64.90 ± 0.91 ^c	64.80 ± 0.84 ^c	63.49 ± 0.89 ^b	29.27 ± 0.76 ^b
70	7	456	63.52 ± 1.12 ^c	63.15 ± 1.05 ^c	61.88 ± 1.20 ^b	28.14 ± 0.95 ^b
80	7	211	54.64 ± 1.44 ^d	55.38 ± 0.72 ^d	54.48 ± 0.86 ^c	23.57 ± 0.90 ^c

Data were presented as LS-means ± SEM. Different letters within the same column indicate significant differences ($p < 0.05$); identical letters indicate no significant difference ($p > 0.05$, linear regression models followed by pairwise comparisons using Tukey's HSD test). All other parameters were set to their refined values as determined in Experiments 1–6.

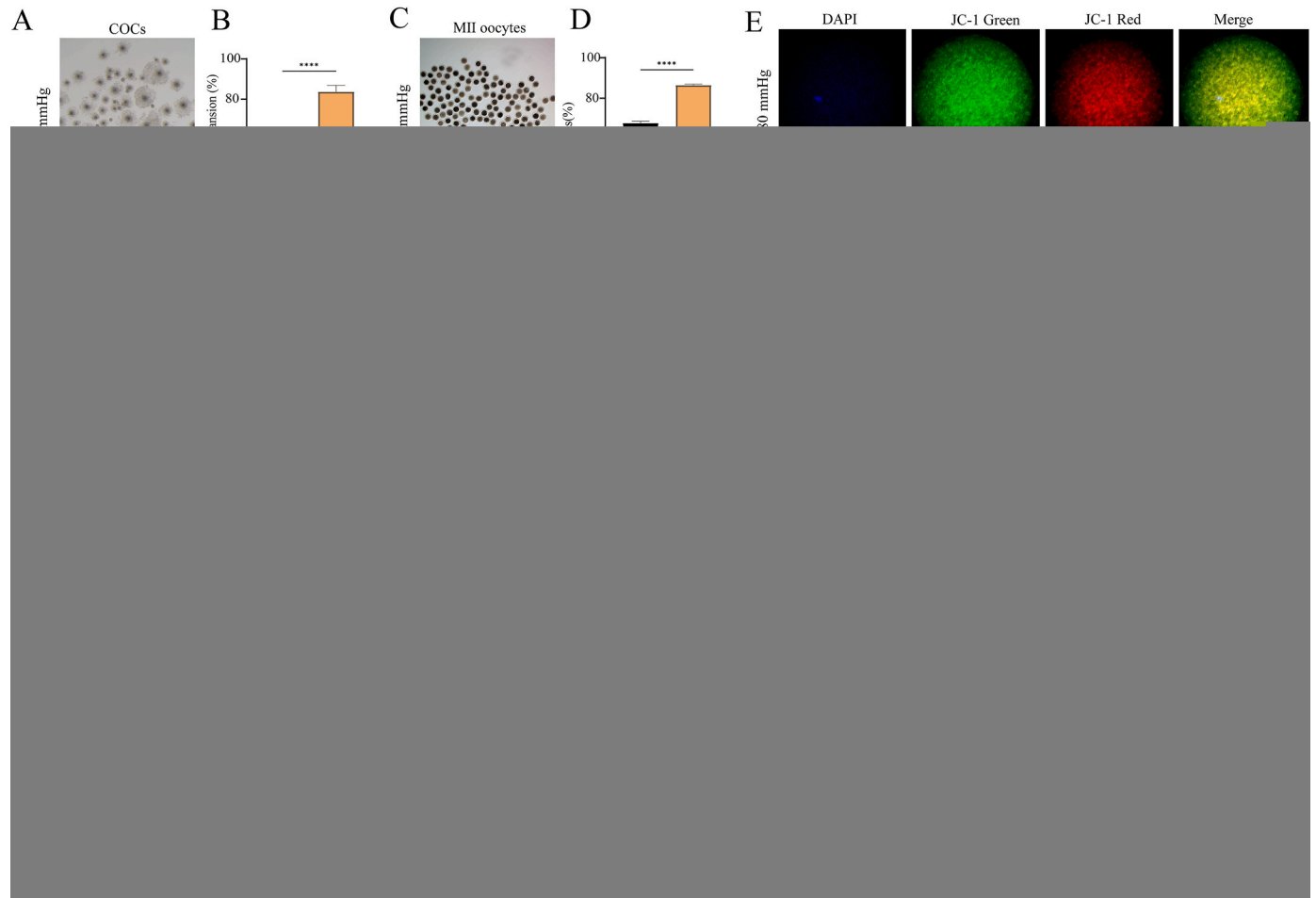


Fig. 1. Effects of vacuum pressure (50 mmHg vs. 80 mmHg) on oocyte nuclear and cytoplasmic maturation. (A) Representative bright-field images of cumulus cell expansion degree, bar = 200 μ m; (B) Statistical analysis of cumulus cell expansion degree; (C) Representative bright-field images of oocyte maturation, bar = 200 μ m; (D) Data analysis of the first polar body extrusion rate in oocytes; (E) Representative fluorescence images of JC-1-stained bovine oocytes, bar = 50 μ m; (F) Fluorescence intensity in oocytes after JC-1 staining; (G) Representative fluorescence images of CGs in bovine oocytes, bar = 50 μ m; (H) Fluorescence intensity in oocytes after CGs staining; (I) Representative fluorescence images of Annexin V-stained bovine oocytes, bar = 50 μ m; (J) Apoptosis-positive fluorescence intensity in oocytes; (K) Representative fluorescence images of Mito-Tracker Red-stained bovine oocytes, bar = 50 μ m; (L) Fluorescence intensity in oocytes after Mito-Tracker Red staining. * indicated a significant difference ($p < 0.05$); ** indicated a highly significant difference ($p < 0.01$). Significance was determined by Student's t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.9. Pregnancy outcome following transfer of embryos produced under refined OPU conditions

To determine whether the sequentially refined OPU protocol translated into improved in vivo developmental outcomes, we compared pregnancy rates following transfer of Grade A blastocysts produced under the pre-optimization protocol ($n = 605$) versus the post-optimization protocol ($n = 819$). Fresh Grade A blastocysts were

transferred into synchronized Holstein heifers, with one blastocyst per recipient. Pregnancy was diagnosed by transrectal ultrasonography at Day 35 post-transfer. The pregnancy rate at Day 35 was numerically higher in the post-optimization group (46.28%, 379/819) compared with the pre-optimization group (41.65%, 252/605), representing an absolute improvement of 4.63 percentage points (Table 9). However, this difference did not reach statistical significance ($\chi^2 = 3.131$, $p = 0.077$). These findings suggest a numerical increase in pregnancy

Table 9

In vivo developmental competence of Grade A blastocysts produced under pre- and post-optimization OPU conditions.

OPU protocol	Transferred blastocysts (n)	Recipients (n)	Pregnancy (n)	Pregnancy rate at D35 (%)
Pre-optimization	605	605	252	41.65 ^a
Post-optimization	819	819	379	46.28 ^a

Identical superscript letters indicate no significant difference ($p > 0.05$, Chi-square test). Pre-optimization protocol: 18 G needle, one-half follicle depth, non-vertical insertion, silicone tubing of 1.5 m, 70 mmHg vacuum pressure. Post-optimization protocol: 18 G needle, 90°, one-third to one-half follicle depth, bevel downward, 1.5 m PVC tubing, 50 mmHg vacuum pressure.

rate following sequential OPU parameter refinement, although the difference did not achieve the conventional threshold for statistical significance.

4. Discussion

The present study establishes a comprehensive and field-validated sequential refinement framework for bovine OPU, systematically integrating aspiration-associated biomechanical parameters that regulate both oocyte recovery efficiency and developmental competence in Holstein cows. By cumulatively refining individual technical variables across sequential experiments, we identified an refined parameter combination consisting of an 18 G aspiration needle, 90° to one-third to one-half follicular depth, downward bevel orientation, 1.2–1.5 m PVC tubing, and a vacuum pressure of 50 mmHg (corresponding to a flow rate of 10–15 mL/min). Importantly, this refined parameter combination not only improved COCs recovery efficiency but also preserved oocyte nuclear and cytoplasmic maturation, thereby enhancing downstream embryonic developmental competence. The recovery and blastocyst outcomes achieved under these refined conditions compare favorably with those reported in previous field-based OPU studies [4,5], highlighting the importance of integrated technical standardization for improving the reproducibility, scalability, and overall efficiency of commercial bovine OPU-IVP systems.

The sequential optimization strategy employed in the present study was designed to reflect the practical workflow of commercial OPU refinement, in which technical parameters are typically adjusted progressively rather than simultaneously. Although a full factorial design could theoretically evaluate all parameter interactions at once, such an approach would require impractically large numbers of donor animals and experimental combinations under commercial field conditions. Therefore, a cumulative sequential design was adopted to maintain both biological relevance and operational feasibility. An important feature of this approach is that parameters refined in later experiments were initially maintained at baseline settings during earlier stages of the study. These baseline conditions for Experiments 1–6 were derived from a previously published OPU protocol [13], which specified the use of an 18 G needle, insertion to approximately one-half follicular depth, silicone tubing, and a vacuum pressure of 70 mmHg. Because insertion angle and bevel orientation were not defined in that protocol, these procedures followed the routine clinical practice at the time, namely non-vertical insertion and bevel-up orientation. Subsequent systematic comparisons demonstrated that 90° (Experiment 2), downward bevel orientation (Experiment 4), PVC tubing (Experiment 5), a tubing length of 1.2–1.5 m (Experiment 6), and reduction of vacuum pressure to 50 mmHg (Experiment 7) each contributed to improved OPU outcomes. These findings demonstrate that even widely adopted OPU procedures can be substantially refined through rigorous parameter-by-parameter optimization. [2,12]. Importantly, because all treatment groups within each individual experiment were evaluated under identical conditions, the relative comparisons and identification of refined parameter settings remain internally valid and biologically robust. The progressive increase in COCs recovery efficiency across sequential experiments, further supports the effectiveness of the cumulative refinement framework and highlights the value of integrated biomechanical refinement for improving commercial bovine OPU-IVP performance.

The superior COCs recovery observed with the 18 G needle is consistent with fundamental principles of aspiration fluid dynamics. According to Poiseuille's law, under laminar flow conditions, flow rate through a cylindrical lumen is proportional to the fourth power of the internal radius [9,14,15]. Accordingly, the larger internal diameter of the 18 G needle (1.07 mm vs. 0.5 mm for the 22 G needle) would be expected to reduce flow resistance during follicular aspiration, thereby facilitating more efficient follicular evacuation. Importantly, the larger needle diameter did not compromise oocyte quality, as evidenced by the comparable proportions of grade A/B COCs between the 18 G and 20 G groups. This observation suggests that the wider lumen may reduce aspiration-associated mechanical stress on COCs despite the larger puncture diameter. Our findings are consistent with those reported by Bols et al. [10], who demonstrated that 18 G needles yielded the highest oocyte recovery efficiency across different vacuum pressure settings, and by Hashimoto et al. [12], who identified needle diameter as a major determinant of bovine COCs recovery. Similar observations were also reported by Sasamoto et al. [16], who found that aspiration needle type significantly affects OPU efficiency. In contrast, no significant differences were observed between short and long needle variants within the same gauge, suggesting that needle length within the tested range has limited influence on OPU performance under standard aspiration conditions. Larger-diameter needles such as 16 G and 17 G were not included in the present study because excessively large puncture diameters may increase tissue trauma and compromise donor recovery following repeated OPU procedures.

The importance of precise needle manipulation was highlighted by the significant effects of insertion angle, insertion depth, and bevel orientation on COCs recovery efficiency. 90° likely improves alignment between the needle bevel and follicular cavity, thereby facilitating more efficient follicular fluid evacuation while reducing the likelihood of bevel occlusion by surrounding follicular tissue. Similarly, insertion to one-third to one-half of the follicular diameter appeared to provide the most favorable intrafollicular positioning for COCs recovery. Shallower insertion depths may result in incomplete follicular aspiration, whereas excessively deep insertion may increase tissue disruption and blood contamination within the follicular environment. In contrast to needle depth and angle, bevel orientation likely influences local aspiration flow dynamics during follicular evacuation. The superior recovery observed with the downward-facing bevel may reflect gravity-associated positioning of COCs within the follicular cavity, allowing more efficient capture during aspiration. The physical basis underlying these effects is consistent with previous analyses of aspiration hydrodynamics during transvaginal follicular aspiration [9]. However, relatively few studies have systematically evaluated the effects of insertion angle, insertion depth, and bevel orientation on bovine OPU outcomes, and published findings remain inconsistent, likely reflecting variability in operator technique and procedural standardization [2]. Therefore, the present study provides one of the first systematic field-based evaluations integrating these handling-associated biomechanical parameters under commercial OPU conditions.

Among the evaluated tubing materials, PVC capillary tubing demonstrated the best overall OPU performance. Its combination of moderate flexibility and a smooth internal surface likely provide an refined balance between buffering aspiration-associated mechanical fluctuations and minimizing COCs adhesion during follicular fluid

transport. In contrast, silicone tubing, despite its greater elasticity, may be more susceptible to COCs adhesion and blood clot accumulation under subrefined temperature conditions. PTFE tubing, although characterized by low surface adhesion, lacks elasticity and may transmit aspiration-associated mechanical fluctuations more directly to COCs, potentially contributing to the significantly reduced proportion of grade A/B COCs observed in this group. The importance of tubing characteristics for preserving oocyte quality during OPU has been recognized in practical recommendations for procedural standardization. Therefore, the finding that PTFE tubing significantly reduced COCs quality may have important practical implications, particularly because PTFE tubing is incorporated into certain commercial OPU systems. Interestingly, a recent successful feline OPU protocol also utilized PVC tubing, further supporting the suitability of this material for oocyte collection across species [17]. The refined tubing length of 1.2–1.5 m appears to reflect a balance between aspiration efficiency and operational stability. Shorter tubing reduces flow resistance and shortens COCs transit time; however, excessively short tubing may prevent stable positioning of the collection tube within the temperature-controlled warming system, thereby increasing the risk of temperature fluctuation during follicular fluid transport. In contrast, tubing lengths exceeding 1.7 m markedly increased flow resistance and prolonged COCs transit, likely increasing cumulative aspiration-associated mechanical stress and contributing to the observed decline in both recovery efficiency and oocyte quality. These findings are consistent with fundamental principles of fluid transport mechanics, whereby increased tubing length proportionally elevates flow resistance during aspiration [9].

Vacuum pressure appeared to be a critical parameter influencing COCs recovery efficiency and subsequent developmental outcomes under the sequential evaluation framework used in this study. The pressure identified in the present study (50 mmHg) appeared to provide a favorable balance between follicular evacuation efficiency and aspiration-associated mechanical stress under the experimental conditions. Compared with the previously established protocol using 70 mmHg vacuum pressure [13], the 50 mmHg condition resulted in higher COCs recovery and blastocyst formation rates under the conditions evaluated in this study. These findings are consistent with previous studies in cattle and swine identifying vacuum pressure as a major determinant of OPU efficiency and oocyte quality [10,11]. The observed alterations in mitochondrial activity, cortical granule organization, mitochondrial localization, and apoptosis-related signals suggest that excessive mechanical stress during aspiration may affect multiple aspects of oocyte cytoplasmic maturation. However, because these analyses were performed after IVM, whether these subcellular alterations represent reversible stress responses or persistent cellular damage remains unclear. High aspiration pressure may increase mechanical stress experienced by cumulus-oocyte complexes, potentially disturbing intracellular organization and reducing the capacity of oocytes to complete normal maturation. These observations extend previous reports by providing direct ultrastructural evidence linking excessive aspiration pressure to impaired oocyte developmental competence [11]. Although the refined absolute pressure setting may vary depending on the vacuum pump system used, calibration based on a follicular fluid flow rate of approximately 10–15 mL/min may provide a practical reference range for further optimization of commercial OPU procedures.

The *in vitro* embryo transfer validation provided additional information regarding the practical applicability of the sequentially refined OPU protocol. The pregnancy rate following transfer of Grade A blastocysts was numerically higher in the post-refinement group compared with the pre-refinement group, although this difference did not reach statistical significance. This numerical increase may be associated with

improved oocyte cellular conditions observed under refined aspiration conditions, including preserved mitochondrial membrane potential, cortical granule organization, and reduced apoptosis; however, the present study does not establish a direct causal relationship between these subcellular characteristics and pregnancy outcome. The numerical difference of 4.63 percentage points, if reproduced in larger studies, may indicate a potential improvement in commercial embryo transfer efficiency. The lack of statistical significance may partly reflect the variability associated with embryo transfer outcomes, including recipient-related factors such as uterine environment, body condition, and nutritional status. Future large-scale embryo transfer studies with improved control of recipient-associated variability will be required to determine whether the observed numerical tendency reflects a biologically meaningful improvement in post-transfer developmental outcomes associated with refined OPU parameters. The field-validated OPU protocol established in the present study offers several important practical advantages for commercial bovine IVP systems. The refined parameters are standardized, quantifiable, and operationally reproducible, thereby reducing operator-dependent variability that commonly affects experience-based OPU procedures [2,6]. Importantly, the protocol was validated under field conditions using live donor animals, enhancing its translational applicability to commercial embryo production programs. Furthermore, the present study highlights the importance of integrated system-level optimization during OPU. Rather than evaluating technical variables in isolation, the cumulative sequential optimization strategy enabled coordinated refinement of multiple aspiration-associated biomechanical parameters, resulting in improved overall system performance and preservation of oocyte developmental competence [12]. When combined with evidence-based donor management strategies—including refined body condition, younger donor age, lower parity, and appropriate postpartum interval [4,5]—the refined protocol established here provides a practical and scalable framework for improving the efficiency, reproducibility, and developmental outcomes of commercial bovine OPU-IVP programs.

This study has several limitations that should be considered when interpreting the findings. First, the sequential optimization design means that parameters refined later in the study were maintained at baseline settings during earlier experiments. Consequently, the absolute recovery efficiencies reported in Experiments 1–6 may underestimate the maximal performance achievable under fully refined conditions. Nevertheless, because all treatment groups within each experiment were evaluated under identical conditions, the relative comparisons and identification of refined parameter settings remain internally comparable and biologically interpretable. Importantly, the progressive improvement in recovery efficiency across sequential experiments, from 53.05% to 70.27%, further supports the effectiveness of the cumulative refinement framework. Second, the baseline conditions used in Experiments 1–6, including 70 mmHg vacuum pressure, non-vertical insertion, and bevel-up orientation, were derived either from a previously published protocol from our laboratory [13] or from the routine clinical practices used at the time. Although subsequent optimization identified superior alternatives, these baseline settings provided a field-validated and reproducible reference framework for systematic parameter evaluation. The ability to significantly improve outcomes through cumulative refinement further emphasizes the importance of critically re-evaluating even widely adopted OPU procedures. Third, optimization was performed using a specific OPU configuration (BCF Easi-Scan ultrasound system combined with a Fujihira vacuum pump). Although the underlying biomechanical and fluid transport principles are expected to be broadly applicable, the refined absolute pressure setting may vary slightly among different vacuum systems. Therefore, calibration based

on follicular fluid flow rate (approximately 10–15 mL/min) may provide a more transferable reference standard across platforms. Fourth, the present study was conducted exclusively in Holstein cows. Although the physical principles governing follicular aspiration are likely conserved across breeds, additional validation in beef cattle and other donor populations would strengthen the broader applicability of the refined protocol. Fifth, the long-term effects of repeated OPU using the refined procedure on donor ovarian function, reproductive longevity, and subsequent fertility were not evaluated. Future studies incorporating longitudinal assessment of donor reproductive health and welfare would further validate the long-term safety and sustainability of the refined procedure. Finally, although the *in vivo* embryo transfer validation demonstrated a numerically higher pregnancy rate in the post-optimization group, this difference did not reach statistical significance. The observed trend toward improved *in vivo* developmental competence is consistent with the enhanced ultrastructural preservation and *in vitro* developmental outcomes achieved under refined aspiration conditions. Larger-scale transfer studies with recipient factors specifically controlled may be required to definitively establish whether the numerical improvement represents a statistically and biologically meaningful enhancement in post-transfer developmental competence resulting from OPU technical parameter optimization.

5. Conclusion

The present study provides a field-based biomechanical refinement framework for bovine OPU through sequential refinement of aspiration-associated technical parameters, including needle configuration, tubing characteristics, and vacuum pressure. The refined parameter combination—18 G aspiration needle, 90°, one-third to one-half follicular depth, downward bevel orientation, 1.2–1.5 m PVC tubing, and a vacuum pressure of 50 mmHg (corresponding to a flow rate of 10–15 mL/min)—resulted in improved COCs recovery while maintaining oocyte nuclear and cytoplasmic maturation under the tested conditions. Excessive aspiration pressure was associated with subcellular alterations, including reduced mitochondrial activity, altered cortical granule organization, and increased apoptotic susceptibility, which may contribute to impaired oocyte developmental competence. Collectively, these findings indicate that aspiration-associated biomechanical stress is an important factor influencing OPU-IVP efficiency and oocyte developmental characteristics under the tested conditions. However, because the parameters were optimized sequentially, the present study cannot determine potential interactions among individual technical factors, and the identified parameter combination should be regarded as a field-validated refinement rather than a universally optimal protocol. Future studies employing factorial designs or response surface approaches are warranted to further define parameter interactions and optimize OPU procedures. Integration of the refined protocol with appropriate donor selection and management strategies may contribute to improving the reproducibility and efficiency of commercial bovine IVP programs.

CRediT authorship contribution statement

Heqiang Li: Writing – original draft, Software, Methodology, Conceptualization. **Baobao Zhao:** Writing – original draft, Software, Methodology, Conceptualization. **Jingya Wang:** Writing – original draft, Software, Methodology, Conceptualization. **Xuexiao Song:** Writing – review & editing, Validation, Data curation. **Dexiang Fan:** Writing – review & editing, Validation, Data curation. **Yong Wang:** Writing – review & editing, Validation, Data curation. **Jieyu Wang:** Writing – review & editing, Validation, Data curation. **Yang Li:** Writing – review & editing, Validation, Data curation. **Zihan Xu:** Writing – review & editing, Validation, Conceptualization. **Zheng Wang:** Writing – review & editing, Validation, Conceptualization. **Zhuangsheng Wang:** Writing – review & editing, Supervision, Resources, Methodology.

Suzhu Qing: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yaping Jin:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yongsheng Wang:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data availability statement

The data involved in this study will be made available upon reasonable request to the corresponding author.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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