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A novel microfluidic device for human sperm separation based on rheotaxis

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Research Article

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Abstract

This study explores the efficacy of a novel microfluidic device in isolating rheotactic sperm and assesses their advantages compared with other motile sperm. Two microfluidic devices were used in this study: the microfluidic device we designed to separate sperm based on rheotaxis and a simple passive microfluidic device. We compared the results with the density gradient centrifugation technique. Sperm attributes including concentration, morphology, viability and motility were assessed using related procedures. Statistical analyses were conducted using one-way analysis of variance. Results showed differences in sperm concentration, motility, morphology and vitality using different sperm separation techniques. The sperms separated using our microfluidic device demonstrated the highest motilities, normal morphology percentages and higher sperm vitality but significantly lower sperm concentrations. These findings suggest the potential of our microfluidic design in enhancing sperm quality. Our findings are in agreement with previous research, emphasizing the capability of microfluidics in enhancing sperm quality. Specifically, our designed microfluidic device exhibited exceptional efficacy in isolating highly motile sperm, a critical factor for successful fertilization.

Introduction

Infertility is a prevalent issue affecting approximately 70 million couples of reproductive age. Male factors alone account for 30% of infertility cases, while a combination of male and female causes contributes to 50% (Yan *et al.*, 2020). It is not possible to simultaneously assess all factors that may cause infertility. Furthermore, routine evaluations including sperm concentration, motility and morphology do not always provide accurate predictions regarding the success rates of natural fertility or assisted reproductive technology (ART) treatments.

The technology in the field of reproductive biology is yet emerging. Since the landmark birth of Louise Brown in 1978, the field of infertility treatments has undergone a remarkable journey spanning over four decades, marked by a persistent pursuit of improvement despite encountering many challenges (Alias *et al.*, 2021). Researchers have made substantial progress in overcoming infertility, including introducing new culture systems, improving the quality and adaptability of culture media, developing medications to stimulate ovulation and enhancing our understanding of ovulation mechanisms to boost egg quality (Gardner and Lane, 2017; Gardner *et al.*, 2012). Over the years, researchers have actively strived to create *in vitro* conditions that closely emulate *in vivo* environments.

Microfluidics, the scientific discipline dedicated to studying and manipulating fluids on a microscale, empowers us with extraordinary precision in governing spatiotemporal dynamics within this realm (Whitesides, 2006). Since the introduction of microfluidics in sperm isolation in 2003 (Schuster *et al.*, 2003), this knowledge has made its way into reproductive biology. Subsequent efforts have focused on utilizing this technology in various areas, such as sperm isolation (Ataei *et al.*, 2021; Nagata *et al.*, 2018; Nosrati *et al.*, 2014), oocyte preparation for fertilization (Han *et al.*, 2010), fertilization itself (Huang *et al.*, 2015), embryo culture (Le Gac and Nordhoff, 2017), embryo cryopreservation (Guo *et al.*, 2019), sperm analysis (Wu *et al.*, 2017) and even modelling an artificial uterus (Ahn *et al.*, 2021; Nosrati *et al.*, 2017). Reports on sperm isolation using microfluidic methods suggest that sperm isolated through these techniques exhibit improved morphology and DNA health (Gonzalez-Castro *et al.*, 2018; Parrella *et al.*, 2019; Shirota *et al.*, 2016; Wei-Xuan *et al.*, 2013). Since conventional sperm washing methods involve centrifugation and carry the risk of sperm damage, microfluidic-based sperm separation has gained significant attention (Twigg *et al.*, 1998).

In the physiological environment of the uterus, sperm cells must actively navigate against fluid flow to reach the oocyte. This counterflow movement is known scientifically as *rheotaxis* (Zhang *et al.*, 2016). Several studies have endeavoured to employ microfluidics to isolate rheotactic sperm and evaluated the characteristics of isolated sperm (Ataei *et al.*, 2021). Until

now, the main challenge in isolating rheotactic sperm has revolved around developing a chip capable of efficiently segregating a substantial quantity of sperm cells from a heterogeneous pool characterized by diverse traits. This study presents the design and fabrication of a novel microfluidic chip to isolate a significant proportion of rheotactic sperm effectively. We evaluated some capabilities and characteristics of the isolated sperms. We analysed the sperm separated using our rheotactic sperm separator microfluidic (RSM) device, a simple passive microfluidic device (PMD) and density gradient centrifugation (DGC) as a conventional method.

Materials and methods

Sample collection and ethics

Semen samples were obtained from male patients visiting the andrology department at Avicenna Centre for Infertility and Recurrent Miscarriage Treatment in Tehran, following a period of abstinence ranging from 2 to 5 days. These samples consisted of the remaining semen following routine clinical assessments and were collected exclusively for research purposes. Informed consent was obtained from all participants prior to sample collection in accordance with established ethical guidelines.

To ensure consistency and reliability in the study, we applied strict inclusion criteria for the selection of sperm samples. Only samples meeting the following criteria were included: a. sperm concentration exceeding 50×10^6/ml, b. normal morphology exceeding 1%, c. progressive motility exceeding 30%, d. leukocyte count below 1×10^6 /ml, e. absence of sperm aggregates. To mitigate potential confounding factors, we excluded patients based on several criteria: a. smoking, b. alcohol consumption, c. hormonal disorders, d. age over 40.

The study design was subjected to rigorous ethical review and was approved by the esteemed Ethics Committee of Avicenna Research Institute, ensuring that all experimental procedures adhered to the established ethical guidelines and regulations.

Experimental design

Ten semen specimens were utilized in this study. Each specimen was divided into three portions, with each portion subjected to one of three different sperm separation techniques: two distinct microfluidic devices and DGC. After the separation, each sample attributes were quantified and compared with the unprocessed semen. Concentration, morphology, viability and motility along with specific sperm motility parameters (Figure 1) were evaluated.

Microfluidic device design and fabrication

The device geometry was designed using CorelDRAW. The design was then imported into COMSOL Multiphysics to simulate fluid flow. The design parameters were changed to reach the fluid velocity of 50–90 µm/s at the centre of the designated rheotactic area. Subsequently, the final design was printed on a transparency to form the UV-lithography mask.

To fabricate the mould, a Si wafer was cleaned followed by deposition of a uniform 80-µm layer of SU-8 photoresist using a spin coater. The wafer was then pre-baked at 65ºC and 90ºC. The wafer was exposed to UV using a mask aligner to transfer the designed pattern into the SU-8 layer. A post-exposure bake was then conducted at 65ºC and 90ºC to complete the polymerization process. The wafer was then placed in the developer so that the unexposed photoresist was removed. After the cleaning using isopropanol and DI water, the mould was ready to be used.

Soft lithography was then used to make the devices out of polydimethylsiloxane (PDMS). The elastomer and curing agent were mixed (10:1 ww) and degassed to remove air bubbles. The degassed mixture was then poured onto the prepared mould covering the entire surface evenly. The process was continued by curing PDMS at 70°C for 3 h. The cured PDMS layer was carefully peeled off from the mould. The extra parts were cut out, and the inlet and outlet ports were punched to provide the way for the introduction of samples into the devices and taking out the processed samples. The prepared PDMS layer was then bonded to a glass substrate using oxygen plasma.

The design of the RSM device comprised several key components, including a single bifurcating channel for semen flow, four containers dedicated to collecting mixed sperm samples (waste), four rheotactic areas and one container at the centre designed explicitly for collecting rheotactic sperm (Figure 2). All ports are 8 mm in diameter and 3 mm high. Rheotactic areas are 500 µm wide and sperm entry channels are 100 µm wide. All internal parts are 70 µm high. The device design incorporated four rheotactic areas to facilitate rapid sperm separation and enhance efficiency to achieve higher sperm concentrations.

The PMD was designed based on FERTILE© containing a wide channel bridging the inlet and outlet. The channel is 1.5 cm long, 5 mm wide and 50 µm high. Notably, the device encompasses multiple identical channels to increase the amount of output.

Sperm separation using density gradient centrifugation (DGC)

DGC is a widely used technique in ART for sperm preparation and selection, enabling the separation of highly motile and morphologically normal spermatozoa from the rest of the semen sample, the procedure was reported earlier by Gode and colleagues (Gode *et al*., 2019). Briefly, a density gradient was established using two layers of PureSperm© (Nidacon, Sweden), the denser (80%) at the bottom and the 40% layer on top, each 1 ml, diluted in Ham's F10 medium containing 0.5% human serum albumin (HAS). The semen sample, after liquefaction, was carefully layered on top of the upper layer of the density gradient. The sample was centrifuged at 300 *g* for 15 min. During this process, motile and morphologically normal sperm swim down through the gradient. In contrast, abnormal or dead sperm, cellular debris and immotile sperm remain in the upper layers due to their inability to penetrate the denser medium. Post-centrifugation, the lower portion of the bottom layer, which contains the highest quality sperm, was aspirated into a 5 ml centrifugation tube without disturbing the other layers. The separated sperm fraction was then typically washed with the Ham's F10 medium by centrifuging again to remove any remaining gradient medium. The supernatant was discarded, and the sperm pellet was resuspended in the Ham's F10 medium.

Sperm separation using RSM

For the device to reach equilibrium, it was subjected to 37°C, 5% CO_2 and 99% humidity within an incubator for 1 h before utilization. Subsequently, Ham's F10 culture medium, supplemented with 0.5% HAS, was introduced into the channels using a 2 ml syringe and a 2 cm long silicone hose connected to the semen sample introduction reservoir. The excess medium within the reservoir was carefully aspirated.

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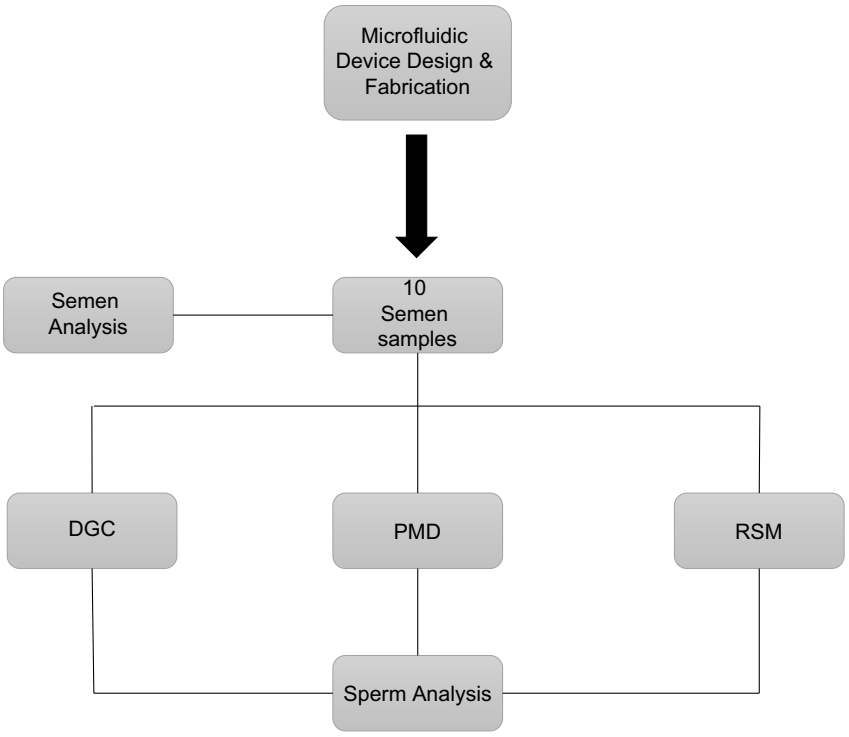

Figure 1. Experimental design. Ten semen specimens were included in this study. Each specimen was divided into three aliquots, and each aliquot was subjected to one of three different sperm separation techniques: two distinct microfluidic devices and density gradient centrifugation (DGC). Following separation, the characteristics of each sample were analysed and compared with the unprocessed semen. Parameters such as concentration, morphology, viability, motility and specific sperm motility indices were assessed.

150 µl of Ham's F10 medium was added to all four waste sperm reservoirs using a micropipette followed by the addition of 200 µl of the medium to the sperm collection reservoir, establishing a gentle outgoing flow. This was followed by the addition of 400 µl of semen into the silicone hose connected to the semen sample introduction reservoir. The silicone hose was used to increase the speed of fluid flow in the rheotactic area (50–90 µm/s), which was achieved by increasing the fluid pressure inside the inlet.

The microfluidic device was then examined via an inverted microscope (Olympus IX70, Japan) to confirm the successful establishment of a rheotaxis flow of sperm cells (Video S1). After the inspection, the device was again placed within the incubator for 45 min.

Afterwards, 150 µl of the culture medium was collected from the sperm collection reservoir and set aside for subsequent analyses.

It is paramount that the introduced semen sample is completely free of sperm aggregates and leukocytes. Additionally, the sample must be fully liquefied and free of gelatinous components. Adherence to these criteria ensures both the optimal operation of the microfluidic device and the accuracy of the resultant data.

Sperm separation using PMD

After achieving equilibrium in the device, the PMD was primed by injecting 25 µl of Ham's F10 medium into the inlet, followed by the injection of 10 µl of raw semen into the inlet of each designated channel. To prevent media evaporation, the inlets and outlets were sealed with light mineral oil. Subsequently, the device was incubated for 45 min. Following incubation, 10 µl of sperm-rich medium was collected from the outlet of each channel for the subsequent evaluation of sperm quality.

Sperm motility analysis

A computer-assisted sperm analysis (CASA) system was employed for the evaluation of sperm motility. For the analysis, a drop of the fully liquefied semen sample was positioned onto a Makler counting chamber purposed for the CASA system using a micropipette. Formation of air bubbles should be avoided to prevent the possible interferences. The prepared Makler chamber was then installed onto the microscope stage that was interfaced with the CASA software. The software had been pre-programmed to capture multiple fields of view to furnish a representative sample evaluation. It calculated a spectrum of parameters, which included total and progressive motility percentage, average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement, beat-cross frequency (BCF), straightness (STR) and linearity (LIN). The CASA system was carefully calibrated prior to the study to ensure precise measurement of sperm motility parameters.

Sperm concentration assessment

To assess the concentration of sperm, a Neubauer chamber, a type of haemocytometer, was utilized. A careful 1:10 dilution of the semen was then prepared for raw semen and DGC groups, using sodium bicarbonate formalin to prevent further sperm motility that could influence the counting.

Once the sample was adequately mixed, 10 µl of the diluted semen or separated semen in the case of microfluidic devices was gently loaded into the Neubauer chamber using a micropipette and subsequently covered with a coverslip, allowing the sperm to settle for a few minutes.

The chamber was then positioned onto the microscope stage, and the sperm cells within the 1 mm^2 square (divided into 25 smaller squares) were counted at 400× magnification. The average number of sperm cells in five of the 0.04 mm^2 squares was used to calculate the overall concentration per ml of the original semen sample. This was done by multiplying the average count per square by the dilution factor and then by 25,000 (the conversion factor for the counting chamber) (Asare-Anane *et al.*, 2016).

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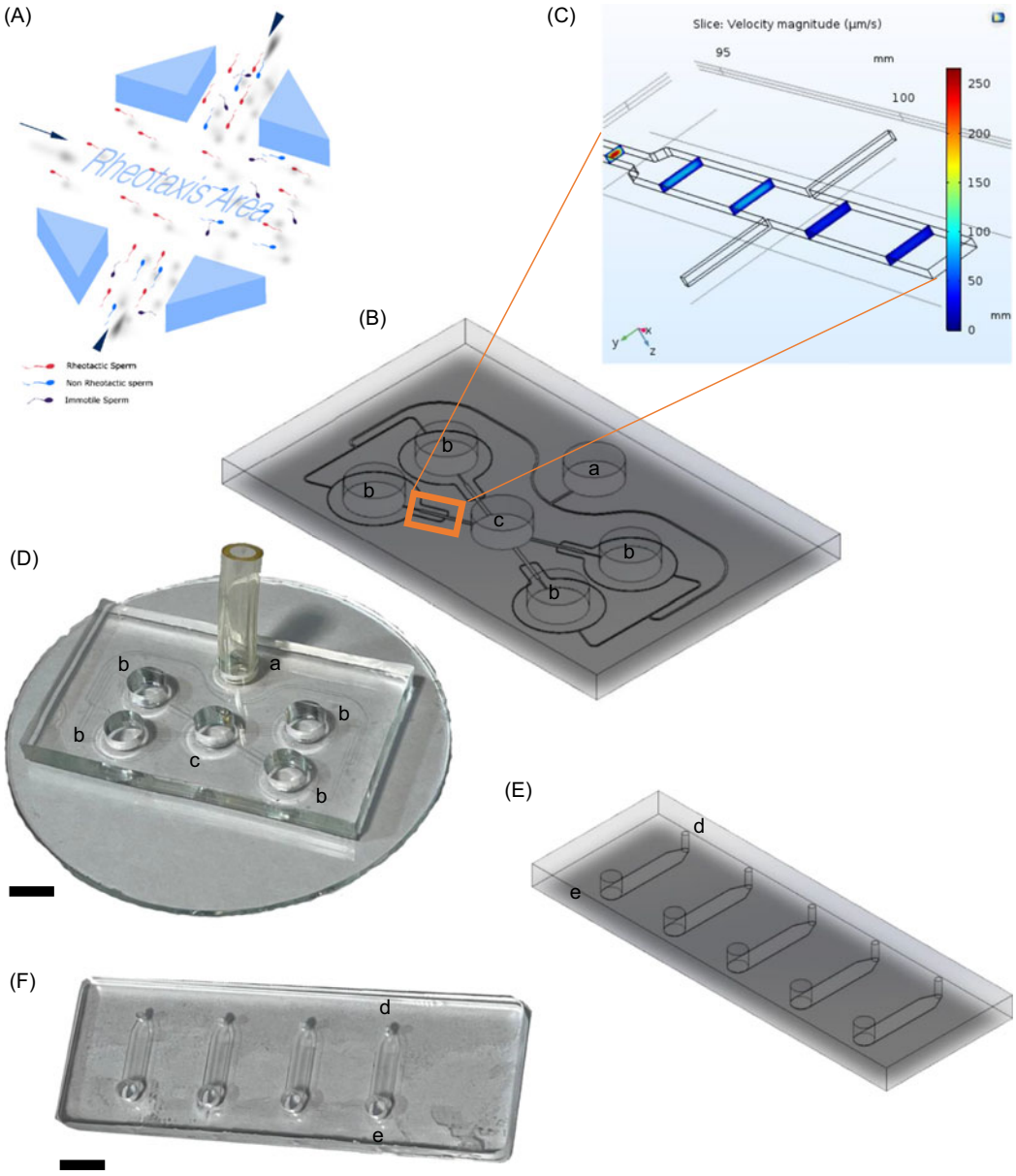

Figure 2. Operation and simulation of microfluidic devices. (A) Schematic picture of sperm entry and separation inside each rheotactic area. The arrow shows the direction of fluid flow and the arrowheads show the mixed sperm entry into the rheotactic area. (B) Schematic picture of the rheotactic sperm separator microfluidic (RSM) device. a, b and c show semen introduction reservoir, waste sperm collection reservoir and rheotactic sperm collection reservoir, respectively. The magnified box shows one of four rheotactic areas. (C) Three-dimensional simulation of fluid flow velocity in the rheotactic zone, revealing speeds ranging from 50 to 90 µm/s in the central region of the channel at the rheotactic sperm separation zone. (D) Actual image of the RSM device. a, b and c show semen introduction reservoir, waste sperm collection reservoir and rheotactic sperm collection reservoir, respectively. The silicone hose is inserted into reservoir a, to increase the fluid pressure and speed. (E) Schematic picture of passive microfluidic device (PMD). The device contains multiple channels to increase the output volume. d and e show the introduction and collection reservoirs, respectively. (F) Actual image of PMD. d and e show the introduction and collection reservoirs, respectively. Scale bars are equal to 1 cm.

Sperm morphology analysis

Sperm morphology was evaluated using the Diff Quik staining method (Moghadam *et al.*, 2019), a rapid staining procedure widely used for such analyses.

Once collected, the semen samples were allowed to liquefy at 37°C for approximately 30 min. Following complete liquefaction, a smear was prepared by placing a small drop of the semen on a clean, grease-free glass slide. The sample was spread thinly across the slide with the edge of another slide, ensuring a uniform distribution of sperm cells. After this step, the smeared slide was air-dried for about 30 min at room temperature.

The staining procedure was initiated once the smear was fully dried. The slide was first immersed in the Diff Quik fixative solution for approximately 5 min. The slide was then rinsed gently with distilled water and allowed to be air-dried briefly.

Following the initial fixation step, the slide was stained with the Diff Quik eosinophilic (red/pink) solution for 30 s and rinsed again with distilled water. After a brief air-drying period, the slide was immersed in the Diff Quik basophilic (blue) solution for another 30 s, followed by another rinse with distilled water. The slide was then air-dried thoroughly.

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The stained sperm samples were then examined under a light microscope (100× oil immersion objective). Sperm morphology was evaluated based on the standards outlined by the World Health Organization, focusing on characteristics such as sperm head morphology, midpiece structure, tail morphology and the presence of cytoplasmic droplets.

Sperm viability assessment

The vitality of sperm was determined utilizing the one-step Eosin-Nigrosin staining method described by Björndahl and colleagues (Björndahl *et al.*, 2003). Following collection, the samples were permitted to liquefy at 37°C for 30 min. A mixture was subsequently prepared by combining an equal volume of the semen sample and the premixed Eosin-Nigrosin stain to ensure adequate staining of sperm cells.

A 10 µl drop of the stained semen mixture was then placed onto a clean, grease-free microscope slide, and a cover slip was carefully placed over the drop and assessed using a 400× microscope objective immediately. Sperm cells appearing pink were considered nonviable as they had absorbed the Eosin stain due to compromised plasma membrane integrity. In contrast, viable sperms were identified by their resistance to staining, indicative of intact plasma membranes. A total of 200 spermatozoa were assessed from each experimental group, and results were reported as the ratio of live spermatozoa to the cumulative count, expressed as a percentage.

The Eosin-Nigrosin stain used in this study consisted of two main dyes: Eosin, a red acidic dye, and Nigrosin, a dark blue or black acidic dye. In this staining procedure, these two dyes were premixed into a single solution. The relative concentrations of Eosin and Nigrosin in the stain involved a formulation of 1% Eosin and 10% Nigrosin combined in an aqueous 9% NaCl solution.

Statistical analysis

The statistical analysis of the datasets was conducted using SPSS software (version 16.0; SPSS Inc., Chicago, Illinois, USA). Initially, the Kolmogorov–Smirnov test was employed to assess the normality of distribution for each dataset. If the data exhibited a normal distribution, a one-way analysis of variance was employed to compare mean values among different groups. Subsequently, *post hoc* tests were performed for specific pairwise comparisons. In cases where the data did not follow a normal distribution, the nonparametric Kruskal–Wallis test was used.

The results are presented as mean values along with their corresponding standard deviations. Statistical significance was determined at a p-value threshold of less than 0.05.

Results

Sperm concentration

In the present investigation, the examination of sperm concentration revealed different outcomes across the four study groups. In the raw semen samples (RS), the mean sperm concentration was documented at 70.06 ± 20.51 million sperm/ml. This initial concentration was substantially attenuated following the application of the DGC method, where the mean concentration was 20.68 ± 10.07 million sperm/ml. Furthermore, sperm separation using the PMD and RSM device produced even lower mean concentrations of 4.2 ± 1.14 million sperm/ml and 1.95 ± 0.89 million sperm/ml, respectively. Evidently, using microfluidic sperm separation results in lower yields of sperm concentration where the RSM device led to the most substantial reduction (Table 1).

Sperm motility and trajectory

In the evaluation of sperm motility, profound disparities became discernible across the different groups under study: RS, DGC, PMD and RSM device. The total motility percentage in raw semen, DGC, PMD and RSM exhibited averages of 33.96 ± 8.42, 64.19 ± 14.91, 66.35 ± 16.87 and impressively 91.99 ± 8.7%, respectively. These distinctions became even more palpable in examining the progressive motility percentage, where raw semen, DGC, PMD and RSM recorded mean values of 31.32 ± 7.10, 59.49 ± 14.95, 60.26 ± 18.10 and 88.29 ± 10.04%, respectively. When assessing the rapid progressive motility percentage, raw semen, DGC, PMD and RSM recorded mean values of 19.85 ± 5.43, 36.04 ± 15.00, 36.54 ± 19.47 and notably 72.22 ± 10.99%, respectively.

Digging deeper into the assessment of sperm trajectories, the examination of VCL yielded averages of 15.84 ± 5.10, 35.88 ± 6.90, 37.91 ± 8.83 and 53.81 ± 3.04 µm/s for raw semen, DGC, PMD and RSM, respectively. VSL had mean values of 7.97 ± 2.31, 17.38 ± 5.67, 18.65 ± 7.53 and 31.43 ± 6.39 µm/s across raw semen, DGC, PMD and RSM, respectively. The VAP in the four groups corresponded to mean values of 10.51 ± 3.34, 22.46 ± 5.61, 24.06 ± 7.44 and 36.44 ± 4.85 µm/s, respectively. BCF demonstrated average values of 1.96 ± 0.55, 4.63 ± 1.36, 5.38 ± 2.13 and 8.33 ± 1.43 Hz for raw semen, DGC, PMD and RSM, respectively. LIN showed mean values of 17.44 ± 3.58, 32.88 ± 11.48, 34.17 ± 14.21 and 56.50 ± 11.97% across the groups. Lastly, STR came out with averages of 24.94 ± 5.13, 48.70 ± 13.84, 50.09 ± 15.72 and 78.41 ± 7.68% for raw semen, DGC, PMD and RSM, respectively.

The results revealed that applying the RSM method for sperm separation led to the acquisition of sperm of demonstrably superior performance in all measured motility parameters. It became evident that this method facilitated the selection of sperm with outstanding motility and trajectory. A more detailed overview of the motility and trajectory parameters across the experimental groups is depicted in Table 1.

Sperm morphology

Using Diff Quik staining procedure, the disparities in sperm morphology among the four designated cohorts were analysed. The raw semen, RS, demonstrated a slender 2.3 ± 1.41% of spermatozoa exhibiting a normal morphology. A marked elevation was noted in the DGC group, with an average of 5.4 ± 2.11% spermatozoa showing normal morphology. After the PMD process, this average ascended to 8.2 ± 2.29%. On the contrary, the RSM process produced a significantly superior result, with 17.1 ± 3.95% of spermatozoa showcasing normal morphology (Table 1).

Impact on spermatozoa viability

The investigation into the viability of the spermatozoa across four groups was carried out by implementing the Eosin-Nigrosin staining method. The raw semen sample revealed a mean viability percentage of 64.4 ± 6.20, setting the baseline. A notable increase was observed when DGC was employed, the viability escalating to an average of 75.9 ± 9.84%. The viability was further amplified where the PMDs were used, reaching a mean of 85.0 ± 7.58%. However, it was the RSM technique that exhibited a significant leap in viability. An impressive 96.6 ± 5.08% of spermatozoa in this group demonstrated viability, accentuating the distinct advantage

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Table 1. Comparison of sperm quality parameters among different sperm separation methods

Parameters	RS	DGC	PMD	RSM	Significance
Sperm concentration (10^6/ml)	70.06 ± 20.51	20.68 ± 10.07	4.2 ± 1.14	1.95 ± 0.89	P < 0.001
Total motility (%)	33.96 ± 8.42	64.19 ± 14.91	66.35 ± 16.87	91.99 ± 8.7	P < 0.001
Progressive motility (%)	31.32 ± 7.10	59.49 ± 14.95	60.26 ± 18.10	88.29 ± 10.04	P < 0.001
Rapid progressive motility (%)	19.85 ± 5.43	36.04 ± 15.00	36.54 ± 19.47	72.22 ± 10.99	P < 0.001
VCL (μm/s)	15.84 ± 5.10	35.88 ± 6.90	37.91 ± 8.83	53.81 ± 3.04	P < 0.001
VSL (μm/s)	7.97 ± 2.31	17.38 ± 5.67	18.65 ± 7.53	31.43 ± 6.39	P < 0.001
VAP (μm/s)	10.51 ± 3.34	22.46 ± 5.61	24.06 ± 7.44	36.44 ± 4.85	P < 0.001
MAD (μm)	13.98 ± 3.83	31.42 ± 5.91	33.47 ± 7.71	48.89 ± 7.98	P < 0.001
ALH (μm)	0.83 ± 0.28	1.75 ± 0.28	1.83 ± 0.38	2.39 ± 0.34	P < 0.001
BCF (Hz)	1.96 ± 0.55	4.63 ± 1.36	5.38 ± 2.13	8.33 ± 1.43	P < 0.001
LIN (%)	17.44 ± 3.58	32.88 ± 11.48	34.17 ± 14.21	56.50 ± 11.97	P < 0.001
WOB* (%)	23.18 ± 5.48	42.19 ± 12.25	43.93 ± 14.73	65.18 ± 10.59	P < 0.001
STR (%)	24.94 ± 5.13	48.70 ± 13.84	50.09 ± 15.72	78.41 ± 7.68	P < 0.001
Normal morphology (%)	2.3 ± 1.41	5.4 ± 2.11	8.2 ± 2.29	17.1 ± 3.95	P < 0.001
Vitality (%)	64.4 ± 6.20	75.9 ± 9.84	85.0 ± 7.58	96.6 ± 5.08	P < 0.001

Comparative analysis of sperm parameters across four study groups: raw semen (RS), density gradient centrifugation (DGC), passive microfluidic device (PMD) and rheotactic sperm separator microfluidic (RSM) chip. VCL refers to curvilinear velocity, VSL is straight-line velocity, VAP is average path velocity, MAD is mean amplitude of head displacement, ALH is amplitude of lateral head displacement, BCF is beat-cross frequency, LIN is linearity, WOB is wobble and STR is straightness.
* Wobble is the oscillation of the actual path about the average path, representing the regularity of the sperm movement. It's calculated as VAP/VCL × 100%.

of the RSM method in yielding a significantly higher proportion of viable sperm (Table 1).

Discussion

In the present study, we compared different sperm separation approaches: the DGC, the PMD and our RSM device. The main objective of our study was to separate adequate numbers of sperm cells using RSM and to gauge the influence of these methods on the principal attributes of sperm quality, namely sperm concentration, motility, morphology and viability.

Our findings draw a line of confluence with previous research, confirming that microfluidic methods, although resulting in lower sperm concentrations, could potentially proffer superior sperm qualities compared with more conventional techniques (Gode *et al.*, 2019; Guler *et al.*, 2021; Mirsanei *et al.*, 2022; Parrella *et al.*, 2019; Shirota *et al.*, 2016). However, this potential advantage should be understood as context-dependent and not universally applicable across all procedures used in assisted reproductive technologies.

In this study, we designed an RSM device that operates without any extra facilities to improve user convenience. The primary objective of this design was to facilitate the potential integration of this device into clinical practices related to infertility treatment, streamlining its usage and ensuring its ease of adoption.

The parallelization of the device used in this study was specifically utilized to augment the volume of the processed sample. This represented a significant advancement over the design of previously rheotaxis-based sperm separation devices (Ahmadkhani *et al.*, 2023; Ataei *et al.*, 2021; Nagata *et al.*, 2018). However, the enhanced design did not meet authors' initial expectations regarding the quantity of isolated sperm, underscoring the intricacies and challenges intrinsic to microfluidic sperm separation techniques.

The concentration of sperm retrieved via the DGC method corresponded to the findings of prior studies, yielding a higher concentration of sperm at 20.68 million sperm/ml when juxtaposed against the passive and RSM microfluidic techniques, which only delivered 4.2 and 1.95 million sperm/ml, respectively (Gode *et al.*, 2019; Gonzalez-Castro *et al.*, 2018). The implications of these disparities in sperm concentration become especially pertinent in procedures like intrauterine insemination (IUI), where larger quantities of sperm are needed. In such a scenario, the demand for millions of sperm to bolster the chances of successful fertilization negates the potential benefit of using microfluidic devices like RSM due to their lower yield.

In 2018, Zafarani and colleagues successfully isolated rheotactic sperms in sufficient quantities to conduct a comprehensive study and characterize their movement patterns (Zaferani *et al.*, 2018). It is acknowledged that the superior spermatozoa represent a minority within the primary population, making it challenging to obtain a substantial number of rheotactic spermatozoa through microfluidic techniques (Suarez and Pacey, 2006). Our study findings corroborate this limitation; however, certain literature reports have claimed the successful isolation of a considerable number of rheotactic spermatozoa (Nagata *et al.*, 2018; Wu *et al.*, 2017); Nevertheless, Nagata *et al.* had separated bovine rheotactic sperms which might have different rheotactic capabilities.

The RSM method, however, shone brightly in its remarkable efficacy in isolating sperms that exhibited superior motility. The overall motility stood at an impressive 91.99%, progressive motility at 88.29% and rapid progressive motility at 72.22%. Furthermore, all the parameters assessing sperm movement trajectory, namely, VCL, VSL, VAP, BCF, LIN and STR, were found to be highest in the RSM group. This apparent enhancement in the selection of more motile sperm echoes previous studies that asserted the potential of microfluidic sperm selection in improving sperm motility (Knowlton *et al.*, 2015). Given that sperm motility is a

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pivotal determinant of successful fertilization, this trait can contribute significantly to increasing the chance of conception in assisted reproductive techniques such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), where fertilization occurs *ex vivo* (Duran *et al.*, 2002; Enginsu *et al.*, 1992; Ombelet *et al.*, 2014).

While the impact of sperm morphology on the outcomes of assisted reproductive techniques remains a topic of debate, several studies underscore its significance in enhancing these results (Danis and Samplaski, 2019). However, several studies utilizing microfluidic devices for sperm isolation have indicated that spermatozoa with superior morphology are segregated using microfluidic microchips (Khodamoradi *et al.*, 2021). In the current study, it was also shown that rheotactic sperm have better morphology than other groups. The data of this study also confirm the findings of previous studies.

In 2021, Sarbandi and colleagues introduced a novel device for the separation of rheotactic sperm. This innovative equipment mimics uterine microgrooves, utilizing biomimicry to simulate the natural environment and facilitate the separation process. In the study, the employed device incorporates a pump, generating a stable and controllable flow, enabling the entrapment of rheotactic sperm within the device's grooves. Subsequently, the non-rheotactic sperms are removed from the primary channel through washing. Ultimately, the rheotactic sperms are harvested from the apparatus by flushing the pathway with a slow, regulated flow. The authors acknowledged that while their device efficiently studies sperm movements, its application in clinical settings is constrained due to usability challenges. However, they expressed optimism that the underlying concept has the potential for future integration with lab-on-a-chip technology, potentially enabling the execution of the fertilization process within a microfluidic device (Sarbandi *et al.*, 2021).

There are other studies in the literature that utilized microfluidic devices for rheotactic sperm isolation. However, the clinical application of these systems appears to be challenging due to their complexity of usage and the need for additional equipment (Ahmadkhani *et al.*, 2023; Huang *et al.*, 2023; Mane *et al.*, 2022; Sharma *et al.*, 2022). However, in 2023, Heydari and colleagues presented a novel microfluidic device characterized by its small size, employing symmetrical flow to isolate an adequate quantity of rheotactic sperms. While the device is relatively user-friendly, it still necessitates using a syringe pump to sustain a continuous flow. The article also illustrates that, in terms of sperm trajectory parameters, rheotactic sperm exhibit superior characteristics (Heydari *et al.*, 2023).

In the present research, our objective was to isolate rheotactic spermatozoa employing an innovative device designed to fulfil this purpose. This automated user-friendly microfluidic apparatus allows for the separation without necessitating additional equipment, such as syringe pumps, while simultaneously ensuring the recovery of a substantial volume of processed sperm.

It is crucial to consider the nuances in the application of the RSM method. While it excels in isolating highly motile and morphologically normal sperms, this technique may fall short when it comes to poor-quality sperm samples. In situations where the sperm samples are critically deficient in either quantity, motility or morphology, the RSM may struggle to recover a sufficient number of healthy sperms to ensure successful fertilization. Therefore, while microfluidic methods like RSM are promising, their limitations should also be considered.

Our research has substantial implications in the realm of assisted reproductive technologies. The data suggest that despite yielding lower sperm concentrations, microfluidic methods, especially the RSM, can effectively separate sperms of superior motility, morphology and viability, vital indicators of overall sperm quality and fertility potential. While this finding bodes well for the future of assisted reproductive technologies, it is equally important to recognize the study's limitations.

Our study was conducted in a laboratory setting and may not entirely replicate the *in vivo* conditions, possibly limiting the direct translation of our findings to real-world applications. Moreover, the study did not extend to assessing the effects of these separation techniques on subsequent critical outcomes such as the fertilization rate, embryo development or pregnancy rate.

Considering our findings, future research endeavours should focus on clinical validation of the RSM method and evaluating its effects on the fertilization rate, embryo development and pregnancy outcomes. This would paint a more comprehensive picture of the method's efficacy and applicability in clinical settings. Also, the possible integration of RSM with other laboratory procedures in assisted reproduction, such as ICSI or IVF, could be explored to optimize these treatments further.

However, it is vital to reiterate that the RSM and other sperm distance swimming microfluidic devices, in general, may not be universally applicable, particularly in IUI procedures or cases where sperm samples are of inferior quality. As we strive to optimize assisted reproductive techniques, we must remain cognizant of the constraints these advanced methodologies come with and continue our exploration of potential solutions to these limitations. The evolving landscape of assisted reproductive technologies necessitates further research and a nuanced understanding of the strengths and weaknesses of each method.

Conclusion

Our findings align with prior research, highlighting the potential advantages of microfluidic methods regarding sperm quality. The RSM method demonstrated remarkable efficiency in isolating highly motile sperm, which is crucial for successful fertilization. However, the limitations and contextual considerations should be considered. Future studies should be conducted focusing on clinical validation of the RSM method and evaluating its impact on critical fertility outcomes. Our study contributes to the growing understanding of assisted reproductive technologies, emphasizing the need for a nuanced approach to optimize sperm selection methods.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0967199424000467.

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Competing interests. The authors declare that they have no conflicts of interest related to this research study.

Ethical standards. This study was conducted in full compliance with the ethical standards set forth by the Declaration of Helsinki and the institutional guidelines of the Avicenna Research Institute. Prior to sample collection, informed consent was obtained from all participants, and they were informed about the study's objectives and the use of their biological materials for research. The confidentiality and anonymity of participants were rigorously upheld throughout the research process. The study was approved by the Avicenna Research Institute's Ethics Committee, with the institutional approval ID: IR.ACECR.AVICENNA.REC.1399.010. All procedures performed in this study adhered to the relevant ethical guidelines and regulations.

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