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Original Article

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Comparison of In-House Microfluidic Device and Centrifuge-Based Method Efficacy in Sperm Preparation for Assisted Reproductive Technology

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Abstract

Background: Sperm DNA fragmentation can affect reproductive outcomes in assisted reproductive techniques (ART), and it is a concern in density gradient centrifugation (DGC). By contrast, microfluidic approaches allow the selection of highly motile sperm with low DNA fragmentation index (DFI). The purpose of current study, was to compare the efficacy of a microfluidic device designed in-house in comparison with DGC.

Methods: Nineteen healthy men with normal semen profiles were included in the study. Semen samples were individually aliquoted for three sperm preparation analyses (crude and processed with to either DGC or the microfluidic method). Sperm parameters of the samples were evaluated along with DNA fragmentation using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method.

Results: Sperm processed using the microfluidic method showed a significantly lower DFI than those obtained using DGC and in crude semen, with DFI of 1.1%, 3.5%, and 4.9%, respectively. Although the microfluidic method yielded significantly lower sperm concentrations than DGC, no significant differences were observed in total motility, progressive motility, curvilinear velocity, straight-line velocity, or normal morphology.

Conclusion: Using the in-house microfluidic device, sperm with lower DFI was effectively isolated when compared with DGC. The motility and normal morphology rates were comparable among the samples.

Keywords: Assisted reproductive technology, Density gradient centrifugation, DNA fragmentation, Microfluidics, Sperm.
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Introduction

Infertility is characterized by the failure to establish a clinical pregnancy after 12 months of regular, unprotected sexual intercourse (1). According to the World Health Organization (WHO), approximately 15% of couples are infertile (2), with specific causes including ovulatory dysfunction (20%–40%), tubal and peritoneal pathology (30%–40%), male factors related to the quality or production of spermatozoa (30%–40%), and some unknown factors (3). In such cases, many couples seek to overcome infertility through assisted reproductive technologies (ARTs). Among the available ARTs, intracytoplasmic sperm injection (ICSI) is widely implemented to address male factors, as the sperm is directly injected into the egg.

As ARTs require high-quality sperm, samples can first be subjected to sperm selection, which should ideally be a simple process with a short preparation time. Ultimately, such procedures should yield high-quality sperm following contaminant removal, such as debris and leukocytes (4). Although several sperm selection methods are currently available, density gradient centrifugation (DGC) is the most widely used protocol. However, the procedure also entails risks, as centrifugation can cause oxidative stress that damages the sperm. Specifically, centrifugation is a major iatrogenic cause of reactive oxygen species (ROS) production (5), and its excess during the selection process can lead to quality issues such as sperm DNA fragmentation (SDF) and apoptosis. In addition, low fertilization rates and poor embryo development can substantially hinder the success of ARTs (6-9); for example, high SDF is correlated with low pregnancy and high miscarriage rates (10-14).

The microfluidic method is an alternative approach to sort spermatozoa, in which the sperm flow is manipulated using small amounts of fluid, with the sperm moving in the flow direction. Either active or passive flow has been applied for sperm sorting in previous studies (4, 15-17). In this arrangement, sperm with high motility can quickly reach the end of the equipment. Moreover, the procedure takes less time than other methods and can recover sperm of high quality. Compared with that in DGC, ROS production is reduced in the microfluidic method, as centrifugation is not required and its sorting time is short (the entire process requires just 20–30 *min*). Several studies have shown that sperm, using the microfluidic method, have lower oxidative stress and less DNA fragmentation than those obtained using DGC or swim-up techniques (4, 15-20). Furthermore, the microfluidic method is relatively inexpensive and does not require intensive labor.

In this study, a capillary flow-driven, chemical-free microfluidic device, designed and developed in-house, was tested. Specifically, the novel device uses barriers to create microchannels that allow the entry of sperm with high motility, normal morphology, and low DNA fragmentation following the introduction of fresh semen. The results obtained using the in-house microfluidic device were also compared with those obtained using DGC, which is currently the standard method used for sperm processing at our center.

Methods

Study design and participant selection: This experimental study was approved by the Institutional Ethics Committee of the Faculty of Medicine, Prince of Songkla University (REC.63-123-12-4). All procedures followed in the study were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and the tenets of the Helsinki Declaration of 1964 and its later amendments. The procedures were conducted at the Reproductive Medicine Unit of Songklanagarind Hospital, Thailand, between 1 September 2020 and 28 February 2021. Participants were healthy men aged between 18 and 40 years with normal semen profiles according to the WHO (Geneva) criteria (21). The exclusion criteria were as follows: sexual abstinence for less than 2 days or more than 7 days, semen collected via sexual intercourse or deposited in an inappropriate container, use of lubricant, and semen viscosity that was considered excessive. All participants were provided sufficient information regarding this study, and their signed informed consent forms were obtained before enrolment. Personal data of the participants were also recorded.

The required sample size was calculated from three dependent means for a pair-matched study using α=0.017, and $Z_{\alpha/2}$=2.12, with the power of at least 80%, and with β=0.2 and Z_{β}=0.84. The mean difference between groups was 0.7. The effect size (delta/standard deviation) was 0.7. Therefore, the required sample size for this study was 18.

Sample collection: Of the 27 initial participants recruited, 19 were enrolled in the study and provided semen samples. All samples were produced via masturbation, collected in sterile containers, and allowed to liquefy for 30 *min* at room temperature (approximately 25°*C*). Each sample was then aliquoted into three tubes.

Design and fabrication of microfluidic device

Device design: Our novel microfluidic device was constructed to feature micropillar arrays to mimic the natural filtering characteristics of the female reproductive tract (15, 22, 23). It was passive, non-invasive, and composed with a soft, flexible polymer called polydimethylsiloxane (PDMS). The design consisted of three zones inside the microchannel. The first zone was created to capture any blood cells or other particles that might be present in unprocessed semen. The remaining two zones, which featured pillar arrays, were designed

to separate sperm with high motility and normal morphology. The channel dimensions of the microfluidic chip designed using NX software *vs.* 1953 (Siemens Digital Industries Software, USA) were as follows: 20 *mm* in length, 3 *mm* in width, and 50 *μm* in depth, with circular columns of 50 *μm* in diameter. There were three zones of micropillars with a gap in the middle, as illustrated in figure 1A. In the first zone, the micropillars were arranged in a staggered pattern to separate sperm from debris in raw semen before entering the next zone of the microchannel, whereas the micropillars in the second and third zones for sperm selection had an aligned arrangement, with 50–75 and 20–25-*μm* space between the micropillars, respectively. The mechanism of the device that allowed high motility sperm to move forward to the outlet was similar to the natural female genital tract. To account for sperm swimming behavior in pillar array geometries (15), the microchannels in the second and third zones contained multiple micropillars, which simulated the surface of the genital tract, featuring tiny pores and crypts that would trap sperm, thereby allowing sperm with high motility to move forward towards the outlet. Thus, highly progressive motile sperm with normal anatomy could reach the outlet faster than abnormal sperm. As this method does not involve centrifugation nor chemical substances, it should produce sperm with low SDF. Moreover, sperm with high motility and normal morphology should move through the microchannel toward the outlet. The diameter of the inlet was 1.5 *mm*, which fitted the tip of a micropipette to make it easier to inject the raw semen, and that of the outlet was 3 *mm* to allow for capillary flow, making it simple to collect sorted sperm. The actual image of the device is shown in figure 1B. The morphology of the micropillars observed using scanning electron microscopy is shown in figure 1C.

Device fabrication: The microfluidic chip was fabricated using photolithography and soft lithography. Polydimethylsiloxane (PDMS, Sylgard 184; Dow Corning Corp, USA) as a biocompatible material was used to fabricate the chip owing to its elasticity, transparency, gas permeability, nontoxicity, inexpensive material composition, easy disposability, and safety for use with reproductive cells. The device was fabricated using the following procedure:

1) to create a master mold, a 50-*μm*-thick SU-8 photoresist layer (MicroChem, Germany) was coated on a quartz glass using a spin coater; 2) the coated substrate was exposed to ultraviolet light using direct laser-lithography (Dilase 250; KLOE, France) according to the design; 3) the patterned substrate was developed using the SU-8 developer and rinsed with isopropanol to obtain the master mold; 4) to fabricate a PDMS-based microfluidic chip, a liquid PDMS prepolymer and its corresponding hardener were mixed at a ratio of 10:1 (*w/w*) and poured into the master mold, degreased, and cured for 2 *hr* at 65°*C*; 5) after curing, the inlet and outlet chambers were punched using the tip of a biopsy punch (1.5 and 3 *mm*); and 6) the microfluidic channel was bound on a glass slide using a plasma cleaner (Harrick Plasma, USA), followed by baking at 80°*C* for 30 *min* to improve adhesion to the substrate before use (Figure 2).

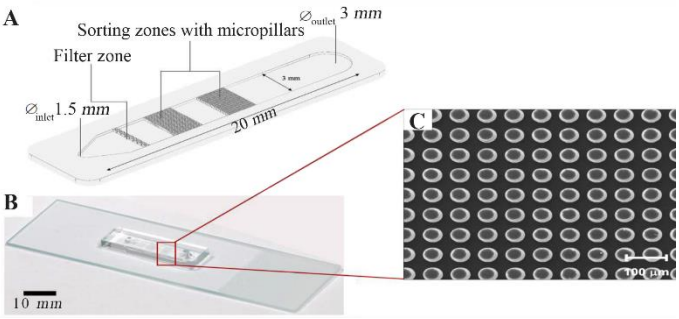

Figure 1. Microfluidic chip model (A) micropillar pattern within the microchannel with three zones, including staggered arrangement and aligned arrangement of circular columns; (B) the actual image of the microfluidic device; and (C) micropillar arrays observed using scanning electron microscopy

Semen analysis: Sperm parameters were evaluated using a computer-assisted sperm analysis (CASA) system (IVOS II; Hamilton Thorne, USA) according to WHO guidelines (5th edition). Sperm motility was analyzed by placing 6 *μl* of sample on a slide and inserting it into the CASA system. Sperm morphology was analyzed by air-drying the sample on a glass slide, and then staining it with Diff-Quick stain. The CASA system was used to analyze the sample at a magnification of 40×. For comparative analysis, each fresh semen sample was aliquoted into three subsamples as follows:

1) an unprocessed sample, 2) a sample for DGC processing, and 3) a sample for microfluidic processing.

Sperm preparation using DGC: DGC was performed with two layers—1 *ml* of 80% Sil-Select

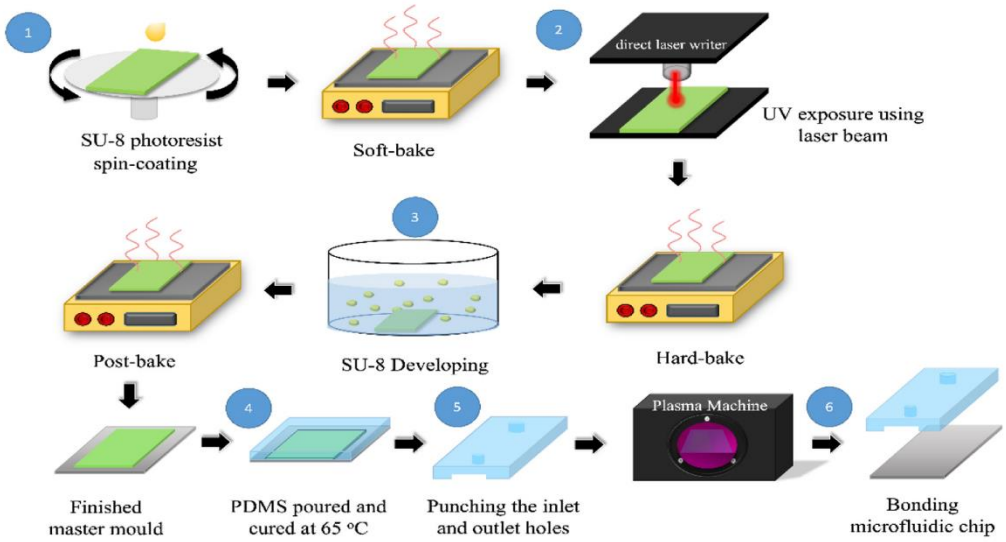

Figure 2. Fabrication process of sperm-sorting microfluidic chips using photolithography for our designed master mold (steps 1–3) and soft lithography for a PDMS-based microfluidic chip (steps 4–6). PDMS, polydimethylsiloxane

STOCK™ solution (Fertipro NV, Belgium) at the bottom and 1 *ml* of 40% Sil-Select STOCK™ solution on the top. A sample of 1 *mm* was then layered on top of the solution and centrifuged at 300× *g* for 15 *min* at room temperature (approximately 25°*C*). After removing the supernatant, the pellet was placed in a new conical tube with 4 *ml* of sperm washing medium (Gibco F-10 Nutrient Mixture; Gibco, USA) and then centrifuged at 300× *g* for 15 *min* at approximately 25°*C*. After removing the supernatant again, the pellet was washed with 4 *ml* of sperm culture medium and centrifuged at 400× *g* for 5 *min* at approximately 25°*C*. Finally, 0.5 *ml* of the suspended pellet was analyzed using the CASA system and subjected to DNA fragmentation assessment.

Sperm preparation using the microfluidic method: After manually filling the microfluidic device with sperm washing medium (Gibco F-10 Nutrient Mixture) using a micropipette, 2 *µl* of unprocessed semen was gently added into the inlet. The device was then placed in an incubator for 30 *min* at 37°*C*. Finally, the sperm were collected at the outlet and subjected to analysis using the CASA system and DNA fragmentation assessment.

Sperm DNA fragmentation analysis: DNA fragmentation was detected using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (Guava TUNEL Kit; Luminex, USA). Sperm were washed with cold phosphate-buffered saline, fixed with 4% paraformaldehyde, and then incubated for 1 *hr* at 4°*C*. Subsequently, ice-cold 70% ethanol was added, and then the samples were stored at −20°*C* for a minimum of 16 *hr*. The fixed samples were centrifuged at 300× *g* for 10 *min*. The supernatant was removed, and the pellets were washed with buffer and then centrifuged at 300× *g* for 10 *min*. After removing the supernatant, the DNA was labeled with a master mix of terminal deoxynucleotidyl transferase (TdT) enzyme reaction buffer, TdT enzyme, 5-bromo-2′-deoxyuridine 5′-triphosphate (BrdU), and sterile water, and incubated at 37°*C* for 1 *hr*. Rinsing buffer was added to the sample, which was then centrifuged at 300× *g* for 10 *min*. Next, a staining master mix of anti-BrdU containing anti-BrdU-TRIC and rinsing buffer was added to the sample; thereafter, the sample was incubated for 30 *min* in the dark at room temperature (approximately 25°*C*). Following incubation, rinsing buffer was added, and the sample was centrifuged at 300× *g* for 10 *min*. Fluorescent labeling was used to identify fragmented sperm DNA. The specimens were placed over a SuperFrost Plus glass slide, and 4′,6-diamidino-2-phenylindole (DAPI) counterstaining was used to identify other cells. In total, 200 sperm were counted from each specimen to calculate the DNA fragmentation index (DFI) under a fluorescence microscope.

Statistical analysis and data representation: Statis-

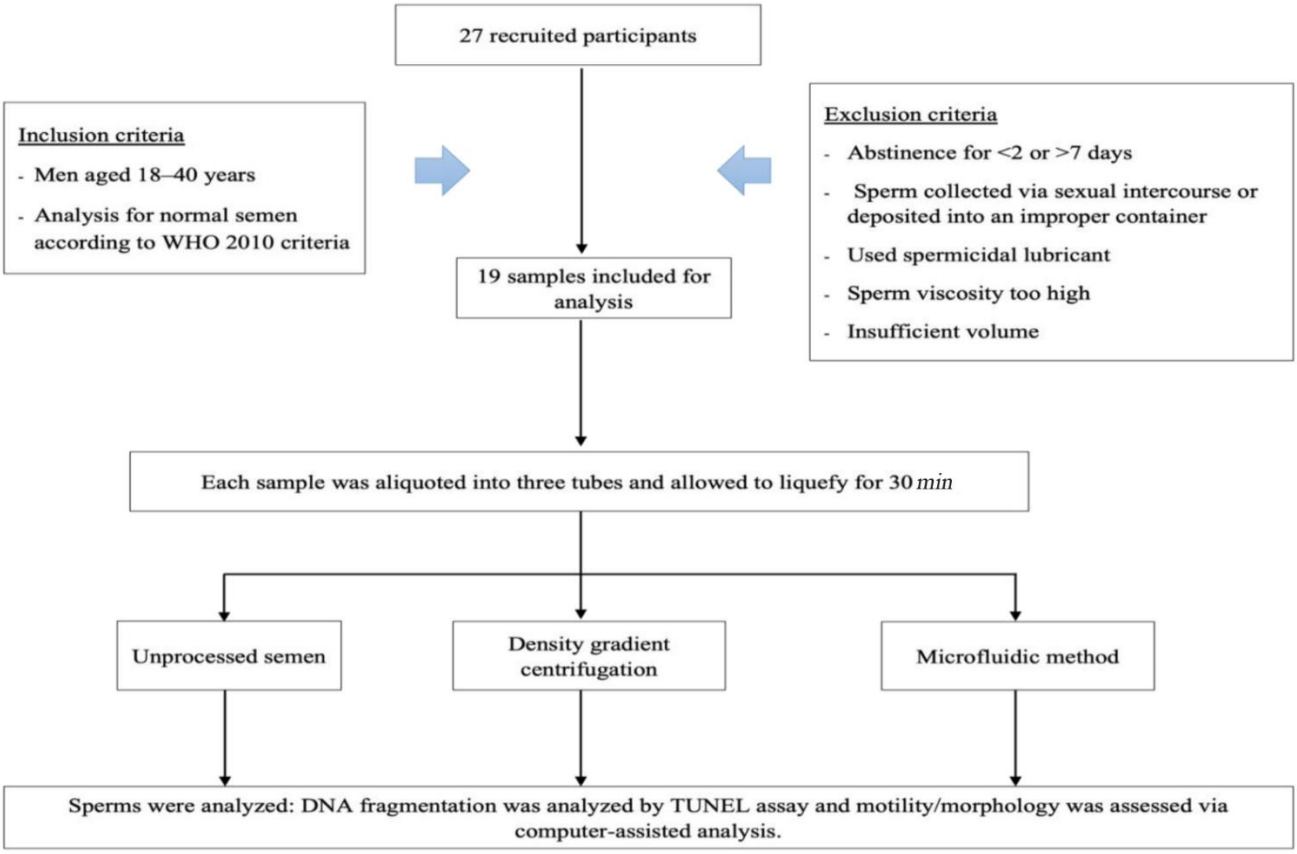

Figure 3. Flowchart outlining the study protocol. WHO, World Health Organization

tical analyses were performed using Stata software *vs*. 14.2 (StataCorp, USA), with baseline characteristics reported as mean±SD. Primary and secondary outcomes were analyzed using the mixed-effect Poisson regression for counted variables and mixed-effect linear regression for continuous variables. A concentration model was developed using log transformation. Statistical significance was determined at $p<0.05$. Figure 3 shows a flowchart outlining the study procedure.

Table 1. Baseline characteristics of the study population

Parameter	Mean±SD (range) (n=19)
Age (years)	29±3.0 (20–40)
Sperm concentration ($\times10^6$/*ml*)	85.7±24.3 (40.6–141)
Total motility (%)	70.8±14.4 (41.6–91.4)
Progressive motility (%)	62.4±14.7 (34–86)
Normal morphology (%)	5.3±1.6 (4–10)
Sperm DNA fragmentation (SDF) (%)	4.9±3.6 (0.47–16.5)

Results

Participant characteristics and baseline semen analysis: After applying the exclusion criteria, 19 participants were enrolled in this study (mean age of 29±3 years). All 19 submitted semen samples were considered normal per the WHO criteria (21). Table 1 lists the basic demographic characteristics of the participants and initial semen analysis results.

Sperm DNA fragmentation: After the TUNEL assay, DNA fragmentation was detected using fluorescence microscopy. Here, a minimum of 200 sperm from each sample were scored using light microscopy. As shown in figure 4, sperm stained with DAPI (blue) were first counted, followed by cells that emitted a red fluorescent signal (TUNEL-positive, highlighted with yellow circles). Figure 4A and 4B show sperm obtained from unprocessed semen, sperm obtained using density gradient centrifugation (Figure 4C, 4D),

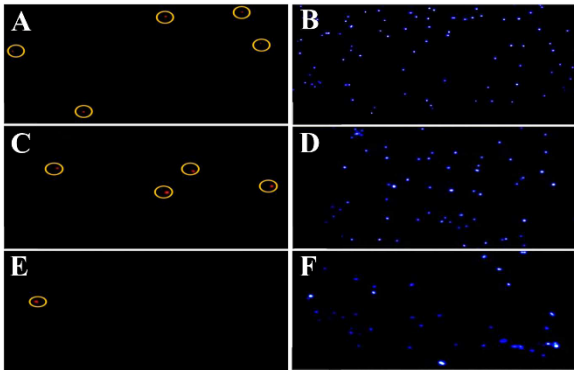

Figure 4. Detection of DNA fragmentation using the TUNEL assay with fluorescence microscopy. Sperm cells stained with DAPI (blue) were counted first, followed by cells that emitted red fluorescence (TUNEL-positive, highlighted with yellow circles); (A, B) sperm from unprocessed semen, (C, D) sperm obtained using density gradient centrifugation, and (E, F) sperm obtained using the microfluidic method. TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling

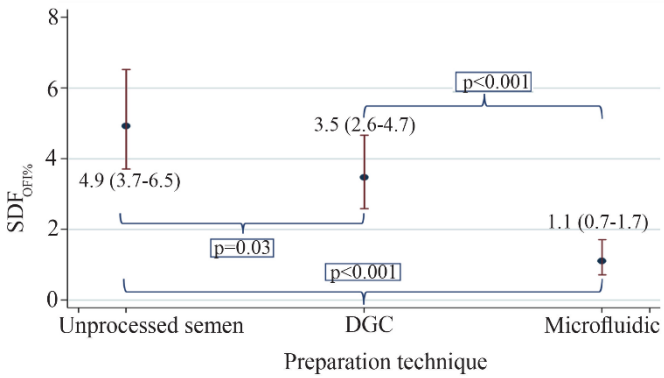

Figure 5. Sperm DNA fragmentation (SDF) in unprocessed semen, semen processed using density gradient centrifugation (DGC), and semen processed using the microfluidic method. Data are presented as mean (95% confidence interval). Intergroup comparisons were performed using the Poisson regression model (statistical significance determined at $p<0.05$)

and sperm obtained using the microfluidic method (Figure 4E, 4F). The number of positive tests was assessed as the percentage of the total count. Sperm cells collected using the microfluidic method showed a significantly lower SDF than those collected using DGC and in the unprocessed semen, median SDF values were 1.1%, 3.5%, and 4.9%, respectively (Figure 5).

Sperm concentration, motility, and morphology: Both DGC and the microfluidic method yielded a lower sperm concentration than that in fresh semen. Although the microfluidic method yielded a significantly lower sperm concentration than DGC, there were no significant differences in total motility, progressive motility, curvilinear velocity, straight-line velocity, or normal morphology among the samples (Table 2).

Discussion

In this study, a microfluidic device, designed and fabricated in-house, was successfully developed that yielded sperm with considerably lower SDF values in comparison to sperm obtained from unprocessed semen or those obtained using DGC. This finding reinforces the efficacy of microfluidic devices in sperm selection, consistent with the findings of previous studies (15, 18-20), which used microfluidic devices and reported lower SDF values in comparison to those in which sperm were obtained using the swim-up technique involving centrifugation. Moreover, this finding highlights the efficacy of our device's unique mechanism, which guides activated sperm towards the outlet zone using microchannel geometries. Our microfluidic device worked by leveraging sperm-swimming behavior and competition-

Table 2. Semen parameters before and after density gradient centrifugation (DGC) and after using the microfluidic method

Parameter	Before DGC	After DGC	After using the microfluidic method
Concentration ($\times10^6$/*ml*)	81.40 (56.83–117.92) [a]	19.30 (13.33–27.67) [b]	0.54 (0.37–0.77) [c]
Total motility (%)	70.78 (64.31–77.26) [a]	92.18 (89.80–94.57) [b]	91.52 (86.49–96.55) [b]
Progressive motility (%)	62.41 (55.82–69.01) [a]	89.22 (86.18–92.23) [b]	87.93 (82.64–93.22) [b]
Curvilinear velocity (*μm/s*)	70.65 (60.61–81.14) [a]	114.62 (104.13–125.118) [b]	118.14 (107.64–128.63) [b]
Straight-line velocity (*μm/s*)	32.82 (27.91–37.74) [a]	63.51 (58.60–68.43) [b]	64.73 (59.82–69.65) [b]
Normal morphology (%)	5.33 (4.59–6.06) [a]	10.45 (7.84–13.06) [b]	11.03 (7.58–14.47) [b]
Sperm DNA fragmentation (SDF) (%)	4.9 (3.7–6.5) [a]	3.5 (2.6–4.7) [b]	1.1 (0.7–1.7) [c]

Notes: Data are presented as mean (95% confidence interval). Superscript letters (a–c) indicate significant differences between subgroups

based motility for sperm selection. Hence, sperm with abnormal morphology and low motility were unable to reach the outlet in the microchannel, whereas highly motile sperm could reach the outlet.

Our device had a simple fabrication process which was designed in-house; moreover, it was easier to use than devices currently commercially available. Notably, the microfluidic method used in this study produced sperm with motility similar to that of sperm obtained using DGC (18, 19). Thus, similar percentages of motile sperm were recovered using microfluidic and DGC methods, demonstrating comparability with widely implemented standardized techniques. Following the Kruger's strict criteria, the in-house microfluidic chip also produced sperm with normal morphology at a rate of approximately 10%, which is equivalent to that achieved with DGC. Owing to the passive-dependent mechanism and short sorting time of sperm, less DNA damage from the sperm preparation process could be expected. This method caused only 1.1% of median SDF in the sperm. A similar finding was reported in a previous study that used a microfluidic device containing micropillars; the results demonstrated better outcomes than the swim-up method, leading to low SDF and greater number of sperm with high motility and normal morphology (15). Another study also demonstrated a higher number of sperm with low SDF and good motility which were recovered after sorting using a microfluidic device based on motility and thigmotaxis (24).

The in-house microfluidic device constitutes an ideal sperm selection method for use in ARTs. As demonstrated in this study, it is simple to use, requires less time, does not entail intensive labor, and consistently selects sperm with low SDF, high motility, and normal morphology. Currently, DGC and swim-up are the most commonly used methods of sperm preparation before application in in vitro fertilization and intracytoplasmic sperm injection. However, the DNA integrity of motile sperm recovered through these methods may be compromised (25). A recent study showed that 20–50% of viable sperm obtained using the DGC and swim-up methods exhibited SDF (26). In contrast, the microfluidic method produced considerably less SDF, with values ranging between 4–15% in a previous study (15). Notably, the in-house microfluidic chip used in the present study showed an SDF value of only 1.1% after complete sperm sorting. This finding is important in the context of clinical practice, as DNA damage in sperm cannot be detected before insemination. Several studies have shown that SDF adversely affects ART outcomes, resulting in low fertilization rates (8, 27), poor blastocyst development (28, 29), low implantation rates (8, 30), and high miscarriage rates (10, 11, 13, 30, 31). In contrast, accumulating evidence indicates that the microfluidic method can be used to isolate sperm with low SDF rates (15, 19, 20), resulting in improved ART outcomes.

Conclusion

The in-house microfluidic device described herein can successfully isolate sperm with considerably less SDF than those with DGC, with comparable motility and normal morphology rates between the samples. Regarding other important aspects, the device is not complicated to operate, labor-intensive, nor time-consuming, and is cost-effective owing to its unique in-house design and massive-scale fabrication process. However, the current design is limited by its microscale qualities, which result in a low number of recovered sperm. Consequently, the device is more suited to intracytoplasmic sperm injection. In the future, the design may be improved to select a higher number of sperm for use in either in vitro fertilization or intrauterine insemination.

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Conflict of Interest

The authors declare that they have no competing interests.

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