



Live motile sperm sorting device for enhanced sperm-fertilization competency: comparative analysis with density-gradient centrifugation and microfluidic sperm sorting

Cheng-Teng Hsu¹ · Chun-I. Lee^{2,3,4,5} · Fong-Sian Lin¹ · Fang-Zong Wang¹ · Hui-Chen Chang¹ · Tse-En Wang¹ · Chun-Chia Huang^{2,5} · Hui-Mei Tsao² · Maw-Sheng Lee^{2,3,4,5} · Ashok Agarwal^{6,7,8}

Received: 23 February 2023 / Accepted: 23 May 2023

© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2023

Abstract

Purpose A live motile sperm sorting device (LensHooke[®] CA0) developed to prevent the deleterious effects of centrifugation was evaluated comparatively with conventional density-gradient centrifugation (DGC) and microfluidic-based device (Zymot) in sperm selection.

Methods Semen samples from 239 men were collected. CA0 under different incubation intervals (5, 10, 30, and 60 min) and temperatures (20, 25, and 37°C) was conducted. The sperm quality in CA0-, DGC-, and Zymot-processed samples was then comparatively evaluated. Semen parameters included concentration, motility, morphology, motion kinematics, DNA fragmentation index (DFI), and the rate of acrosome-reacted sperm (AR).

Results Total motility and motile sperm concentration increased in a time- and temperature-dependent manner and the total motility peaked for 30 min at 37°C. In paired analysis, CA0 showed significantly higher total motility (94.0%), progressive motility (90.8%), rapid progressive motility (83.6%), normal morphology (10.3%), and lower DFI (2.4%) and AR (4.7%) than the other two methods in normozoospermic samples (all $p < 0.05$). For non-normozoospermic samples, CA0 had significantly better results than the other two methods (total motility 89.2%, progressive motility 80.4%, rapid progressive motility 74.2%, normal morphology 8.5%, DFI 4.0%, and AR 4.0%; all $p < 0.05$).

Conclusion CA0 yielded spermatozoa with enhanced sperm fertilization potentials; DFI was minimized in samples processed by CA0. CA0 was effective for both normal and abnormal semen samples due to its consistent selection efficiency.

Keywords Sperm preparation · Sperm motility · Sperm DNA fragmentation · Sperm quality · Sperm retrieval

Introduction

Worldwide, approximately 190 million individuals in the reproductive age group are affected by infertility [1–3]. Up to 30% of infertility cases are attributed solely to male

factor infertility, which contributes to approximately 50% of infertility in combination with female infertility. The most prominent abnormalities found in semen are low sperm concentration, poor sperm motility, and abnormal sperm morphology [4]. To overcome sperm abnormalities in assisted

Cheng-Teng Hsu and Chun-I Lee are joint first authors by virtue of their contributions to this work.

✉ Ashok Agarwal
Agarwaa@GlobalAndrology.org

¹ Center for Research and Development, Bonraybio Co., Ltd, Taichung, Taiwan

² Division of Infertility Clinic, Lee Women's Hospital, Taichung, Taiwan

³ Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan

⁴ Department of Obstetrics and Gynecology, Chung Shan Medical University, Taichung, Taiwan

⁵ Department of Post-Baccalaureate Medicine, National Chung Hsing University, Taichung, Taiwan

⁶ Global Andrology Forum, American Center for Reproductive Medicine, OH 44022 Moreland Hills, USA

⁷ Cleveland Clinic Foundation, Cleveland, OH 44195, USA

⁸ Global Andrology Forum, 130 West Juniper Lane, Moreland Hills, OH 44022, USA

reproductive technology (ART), various in vitro sperm processing techniques have been developed to improve the ART success rate. Swim-up and density-gradient centrifugation (DGC) are conventional sperm-preparation techniques that rely on the principle of sedimentation or migration to enable the separation of spermatozoa. These routine sperm separations appear effective in terms of the recovery rate, and comparably yield sperm with higher motility and better morphology [5]. However, DGC-associated centrifugation induces oxidative stress and the subsequent increase in sperm DNA fragmentation negatively affect ART outcomes and decrease the probability of pregnancy [6–8]. On the other hand, while conventional swim-up relies on sperm motility, the procedure is time consuming and eventually prolongs the exposure of motile sperm to seminal plasma. Thus, the swim-up method is inevitably accompanied by centrifugation and is commonly used in clinical practice [30, 31]. To overcome the limitations of centrifugation-based methods, several advanced sperm-selection methods have been developed to provide a broad range of possibilities for improving the sperm-fertilizing potential. Among advanced sperm-selection techniques, the magnetic-activated cell sorting (MACS) system enables the selection of non-apoptotic motile spermatozoa based on the affinity between Annexin-V-conjugated microbeads and the apoptotic phenotype (externalized phosphatidylserine). However, MACS users tend to combine this procedure with DGC to eliminate contamination by leukocytes and immature germ cells, and this makes the process laborious and time consuming [9]. Sperm selected via a hyaluronan-binding assay have shown improved motility, apoptosis, acrosome integrity, sperm DNA fragmentation, and normal morphology; however, the procedure is time consuming and affects ART efficiency [10, 11]. Motile sperm organelle morphology examination (MSOME) selects sperm with normal morphology by using high-power differential interference contrast optics; however, the limited correlations of sperm ultrastructure with sperm physiological functions and fertilization potential are indicated [12–14].

In the last decade, several medical devices for sperm separation have been introduced. For instance, the sperm sorter Qualis® (Menicon, Kasugai, Japan) based on microfluidic flow theory enables the selection of spermatozoa with low DNA fragmentation index (DFI) and higher motility; however, the device only accommodates 65 µL of a semen sample, which limits its implementation in ART [15]. The Zech-selector (AssTIC Medizintechnik GmbH, Leutsch, Austria) sorts motile spermatozoa via a capillary bridge connected by two concentric wells and improves sperm motility, DFI, and normal morphology; however, the device is impaired due to the improper filling of a U-ring in the center chamber, which leads to inconsistent selection performance [16, 17]. Miglis® (Menicon Life Science, Japan) comprises four parts (outer lid, inner lid, spacer, and base) and sorts sperm using

the migration-sedimentation technology. Miglis® sperm separation for 1 h improved parameters such as sperm motility, DNA integrity, and mitochondrial functionality [18]. However, Miglis® is not routinely used in clinics due to the prolonged processing time. ZyMöT® (DxNow, Gaithersburg, MD) is a sperm separation device which sorts sperm within a space-constrained microfluidic sorting chip and involves two manipulation steps: loading semen samples into the inlet sample chamber, and retrieving processed samples from the outlet port by using a syringe. While the single-use chip makes sperm separation more user friendly and potentially improves sperm characteristics including sperm motility and DNA integrity [19–21], the isolated sperm fraction carries the risk of contamination by immotile sperm and/or cell debris due to the use of the external syringe force. In addition, it is not cost-effective because this device requires an additional Petri dish to cover and maintain the humidity. We summarized noninvasive sperm separation devices mentioned above in Supplementary Table 1. Collectively, there is a need for a further improved sperm-preparation technique that enables the isolation of high-quality spermatozoa and at the same time satisfies the requisite optimal conditions, including noninvasiveness (centrifugation free), time efficiency, cost-effectiveness, and ease of operation.

Herein, we developed a noninvasive sperm-separation technique using the LensHooke® CA0 device (CA0) wherein we incorporate the natural principle of sperm motility and select self-propelling spermatozoa within a microenvironment that comprises a built-in microporous filter membrane. Based on the principle of live motile sperm sorting, we sought to evaluate (1) CA0 usability under different incubation times and at various incubation temperatures and (2) to comparatively evaluate the post-selection sperm quality of CA0 with that of DGC and Zymot.

Methods and materials

Ethics statement

The study was approved by the Institutional Review Board of Chung Shan Medical University and was registered with the reference number CS2-22039. All participants were fully counseled and provided informed written consent prior to their participation in the study.

Participant enrollment

We recruited male partners of infertile couples who went through ART treatment at Lee Women's Hospital (Taichung, Taiwan) from June to November 2022. The eligibility criteria were age 20–65 years and provision of written informed consent for participation in the study. The exclusion criteria

included azoospermia and/or retrograde ejaculation and total volume < 1.5 mL. Semen samples from 120 patients were collected for the first part of this study and were categorized as normozoospermic or non-normozoospermic semen samples according to the World Health Organization 5th edition guideline (WHO, 2010) lower reference limits, including sperm concentration < 15 million/mL, or total motility < 40%, or normal morphology rate < 4%, or combined [4]. Subsequently, semen samples from an additional 119 participants were collected and investigated in the second part of the study. Raw specimens (neat) were allocated in three aliquots for processing by LensHooke CA0, density gradient centrifugation, and microfluidic-based device. As a total of 2.85 mL was required for the paired analysis, samples with less than 2.85 mL were brought up to this volume with mHTF medium to complete split procedure.

Sperm preparation

The semen samples were collected after an abstinence period of 2–5 days and deposited into sterile cups by masturbation. Samples liquefied at room temperature within 30 min. After liquefaction, an aliquot of the semen sample was deposited into a 10- μ m-deep chamber slide by using CEROS II (Hamilton-Thorne, Danvers, MA, USA). Sperm concentration, total motility, progressive motility, rapid progressive motility (average path velocity, $VAP \geq 25$ μ m/s), slow progressive motility (VAP : 5 to < 25 μ m/s), and

various sperm motion kinematics were evaluated according to the WHO 5th edition guideline [22]; the remaining semen sample was subjected to sperm separation. Sperm motion kinematic parameters, including velocity along the curvilinear path (VCL; μ m/s), amplitude of the lateral displacement of the head (ALH; μ m), and linearity (LIN; %), were evaluated to determine whether the selected sperm had an advanced swimming trajectory.

Sperm separation

CA0 assembly and sperm-separation procedure

The LensHooke® CA0 sperm-separation device (Bonraybio, Taichung, Taiwan) was assembled from three components: lower chamber, upper chamber, and cover. The upper chamber has a built-in polycarbonate membrane filter and is compartmentalized into a retrieval port for sperm recovery (Fig. 1a). The separation procedure was as follows: 1.0 mL of neat semen sample was filled into the lower chamber and placed on a work bench and then attached to the upper chamber. The upper chamber was then filled with 0.9 mL modified human tubal fluid (mHTF) medium [23] supplemented with 1% BSA; the cover was placed over the two-chamber device and the assembly was incubated at 37°C. After incubation, 0.5 mL sperm suspension was aspirated from the retrieval port of the upper chamber for further analysis (Fig. 1b).

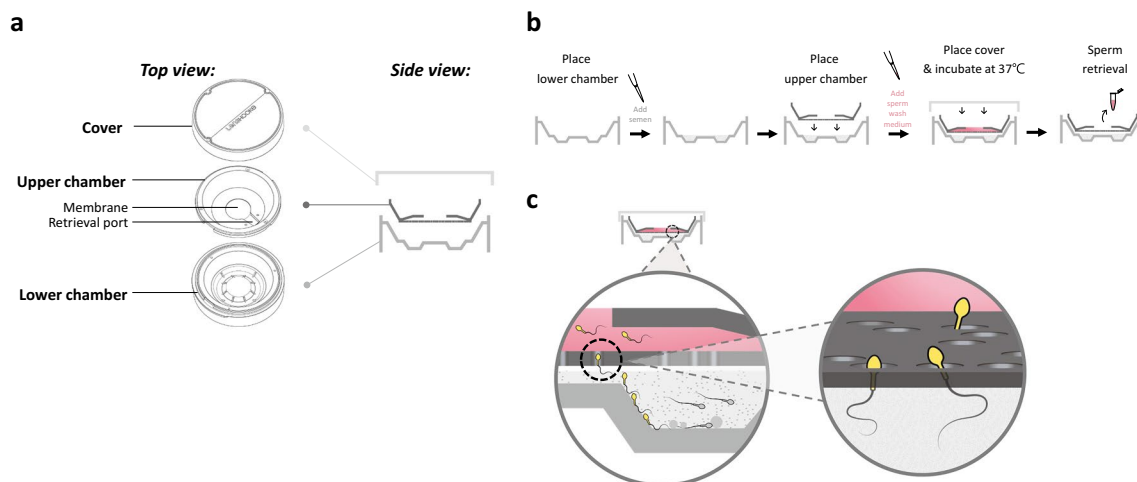


Fig. 1 CA0 sorts neat semen sample based on sperm motility. **a** The LensHooke® CA0 sperm-separation device consists of three components: lower chamber (gray), upper chamber (dark gray) with a built-in membrane filter and fabricated retrieval port, and a cover (light gray). **b** The separation steps are as follows: (1) place the lower chamber on a flat surface, (2) add 1 mL neat semen sample (shown in gray) until the lower chamber is filled, (3) place the upper chamber on top of the lower chamber, (4) add 0.9 mL sperm wash media (shown in pink) into the upper chamber, (5) close the CA0 device

with the cover and incubate the assembly at 37°C, and (6) aspirate 0.5 mL sperm suspension from the retrieval port of the upper chamber. **c** Motile sperm (sperm head shown in yellow) vertically swim along the wall of the collection area within the lower chamber. Self-propelling sperm pass through a built-in microporous membrane filter in the upper chamber, whereas immotile sperm (head in gray) and debris (grayish round structures) sediment go to the bottom of the lower chamber

Evaluation of CA0 feasibility

Fifty-nine neat semen samples were separated using CA0 at different incubation times of 5, 10, 30, and 60 min, respectively, and each condition was performed at 37°C. Similarly, 61 neat semen samples were separated following CA0 under different incubation temperatures of 20, 25, and 37°C, respectively, and each condition was performed for 30 min. After conditional CA0 separation, the concentration (million/mL), total motility (%), and motile sperm concentration (MSC, million/mL) of the recovery sperm suspension were measured.

Density gradient centrifugation

The density gradient medium was prepared by layering 1 mL 40% (v/v) PureSperm (Nidacon, Gothenburg, Sweden) over 1 mL 80% (v/v) density-gradient medium in a 15-mL conical tube. One milliliter neat semen sample was layered over the density gradient medium and centrifuged at $300\times g$ for 20 min at room temperature. After centrifugation, the sperm pellet was washed and gently resuspended in 5 mL mHTF medium and subsequently centrifuged at $200\times g$ for 10 min. The washing procedure was repeated for a total of two times. The final pellet was suspended in 1 mL mHTF medium by gentle mixing and used for further analysis.

Microfluidic-based selection

Microfluidic sperm sorting was performed using a commercial FERTILE (Zymot) device (DxNow Inc., Gaithersburg, MD, USA) according to the manufacturer's instruction. Briefly, 850 μ L of neat semen sample was loaded into the inlet of the device by using a syringe, and 750 μ L of mHTF medium was added on top of the microporous membrane. The device was placed in a humidified incubator at 37°C for 30 min. Following incubation, 500 μ L of spermatozoa was recovered from the outlet with a syringe for further analysis.

Semen analyses

Sperm morphological evaluation

The sperm smears were performed using 10 μ L sperm suspension dragged with a coverslip. The air-dried slide was stained using Diff-Quik (Sperm Morphology Quick-Staining Solution, Ref. BA4157B, Baso, People's Republic of China). Briefly, air-dried sperm smears were immersed in Diff-Quik Fix for 50 s, and thereafter, sequentially immersed in Diff-Quik I and Diff Quik II for 20 s each. The stained smears were washed by fresh water and air dried. Sperm morphology is determined using brightfield illumination at $\times 1000$ magnification based on the WHO 5th edition guideline [22].

All slides were read blind by an experienced technician who produced consistent and reliable results. At least 100 spermatozoa were evaluated in each test.

Sperm DNA fragmentation

Sperm DNA fragmentation was evaluated using a recently developed sperm chromatin dispersion LensHooke R10 assay (Bonraybio, Taichung, Taiwan) according to the manufacturer's instructions [24, 25]. To prepare the samples for analysis, an agarose gel-containing tube was heated to 100°C for 5 min and subsequently stabilized at 37°C. Next, 25 μ L of liquefied semen sample (10×10^6 /mL) and 25 μ L acidic denaturant were added to the tube. A 25- μ L portion of the resulting mixture was then placed on a pretreated microscope slide and covered with a 22 \times 22 mm coverslip before undergoing a lysis reaction at room temperature for 10 min. Finally, the features of the compacted nuclei and the dispersed DNA loops (halo) were evaluated after Wright–Giemsa staining. The percentage of sperm with halo was recorded as the DNA fragmentation index (DFI). At least 500 sperm cells per test were examined.

Spontaneous acrosome reaction assessment

Semen samples were diluted with 0.9% NaCl solution and centrifuged three times at $800\times g$ for 10 min each. After centrifugation, the sperm pellets were resuspended in 40 μ L of 0.9% NaCl and then 5 μ L of the sperm suspension was spread on a 1-cm slide. The slide was allowed to partially air dry for 30 min before being fixed with 95% ethanol for additional 30 min. Finally, the slide was air dried for another 30 min. The fixed sperm smear was stained with 25 μ g/mL *Pisum sativum* agglutinin labeled with fluorescein isothiocyanate (PSA-FITC; Sigma Aldrich, St Louis, MO, USA) and incubated overnight at 4°C. Labeled specimens were washed with DPBS three times and then mounted with a drop of glycerol (Sigma Aldrich). To determine acrosome integrity, the number of sperm in each of the following categories were quantified: acrosome intact and acrosome reacted. At least 200 spermatozoa in each replicate were measured under an excitation laser at 488 nm using an epifluorescence microscope (Olympus BX53). The acrosome-reacted sperm rate (AR) was defined as the percentage of spontaneous acrosome-reacted sperm per test.

Statistical analysis

Summary descriptive statistics were calculated with median values and interquartile ranges (IQR) for variables, and the Wilcoxon signed-rank test was conducted to test whether the median values obtained via CA0 separation were equal to the median values of the neat sample and/or

other methods. A two-tailed probability test with $p < 0.05$ was considered statistically significant by using MedCalc Statistical Software version 20.113 (MedCalc Software, Ostend, Belgium).

Results

Evaluation of CA0 feasibility under different conditions

Incubation time

Of the 59 neat semen samples, 19 were categorized as normozoospermic and 40 as non-normozoospermic. In both semen categories, the percentage of total motility significantly increased following CA0 sperm separation, as compared to the neat semen samples. More specifically, the plateaued and maximized total motility observed for 30 min showed a median of 95.7% for normozoospermic samples and 94.3% for non-normozoospermic samples (Fig. 2a, Supplementary Table 1). The MSC increased over time, reaching 0.6 M/mL after 5 min, then 0.7 M/mL after 10 min, 2.4 M/mL after 30 min, and 3.5 M/mL after 60 min for normozoospermic samples; for non-normozoospermic samples, the MSC observed were 0.3, 0.4, 0.8, and 0.8 M/mL, after 5, 10, 30, and 60 min (Fig. 2b, Supplementary Table 2). Taken together, we have determined that an incubation time of 30 min is optimal for selecting good quality spermatozoa, and this cutoff was used for further investigations.

Incubation temperature

Of the 61 neat semen samples, 23 were categorized as normozoospermic and 38 as non-normozoospermic. When comparing total motility of normozoospermic samples under different incubation temperatures, the values obtained were 83.8% at 20°C, 90.6% at 25°C, and 91.9% at 37°C. For selecting non-normozoospermic specimens, the total motility was 55.9% at 20°C, 79.3% at 25°C, and 80.1% at 37°C (Fig. 2c, Supplementary Table 3). The MSC 0.7 M/mL and 1.5 M/mL at the incubation temperatures of 25°C and 37°C, respectively, were significantly higher than the 0.6 M/mL obtained after 20°C incubation in normozoospermic samples. Furthermore, the maximum MSC of 1.1 M/mL was detected at 37°C for non-normozoospermic samples in comparison with that of 0.3 M/mL at 20°C and 0.4 M/mL at 25°C (Fig. 2d, Supplementary Table 4). Accordingly, 37°C was determined as the optimal incubation temperature in terms of recovering total motility and MSC in both types of sample.

Comparison of semen quality following DGC, Zymot, and CA0 and the selection efficiency

For selecting normozoospermic semen sample

Of the 119 semen samples, 34 were categorized as normozoospermic samples. Table 1 shows a summary of the results for 11 semen parameters including sperm concentration, total motility, progressive motility, rapid progressive motility, slow progressive motility, normal morphology, DFI, VCL, ALH, LIN, and AR in neat semen samples and those derived from DGC, Zymot, and CA0, respectively; a summarized index: motile sperm count was indicated as well. The motile sperm count decreased after sperm separation: 5.4 M for DGC, 2.2 M for Zymot, and 2.8 M for CA0 as compared to the neat semen sample (24.2 M). Furthermore, other semen parameters significantly improved in Zymot and CA0, whereas percent AR did not improve in DGC. The comparison of sperm selection methods showed that CA0 process presented a significant highest number of spermatozoa with total motility (94.0%), progressive motility (90.8%), rapid progressive motility (83.6%), and normal morphology (10.3%); in addition, DFI (2.4%) and AR (4.7%) were lowest in CA0 group as compared to DGC and Zymot (all $p < 0.05$).

For selecting non-normozoospermic semen sample

Of the 119 semen samples, 85 were categorized as non-normozoospermic samples. First, when comparing the results obtained from sperm selections to neat semen samples, significant improvements in ten semen parameters (sperm concentration and motile sperm count were excluded) were identified following Zymot and CA0, whereas in samples processed by DGC, the percent AR was similar to that of neat samples; moreover, the median of DFI significantly increased with DGC as compared to neat samples (18.2% vs. 13.9%, $p < 0.05$), suggesting that DGC should be avoided when processing non-normozoospermic specimens. Comparison of the three sperm separation methods showed that CA0 produced the highest levels of total motility (89.2%), progressive motility (80.4%), rapid progressive motility (74.2%), and normal morphology (8.5%) and the lowest DFI (4.0%) and AR (4.0%), compared to DGC and Zymot (all $p < 0.05$); these findings were consistent in the group of normozoospermic samples (Table 2).

Change to neat (%) is obtained and defined as the ratio of the difference between post-selection value and the initial neat value. When comparing the efficiency in improving semen parameters (% change to neat) in the group of normozoospermic sample (Table 1) and non-normozoospermic sample (Table 2), it is worth noting that non-centrifugation sperm selection methods in this study (Zymot and CA0) tended to be more effective (% change to neat) in improving most of the semen parameters in non-normozoospermic specimens

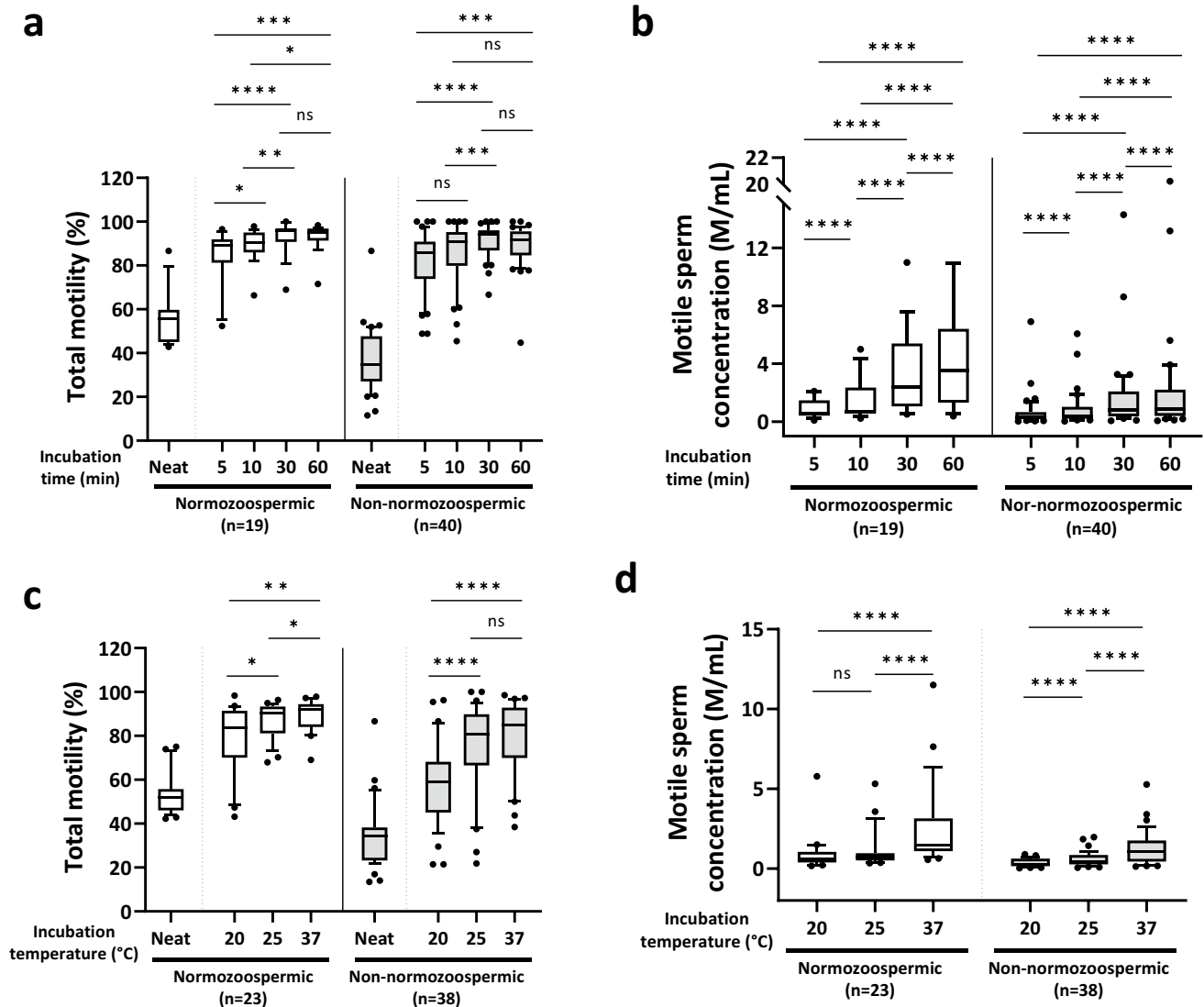


Fig. 2 CA0 performance in total motility and motile sperm concentration with different incubation times and incubation temperatures. Nineteen normozoospermic samples with normal sperm parameters and 40 non-normozoospermic samples with abnormal sperm concentration, total motility, normal morphology, or combined, based on the WHO 5th lower reference limit, were processed. Each semen sample was split into five aliquots. One aliquot was allocated as the control without sperm separation (Neat), and other four aliquots were placed into the CA0 device and incubated for 5, 10, 30, and 60 min, respectively. Values of **(a)** total motility (%) and **(b)** motile sperm concentration (M/mL) were measured. Next, 23 normozoospermic samples

and 38 non-normozoospermic samples were prepared. A liquefied semen aliquot was placed in the collection tube as the control (Neat); three liquefied semen aliquots were placed into the CA0 device and incubated at 20, 25, and 37°C for 30 min respectively. Values of **(c)** total motility (%) and **(d)** motile sperm concentration (M/mL) were measured. Box plots illustrate the data distribution, with five horizontal lines representing the 10th, 25th, 50th, 75th, and 90th percentiles of a variable. The values outside the 10th and 90th percentile are plotted as individual diamonds (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

as compared to normozoospermic specimens. In contrast, the efficiency in eliminating sperm DNA appeared to reduce in the selection of non-normozoospermic samples; despite this, CA0 demonstrated a better efficiency in DFI reduction as compared to Zymot (73.0% for CA0 vs. 52.9% for Zymot). Thus, CA0 provides a reliable option for sperm preparation and can minimize the variability arising from sample variations.

Discussion

Herein, we assessed the effectiveness of a novel centrifugation-free CA0 sperm separation device. This device utilizes live motile sperm sorting and enables the selection of spermatozoa with improved fertilization potential while minimizing sperm DNA fragmentation.

Table 1 Assessment of semen quality after DGC, Zymot, and CA0 for processing of normozoospermic specimens

		Normozoospermic (n=34)			
Parameters		Neat	DGC	Zymot	CA0
Concentration	Median (IQR) (M/mL)	38.5 (27.0-56.7)	6.5 (3.1-14.1) ^{*a}	4.8 (2.4-8.5) ^{*b}	5.7 (2.5-8.3) ^{*c}
	Change to neat (%)	-	-85.0 ^a	-89.2 ^b	-87.6 ^b
Total motility	Median (IQR) (%)	56.2 (51.6-68.7)	85.8 (78.7-91.2) ^{*a}	90.7 (76.9-95.5) ^{*b}	94.0 (91.3-95.9) ^{*c}
	Change to neat (%)	-	+40.0 ^a	+46.4 ^b	+57.6 ^c
Motile sperm count	Median (IQR) (M)	24.2 (14.6-34.8)	5.4 (2.4-13.3) ^{*a}	2.2 (0.9-3.6) ^{*b}	2.8 (1.2-3.9) ^{*b}
	Change to neat (%)	-	-79.9 ^a	-92.8 ^b	-90.2 ^b
Progressive motility	Median (IQR) (%)	47.4 (39.5-55.9)	80.6 (69.2-86.4) ^{*a}	85.6 (73.9-92.7) ^{*b}	90.8 (86.7-93.9) ^{*c}
	Change to neat (%)	-	+50.5 ^a	+64.8 ^b	+85.3 ^c
Rapid progressive motility	Median (IQR) (%)	35.7 (25.8-46.7)	75.8 (65.8-82.3) ^{*a}	77.7 (64.5-85.1) ^{*a}	83.6 (77.9-87.5) ^{*b}
	Change to neat (%)	-	+87.6 ^a	+97.7 ^a	+125.3 ^b
Slow progressive motility	Median (IQR) (%)	10.9 (8.7-13.8)	4.4 (3.1-5.4) ^{*a}	7.1 (4.1-8.9) ^{*b}	7.1 (4.8-9.7) ^{*b}
	Change to neat (%)	-	-64.4 ^a	-49.9 ^b	-38.4 ^b
Normal morphology rate	Median (IQR) (%)	5.5 (4.5-8.0)	8.1 (5.6-10.9) ^{*a}	8.1 (7.0-12.0) ^{*b}	10.3 (7.6-14.5) ^{*c}
	Change to neat (%)	-	+22.5 ^a	+47.7 ^b	+57.7 ^c
DFI	Median (IQR) (%)	18.5 (10.5-22.8)	11.8 (6.9-19.8) ^{*a}	3.7 (2.3-6.1) ^{*b}	2.4 (1.6-3.4) ^{*c}
	Change to neat (%)	-	-22.0 ^a	-76.1 ^b	-86.2 ^c
AR	Median (IQR) (%)	13.2 (9.3-17.9)	13.4 (8.0-18.4) ^a	6.5 (4.0-9.0) ^{*b}	4.7 (2.5-6.8) ^{*c}
	Change to neat (%)	-	-7.0 ^a	-51.6 ^b	-62.0 ^c
VCL	Median (IQR) (μm/s)	55.3 (42.6-58.5)	94.3 (79.1-108.0) ^{*a}	86.3 (80.0-92.2) ^{*b}	80.2 (75.3-98.5) ^{*b}
	Change to neat (%)	-	+78.1 ^a	+59.6 ^b	+67.9 ^b
ALH	Median (IQR) (μm)	4.5 (3.8-5.0)	6.2 (5.1-7.1) ^{*a}	6.2 (5.5-6.6) ^{*a}	5.5 (5.1-6.9) ^{*a}
	Change to neat (%)	-	+41.2 ^a	+40.7 ^a	+37.6 ^a
LIN	Median (IQR) (%)	42.8 (41.1-47.3)	35.5 (30.4-41.2) ^{*a}	34.5 (31.5-37.9) ^{*a}	32.5 (29.3-38.7) ^{*a}
	Change to neat (%)	-	-23.2 ^a	-22.6 ^a	-26.5 ^a

Asterisk indicates significant difference between neat and sperm selection methods ($p < 0.05$). Different alphabets indicate a significant difference between two methods ($p < 0.05$)

IQR interquartile range, *DFI* DNA fragmentation index, *AR* acrosome-reacted sperm rate, *VCL* velocity along the curvilinear path, *ALH* amplitude of the lateral displacement of the head, *LIN* linearity

The design of the CA0 is bioinspired by two migratory abilities of motile sperm. We fabricated a total of 12 columnar joints at the lower chamber to promote sperm transition along solid boundaries that mimic obstacle-ridden paths of in vivo environment [26, 27] (Fig. 1a). Then, we applied passive-driven force theory in that the polycarbonate porous membrane in the two-chamber CA0 device separates and passively selects sperm from other cellular and/or non-cellular components (Fig. 1c). With the no-flow selection microenvironment, only highly motile spermatozoa were able to overcome the microchannel geometry; in contrast, immobile objects remained within and/or precipitated in the lower chamber.

An ideal sperm-sorting device should be easy to operate and effective in selecting fertilizing-competent sperm for ART treatment procedures. Given the feasibility of CA0, we inferred an optimal incubation time of 30 min based on the plateau phase in total motility (Fig. 2a). Nevertheless, we found that even a short incubation time of 5 min

can yield a high-quality total motility in normozoospermic samples (89.2%) and non-normozoospermic samples (86.1%); both values are on the 97.5th percentile of the distribution for total motility ($> 81\%$) and are associated with a higher likelihood of pregnancy success, as indicated by the WHO 5th edition guideline [22]. Furthermore, the MSC was 0.6 and 0.3 M/mL in recovered sperm suspensions from normozoospermic and non-normozoospermic samples, respectively. As many of the non-normozoospermic patients would consider ICSI treatment, which requires the relatively fewer spermatozoa than other insemination procedures, the criterion can be satisfied by applying CA0 for 5 min to achieve an adequate amount of high-quality motile sperm. For use in IUI and IVF, we recommend the standard 30-min incubation. Alternatively, by performing repeated CA0 separation using the same device, a sufficient number of post-process sperm can be easily obtained. With regard to the effect of incubation temperature on sperm-separation efficiency, a previous research showed that incubation at

Table 2 Assessment of semen quality following DGC, Zymot, and CA0 for processing of non-normozoospermic specimens

		Non-normozoospermic (n=85)			
Parameters		Neat	DGC	Zymot	CA0
Concentration	Median (IQR) (M/mL)	15.5 (10.2-30.3)	2.5 (1.4-6.6) ^{*a}	1.7 (1.0-3.6) ^{*b}	1.3 (0.7-3.0) ^{*c}
	Change to neat (%)	-	-79.3 ^a	-88.5 ^b	-92.0 ^c
Total motility	Median (IQR) (%)	40.0 (32.3-52.6)	66.8 (38.7-79.7) ^{*a}	78.8 (67.8-89.1) ^{*b}	89.2 (80.9-93.8) ^{*c}
	Change to neat (%)	-	+47.5 ^a	+88.8 ^b	+115.8 ^c
Motile sperm count	Median (IQR) (M)	6.2 (3-12.7)	1.29 (0.57-3.91) ^{*a}	0.61 (0.34-1.44) ^{*b}	0.57 (0.28-1.38) ^{*c}
	Change to neat (%)	-	-70.7 ^a	-88.0 ^b	-89.9 ^c
Progressive motility	Median (IQR) (%)	33.1 (23.8-43.4)	55.3 (28.4-71.7) ^{*a}	70.3 (54.1-84.2) ^{*b}	80.4 (70.6-87.1) ^{*c}
	Change to neat (%)	-	+45.5 ^a	+99.2 ^b	+131.0 ^c
Rapid progressive motility	Median (IQR) (%)	22.8 (13.8-33.0)	50.3 (25.6-66.2) ^{*a}	62.9 (48.8-78.7) ^{*a}	74.2 (63.8-82.0) ^{*b}
	Change to neat (%)	-	+84.2 ^a	+157.9 ^b	+205.1 ^c
Slow progressive motility	Median (IQR) (%)	9.2 (6.1-11.1)	2.3 (0-5.3) ^{*a}	4.1 (0-9) ^{*b}	4.6 (0-9.8) ^{*c}
	Change to neat (%)	-	-79.5 ^a	-55.7 ^b	-43.9 ^c
Normal morphology rate	Median (IQR) (%)	3.0 (2.0-4.5)	5.1 (3.0-7.0) ^{*a}	6.3 (4.0-8.5) ^{*b}	8.5 (6.0-10.8) ^{*c}
	Change to neat (%)	-	+45.9 ^a	+75 ^b	+111.7 ^c
DFI	Median (IQR) (%)	13.9 (9.4-21.2)	18.2 (9.5-35.9) ^{*a}	6.2 (3.0-13.5) ^{*b}	4.0 (2.0-7.4) ^{*c}
	Change to neat (%)	-	+22.3 ^a	-52.9 ^b	-73.0 ^c
AR	Median (IQR) (%)	9.5 (6.5-14.5)	8.5 (5.5-17.5) ^a	5 (3.0-8.5) ^{*b}	4 (2.5-6.0) ^{*c}
	Change to neat (%)	-	-5.4 ^a	-50 ^b	-60 ^c
VCL	Median (IQR) (μm/s)	45.5 (38.7-56.0)	77.9 (66.4-93.4) ^{*a}	75.9 (64.9-90.2) ^{*a}	76.8 (66.0-88.6) ^{*a}
	Change to neat (%)	-	+63.3 ^a	+66.7 ^a	+64.4 ^a
ALH	Median (IQR) (μm)	3.9 (3.2-4.7)	5.2 (4.4-5.9) ^{*a}	5.4 (4.6-6.6) ^{*b}	5.3 (4.7-6.3) ^{*b}
	Change to neat (%)	-	+34.0 ^a	+45.7 ^b	+38.7 ^b
LIN	Median (IQR) (%)	42.2 (39.2-46.8)	35.3 (31.4-40.6) ^{*a}	35.6 (32.4-39.9) ^{*a}	33.7 (29.1-38.2) ^{*b}
	Change to neat (%)	-	-16.3 ^a	-16.4 ^a	-22.6 ^b

Asterisk indicates significant difference between neat and sperm selection methods ($p < 0.05$). Different alphabets indicate a significant difference between two methods ($p < 0.05$)

IQR interquartile range, *DFI* DNA fragmentation index, *AR* acrosome-reacted sperm rate, *VCL* velocity along the curvilinear path, *ALH* amplitude of the lateral displacement of the head, *LIN* linearity

20°C significantly compromises sperm motion kinematics and blocks the development of hyperactivation as compared to 37°C incubation [28]. The thermal effect reflects the relatively low degree of improved total motility when sperm separation was performed at 20°C, particularly limited to 55.9% (50th percentile of the distribution for total motility per WHO 5th edition) when processing non-normozoospermic specimens (Fig. 2c). Therefore, although the WHO laboratory manual suggests that semen handling could be performed at ambient temperatures between 20 and 37°C [22], we recommend a higher handling temperature in such range toward 37°C for sperm separation, to prevent a detrimental thermal effect particularly for centrifugation-free sperm-separation process.

Among the various sperm-separation techniques used in clinics, conventional swim-up seems to have consistent results for decreasing sperm DNA fragmentation [29]. However, given that the procedure is time consuming, a modified swim-up process involving initial centrifugation

is commonly practiced in many clinics [30, 31]. In our preliminary assessment (unpublished internal data), the modified swim-up provides no significant improvement in post-selection semen quality parameters than DGC techniques. Hence, our focus of this study was on the comparative selection performance of CA0, DGC, and Zymot. The paired analysis between CA0 and other two methods may be biased due to difference in initial volume of neat semen sample and final volume of sperm suspension. Herein, we attempted to make a fair comparison following the original protocol recommended by manufacturers for Zymot, and applying the same initial volume for DGC as CA0. Besides comparing basic semen parameters, we ascertained sperm hyperactivation by the sperm motion kinematic index (increased VCL, increased ALH, and decreased LIN) and acrosome integrity by PSA staining. Sperm recovered from all three separation methods tended to have a hyperactivation movement pattern, however, not enough to be recognized as hyperactivation

due to the short period of handling less than the 3–24 h to acquire capacitation. Thus, we indicated that these three sperm-separation methods were effective in selecting spermatozoa with capacitation potential and their effectiveness was comparable among each other. On the other hand, decreased AR were found in the Zymot and CA0 groups, but not in DGC. The phenomenon echoes a previous finding that spermatozoa under DGC are more capacitated and respond more to acrosome reaction than with other methods [32]. Although the effect in DGC can be acceptable for ICSI due to the instant processing [33], the higher probability of premature acrosome reaction is unsuitable for IUI and IVF wherein sperm rely on zona pellucida-induced acrosome reaction [34]. Therefore, centrifugation-free sperm separation devices are more suitable for IUI and IVF, especially the CA0 method which ensures the lowest level of AR.

Regarding the improvement in eliminating spermatozoa with DNA damage, DGC generated inconsistent outcomes showing a decline of DFI (−22.0%) when processing normozoospermic samples and an uptrend of DFI (+22.3%) when separating non-normozoospermic samples. The discrepancy indicated that healthy spermatozoa may be able to tolerate centrifugation stress to some extent, but poor-quality spermatozoa may not. Unlike DGC, both Zymot and CA0 had consistent efficiency in eliminating DNA-damaged spermatozoa; moreover, better DFI elimination efficiency was determined in the group of CA0 than Zymot, regardless of sample conditions (normozoospermic: CA0 −86.2% vs. Zymot −76.1%; non-normozoospermic: CA0 −73.0% vs. Zymot −52.9%).

The limitations of our current study include single-center trial, a relatively small sample size, and the lack of correlation between minimized sperm DNA fragmentation and clinical ART outcomes. To address these limitations, a randomized controlled trial to further evaluate CA0 benefits in various ART applications is needed.

In conclusion, the CA0 separation device provides practical advantages of a centrifugation-free process and ease of operability, as well as a customizable incubation time for different ART treatment applications. Most importantly, CA0 achieves maximal improvement in various indices of sperm quality and ensures minimum sperm DNA fragmentation.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10815-023-02838-4>.

Author contribution C.T.H., C.I.L., M.S.L., and A.A. conceptualized the study and designed all the experiments. C.C.H. and H.M.T. collected samples and curated data. F.S.L., F.Z.W., and H.C.C. conducted all the experiments and statistical analyses. T.E.W. drafted and revised the manuscript.

Data availability The data underlying this article will be shared on reasonable request made to the corresponding author.

Declarations

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the local Ethics Committee of Chung Shan Medical University (No. CS2-22039).

Consent to participate Informed consent was obtained from all individual participants included in the study.

Conflict of interest This study was supported by Bonraybio Co., Ltd. C.T.H. is the owner of Bonraybio Co., Ltd. and F.S.L., F.Z.W., H.C.C., and T.E.W. are employees of Bonraybio Co., Ltd. The employer (C.I.L., C.C.H., and H.M.T.) of Lee Women's Hospital has collaborated with Bonraybio Co., Ltd. with regard to the IRB approval for providing human samples to complete this collaborative project. A.A. reports no financial support from Bonraybio Co., Ltd. Bonraybio Co., Ltd. reports patent applications "Sperm sorting device and method" licensed in Taiwan (application number: 111110406; 111209204), China (application number: 202210427414.7; 202220949418.7; 202222325590.8), the USA (application no: 17/815,484), and Europe (application no: 22188408.3). The remaining authors have no conflicts of interest to disclose.

References

1. Choy JT, Eisenberg ML. Male infertility as a window to health. *Fertil Steril*. 2018;110(5):810–4. <https://doi.org/10.1016/j.fertnstert.2018.08.015>.
2. Boivin J, Bunting L, Collins JA, Nygren KG. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod*. 2007;22(6):1506–12. <https://doi.org/10.1093/humrep/dem046>.
3. Collins J. An international survey of the health economics of IVF and ICSI. *Hum Reprod Update*. 2002;8(3):265–77. <https://doi.org/10.1093/humupd/8.3.265>.
4. Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, Haugen TB, Kruger T, Wang C, Mbizvo MT, Vogel-song KM. World Health Organization reference values for human semen characteristics. *Hum Reprod Update*. 2010;16(3):231–45. <https://doi.org/10.1093/humupd/dmp048>.
5. Rao M, Tang L, Wang L, Chen M, Yan G, Zhao S. Cumulative live birth rates after IVF/ICSI cycles with sperm prepared by density gradient centrifugation vs. swim-up: a retrospective study using a propensity score-matching analysis. *Reprod Biol Endocrinol*. 2022;20(1):60. <https://doi.org/10.1186/s12958-022-00933-2>.
6. Takeshima T, Yumura Y, Kuroda S, Kawahara T, Uemura H, Iwasaki A. Effect of density gradient centrifugation on reactive oxygen species in human semen. *Syst Biol Reprod Med*. 2017;63(3):192–8. <https://doi.org/10.1080/19396368.2017.1294214>.
7. Muratori M, Tarozzi N, Cambi M, Boni L, Iorio AL, Passaro C, Luppino B, Nadalini M, Marchiani S, Tamburrino L, Forti G, Maggi M, Baldi E, Borini A. Variation of DNA fragmentation levels during density gradient sperm selection for assisted reproduction techniques: a possible new male predictive parameter of pregnancy? *Medicine (Baltimore)*. 2016;95(20):e3624. <https://doi.org/10.1097/md.0000000000003624>.
8. Muratori M, Tarozzi N, Carpentiero F, Danti S, Perrone FM, Cambi M, Casini A, Azzari C, Boni L, Maggi M, Borini A, Baldi E. Sperm selection with density gradient centrifugation and swim up: effect on DNA fragmentation in viable spermatozoa. *Sci Rep*. 2019;9(1):7492.
9. Said TM, Grunewald S, Paasch U, Rasch M, Agarwal A, Glander HJ. Effects of magnetic-activated cell sorting on sperm motility and

- cryosurvival rates. *Fertil Steril*. 2005;83(5):1442–6. <https://doi.org/10.1016/j.fertnstert.2004.11.052>.
10. Ye H, Huang GN, Gao Y, Liu DY. Relationship between human sperm-hyaluronan binding assay and fertilization rate in conventional in vitro fertilization. *Hum Reprod*. 2006;21(6):1545–50. <https://doi.org/10.1093/humrep/del008>.
 11. Parmegiani L, Cognigni GE, Bernardi S, Troilo E, Ciampaglia W, Filicori M. “Physiologic ICSI”: hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. *Fertil Steril*. 2010;93(2):598–604. <https://doi.org/10.1016/j.fertnstert.2009.03.033>.
 12. Eliasson R. Semen analysis with regard to sperm number, sperm morphology and functional aspects. *Asian J Androl*. 2010;12(1):26–32. <https://doi.org/10.1038/aja.2008.58>.
 13. Berkovitz A, Eltes F, Ellenbogen A, Peer S, Feldberg D, Bartoov B. Does the presence of nuclear vacuoles in human sperm selected for ICSI affect pregnancy outcome? *Hum Reprod*. 2006;21(7):1787–90. <https://doi.org/10.1093/humrep/del049>.
 14. Komiya A, Watanabe A, Kawauchi Y, Fuse H. Sperm with large nuclear vacuoles and semen quality in the evaluation of male infertility. *Syst Biol Reprod Med*. 2013;59(1):13–20. <https://doi.org/10.3109/19396368.2012.729174>.
 15. Kishi K. Frequency of sperm DNA fragmentation according to selection method: comparison and relevance of a microfluidic device and a swim-up procedure. *J Clin Diagn Res*. 2015. <https://doi.org/10.7860/jcdr/2015/10332.6811>.
 16. Seiringer MMM, Shebl O, Dreier K, Tews G, Ziehr S, Schappacher-Tilp G, Petek E, Ebner T. Efficacy of a sperm-selection chamber in terms of morphology, aneuploidy and DNA packaging. *Reprod Biomed Online*. 2013;27(1):81–8. <https://doi.org/10.1016/j.rbmo.2013.03.013>.
 17. Ebner T, Shebl O, Moser M, Mayer RB, Arzt W, Tews G. Easy sperm processing technique allowing exclusive accumulation and later usage of DNA-strandbreak-free spermatozoa. *Reprod Biomed Online*. 2011;22(1):37–43. <https://doi.org/10.1016/j.rbmo.2010.09.004>.
 18. Meitei HY, Uppangala S, Sharan K, et al. A simple, centrifugation-free, sperm-sorting device eliminates the risks of centrifugation in the swim-up method while maintaining functional competence and DNA integrity of selected spermatozoa. *Reprod Sci*. 2021;28(1):134–43. <https://doi.org/10.1007/s43032-020-00269-5>.
 19. Quinn MM, Jalalian L, Ribeiro S, Ona K, Demirci U, Cedars MI, Rosen MP. Microfluidic sorting selects sperm for clinical use with reduced DNA damage compared to density gradient centrifugation with swim-up in split semen samples. *Hum Reprod*. 2018;33(8):1388–93. <https://doi.org/10.1093/humrep/dey239>.
 20. Parrella A, Keating D, Cheung S, Xie P, Stewart JD, Rosenwaks Z, Palermo GD. A treatment approach for couples with disrupted sperm DNA integrity and recurrent ART failure. *J Assist Reprod Genet*. 2019;36(10):2057–66. <https://doi.org/10.1007/s10815-019-01543-5>.
 21. Yetkinel S, Kilicdag EB, Aytac PC, Haydardedeoglu B, Simsek E, Cok T. Effects of the microfluidic chip technique in sperm selection for intracytoplasmic sperm injection for unexplained infertility: a prospective, randomized controlled trial. *J Assist Reprod Genet*. 2019;36(3):403–9. <https://doi.org/10.1007/s10815-018-1375-2>.
 22. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization; 2010.
 23. Quinn PK, John F, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil Steril*. 1985;44(4):493–8. [https://doi.org/10.1016/s0015-0282\(16\)48918-1](https://doi.org/10.1016/s0015-0282(16)48918-1).
 24. Lin HTWM, Wu WL, Tsai LC, Chen YY, Hung KH, Wu PH, Chen TS, Ou HT, Cheng YS. Incorporating sperm DNA fragmentation index with computer-assisted semen morphokinematic parameters as a better window to male fertility. *Chin J Physiol May-Jun*. 2022;65(3):143–50. https://doi.org/10.4103/CJP.CJP_12_22.
 25. Hsu CT, Lee CI, Huang CC, et al. Development and integration of LensHooke® R10 for automatic and standardized diagnosis for sperm DNA fragmentation. *Andrology*. Mar 3 2023. <https://doi.org/10.1111/andr.13419>.
 26. Eamer LVM, Nosrati R, San Gabriel MC, Zeidan K, Zini A, Sinton D. Turning the corner in fertility: high DNA integrity of boundary-following sperm. *Lab Chip*. 2016;16(13):2418–22. <https://doi.org/10.1039/c6lc00490c>.
 27. Tasoglu SSH, Zhang X, Kingsley JL, Catalano PN, Gurkan UA, Nureddin A, Kayaalp E, Anchan RM, Maas RL, Tüzel E, Demirci U. Exhaustion of racing sperm in nature-mimicking microfluidic channels during sorting. *Small*. 2013;9(20):3374–84. <https://doi.org/10.1002/smll.201300020>.
 28. Marín-Briggiler CITJ, Miranda PV, Vazquez-Levin MH. Effect of incubating human sperm at room temperature on capacitation-related events. *Fertil Steril*. 2002;77(2):252–9. [https://doi.org/10.1016/s0015-0282\(01\)02982-x](https://doi.org/10.1016/s0015-0282(01)02982-x).
 29. Zini A, Finelli A, Phang D, Jarvi K. Influence of semen processing technique on human sperm DNA integrity. *Urology*. 2000;56(6):1081–4. [https://doi.org/10.1016/s0090-4295\(00\)00770-6](https://doi.org/10.1016/s0090-4295(00)00770-6).
 30. Dai XWY, Cao F, Yu C, Gao T, Xia X, Wu J, Chen L. Sperm enrichment from poor semen samples by double density gradient centrifugation in combination with swim-up for IVF cycles. *Sci Rep*. 2020;10(1):2286. <https://doi.org/10.1038/s41598-020-59347-y>.
 31. Yamanaka M, Tomita K, Hashimoto S, et al. Combination of density gradient centrifugation and swim-up methods effectively decreases morphologically abnormal sperms. *J Reprod Dev*. 2016;62(6):599–606. <https://doi.org/10.1262/jrd.2016-112>.
 32. Hernández-Silva G, López-Torres AS, Maldonado-Rosas I, Mata-Martínez E, Larrea F, Torres-Flores V, Treviño CL, Chirinos M. Effects of semen processing on sperm function: differences between swim-up and density gradient centrifugation. *World J Mens Health*. 2021;39(4):740–9. <https://doi.org/10.5534/wjmh.200115>.
 33. Wang J, Tang H, Zou Q, et al. Patient with CATSPER3 mutations-related failure of sperm acrosome reaction with successful pregnancy outcome from intracytoplasmic sperm injection (ICSI). *Mol Genet Genomic Med*. 2021;9(2):e1579. <https://doi.org/10.1002/mgg3.1579>.
 34. Liu DY, Baker HW. Disordered zona pellucida-induced acrosome reaction and failure of in vitro fertilization in patients with unexplained infertility. *Fertil Steril*. 2003;79(1):74–80. [https://doi.org/10.1016/s0015-0282\(02\)04555-7](https://doi.org/10.1016/s0015-0282(02)04555-7).

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.

	Qualis®	Zech-selector	Miglis®	ZyMōt®
Manufacturer	Menicon Life Science (Kasugai, Japan)	AssTIC Medizintechnik GmbH (Leutsch, Austria)	Menicon Life Science (Kasugai, Japan)	DxNow (Gaithersburg, MD, USA)
Core technology	Microfluidic flow	Capillary bridge	Migration- sedimentation	Microfluidic flow
Incubation time (min)	30	60	60	30
Advantages (Improved semen outcomes)	① Sperm motility ② DFI	① Sperm motility ② Morphology ③ DFI	① Sperm motility ② DFI ③ Mitochondria functionality	① Sperm motility ② DFI
Disadvantages	<ul style="list-style-type: none"> Limited implementation in ART (ICSI only) 	<ul style="list-style-type: none"> Unstandardized procedure Time-consuming 	<ul style="list-style-type: none"> Time-consuming 	<ul style="list-style-type: none"> Immotile sperms/cell debris contamination Cost-ineffectiveness

Total motility (%)												
Normozoospermic						Non-normozoospermic						
Incubation time	0 min	5 min	10 min	30 min	60 min	Incubation time	0 min	5 min	10 min	30 min	60 min	
Median	55.5	89.2	90.6	95.7	95.1	Median	34.8	86.1	90.9	94.3	91.7	
IQR	46.7-59.6	81.8-91.3	86.2-94.6	91.5-96.8	91.7-96.5	IQR	28.4-45	74.3-90.6	80.8-94.9	86.9-96	85.6-95.4	
10-90%	44.1-70.3	61.3-94.4	83.5-95.4	85.5-98.4	89.8-97.2	10-90%	20.9-51.3	61.2-95.5	61.3-97.9	82.1-98.2	81-97.4	

MSC (M/mL)												
Normozoospermic						Non-normozoospermic						
Incubation time	0 min	5 min	10 min	30 min	60 min	Incubation time	0 min	5 min	10 min	30 min	60 min	
Median	-	0.6	0.7	2.4	3.5	Median	-	0.3	0.4	0.8	0.8	
IQR	-	0.4-1.4	0.6-2.3	1.1-5	1.6-6.3	IQR	-	0.2-0.6	0.2-1	0.4-1.8	0.4-2.1	
10-90%	-	0.3-1.8	0.4-3.8	0.6-7.3	0.6-10.2	10-90%	-	0.1-1.1	0.1-1.8	0.2-2.6	0.2-3.7	

Total motility (%)									
Normozoospermic					Non-normozoospermic				
Incubation temp. (°C)	Neat	20	25	37	Incubation temp. (°C)	Neat	20	25	37
Median	52.0	83.8	90.6	91.9	Median	31.2	55.9	79.3	80.1
IQR	46-55.6	70.7-90.7	81.4-93	85.4-94.3	IQR	23.2-36	43.3-66.9	63.5-87.1	61.8-91.6
10-90%	46-72.4	51-92.8	78-93.9	81.3-96.9	10-90%	22.2-38	36.5-70.6	38.1-93	50.4-94

Total motility (%)									
Normozoospermic					Non-normozoospermic				
Incubation temp. (°C)	Neat	20	25	37	Incubation temp. (°C)	Neat	20	25	37
Median	52.0	83.8	90.6	91.9	Median	31.2	55.9	79.3	80.1
IQR	46-55.6	70.7-90.7	81.4-93	85.4-94.3	IQR	23.2-36	43.3-66.9	63.5-87.1	61.8-91.6
10-90%	46-72.4	51-92.8	78-93.9	81.3-96.9	10-90%	22.2-38	36.5-70.6	38.1-93	50.4-94