



Enrichment of motile spermatozoa from cattle semen samples by microfluidics method

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ABSTRACT

Motile sperm cell separation is important in sample preparation for both artificial insemination and cryopreservation of semen. A novel microfluidic device consisting of an inlet microchannel, a separating reservoir and two outlet microchannels was developed to enrich the motile sperm cells of cattle semen samples. Sperm separation was performed in this microfluidic device using a continuous flow process based on the swim up behaviour of motile cells. Separating reservoir allows the high motile sperm cells to swim up and pass through the top outlet of the reservoir. Low and non-motile sperm cells pass through the bottom outlet of the reservoir in the direction of fluid flow. The microfluidic device was fabricated using polydimethylsiloxane (PDMS) and semen samples were infused into the microfluidic device through a syringe pump. Sperm motility was analyzed by Computer-assisted sperm analysis (CASA). More than 80% enrichment of motile spermatozoa in the cattle semen samples was observed after their separation in the fabricated microfluidic device.

Keywords: CASA, Cattle, Microfluidics, Motile sperm, Sperm separation

Artificial insemination (AI) is one of the most important assisted reproductive technologies practiced in livestock industry to improve the productivity of animals (Foote 2010). The success rate of AI technology greatly depends upon the quality of semen. One of the major reasons for the failure of AI is the decrease in the viability of the sperm cells on storage and improper maintenance during storage (Rodriguez and Martinez 2012). Sorting out dead and non-motile sperms from live and motile cells of fertile male animals before the insemination could help in achieving the high pregnancy rates. Sperm preparation for assisted reproductive technology (ART) is an important step and involves separation of viable and highly motile spermatozoa from other constituents of ejaculate (Rodriguez and Martinez 2012). Sperm separation techniques such as conventional swim-up separation technique (Mustafa *et al.* 1998), centrifuge assisted washing procedure (Goyal *et al.* 1996), glass wool column filtration (Piperelis *et al.* 2008, Scholkamy *et al.* 2009, Arzondo *et al.* 2012) can improve sperm motility and viability but require multiple interventions and additional sophisticated preparatory stages in standard protocols (Rao *et al.* 2013). Mechanical damage to the spermatozoa and fertility efficiency of sorted

spermatozoa are the major limitations of the conventional sperm sorting methods (Henkel 2012), and there is an urgent need to develop a more rapid, efficient, portable and cost-effective technique for semen upgradation.

Live dynamic microfluidics emerged as a promising alternative to sperm preparation for ART as this technique facilitates the fabrication of microfluidics chips to improve efficiency of sperm separation with minimum mechanical damage (Swain *et al.* 2013). However, microfluidics is one such approach that results in the manipulation of biological fluids within the microchannels and has gained attention of semen biologists due to label free separation of the bacterial and mammalian cells as well as underlying ease of handling small quantity of samples (Knowlton *et al.* 2015).

During the past few years, microfluidics technology has opened up doors for applications in the field of biology (Holmes and Gawad 2010, Velve-Casquillas *et al.* 2010) including handling of the particles for imaging, tracking applications and single cell analysis (Ohno *et al.* 2008, Lecault *et al.* 2012). Sorting in microfluidic is broadly classified into two categories namely active and passive cell sorting (Shields *et al.* 2015). Active sorting relies majorly on fluorescent labels or beads. These label or tagging techniques have a cumbersome process that involves attachment of these labels and later on removal as well. Active cell sorting technique requires advanced tools, which are not only expensive but have high maintenance

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cost as well. Moreover, the unavailability of fluorescent labels limits the utilization of these techniques. The passive or label-free sorting in microfluidics relies on the difference in physical properties of analyte such as size and shape, mass, elasticity, density, magnetic susceptibility, and polarizability. Of all the sorting techniques, label-free separation involves least amount of sample preparation thus rendering this process as an attractive option for sorting (Bhagat *et al.* 2010). Microfluidic chip designs, based on sperm motility (passively driven), fluid flow, chemo-attractant and thermos-toxic properties, have been tested for sorting the functional spermatozoa for ART (Knowlton *et al.* 2015).

Herein, we report a passive sorting method for separation of motile cells of cattle. By using a relatively inexpensive wire assisted technique for the fabrication of polydimethylsiloxane (PDMS) based microfluidic platform, we were able to demonstrate a separation technique that completely relies on swim up behaviour of live cells. In this method, syringe pump was used to infuse the samples into the microfluidic device which consists of an inlet, reservoir and two outlets (Fig. 1). A Patent application has been filed for this microfluidic method (Indian Patent Application Number: 202011008229).

MATERIALS AND METHODS

Ethics statement: All experimental procedures involving animals were approved by the Institute Animal Ethics Committee (IAEC), National Dairy Research Institute, India (No. 42-IAEC-18-30, Dated: 30 June 2018).

Fabrication of microfluidic device: Microfluidic device was fabricated via wire assisted technique. A smooth finished copper wire with the diameter of 450 μm was used. The wire was cut into three pieces with the length of 26.06 mm each. A straw was diced precisely for the reservoir with the dimensions of 3000 μm (in depth) and 1000 μm (in width). Parafilm and double-sided tape were used to seal the reservoir. The three cut pieces of wire were pricked through the reservoir for inlet and outlets and the setup was placed in a plastic mold. The PDMS (Sylgard - 184 silicone

elastomer kit was purchased from Kevin Electrochem, India) and curing agent (10:1) were vigorously stirred to form a well-blended mixture. The mixture was poured into the mold and degassed by keeping it in a desiccator. Once all the air bubbles were released, the mold was kept in the oven at 70°C for 4 h to facilitate cross linking of PDMS and curing agent. After the system cools, the wires were pulled out carefully avoiding damage to the chip leaving behind smooth microchannels connected to the reservoir.

Semen collection and experimental procedure: Fresh ejaculates of Karan Fries bulls were collected from Animal Breeding Research Centre, National Dairy Research Institute, Karnal, Haryana, India. We validated the device efficiency with the repetition of 15 samples. Semen samples collected from bulls were washed with PBS by centrifugation at $275 \times g$ for 5 min. Sperm concentration was adjusted to 40×10^6 sperm/mL with HEPES buffer and it was considered as control sample. The inlet microchannel of microfluidic device was connected to the syringe pump using standard silicone tubing with an internal diameter of 800 μm . Flow rate of the semen sample was maintained at 0.5 ml/min using a syringe pump. The semen samples collected from top outlet and bottom outlet were analyzed for semen quality variables and compared with control sample.

Analysis of semen samples: Total motility, progressive motility, rapid motility, straight linear velocity (VSL), average path velocity (VAP), curvilinear velocity (VCL), average lateral head displacement (ALH), beat cross frequency (BCF) were analyzed by using Hamilton Thorne Sperm Analyser (Hamilton Thorne Research, Beverly, MA, USA). Two microliter (2 μl) each of control semen sample, collected from top outlet and bottom outlet were loaded in a pre-warmed disposable Leja slides and sperm kinetic parameters were measured at 37°C.

Statistical analysis: In order to ensure that the data was normally distributed for statistical treatment, normality tests like Kolmogorov-Smirnov test and Shapiro-Wilk test were performed. Further, homogeneity of error variances was deduced based on Levene's test of equality of error

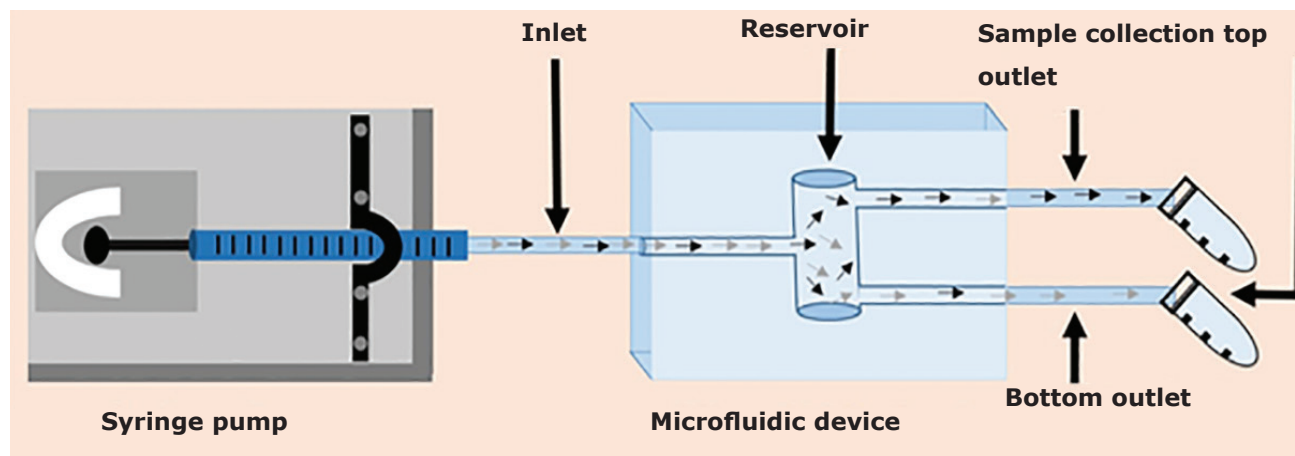


Fig. 1. Microfluidic device setup for sperm separation.

variances. The descriptive statistics were estimated for all the semen parameters. Pearson's correlation test was used to assess the extent of correlation between different parameters. Variance between samples with respect to different semen characteristics was assessed using Analysis of Variance (ANOVA) test followed by post-hoc comparison using Tukey's test. All the statistical analysis was performed using SPSS v22.

RESULTS AND DISCUSSION

In this study, we report fabrication of a highly cost-effective microfluidic device by using polydimethylsiloxane (PDMS) for enrichment of live spermatozoa. The reservoir-based sorting technique allows continuous flow sorting enabling the device to process large volumes compared to the microfluidic sperm sorter (MSS) device reported by Asghar *et al.* (2014) that uses a polycarbonate membrane filter to selectively separate motile sperm from non-motile sperm. It is well known that these membranes eventually end up clogged. Absence of an outlet channel in MSS limits the amount of sample processed in a single run. Another microfluidic device, reported by Matsuura *et al.* (2013) had similar limitations. The device was fabricated by laminating a cyclic olefin polymer-based sorting chip with polyvinylidene chloride films. The device works on a principle similar to the swim up technique. To obtain enriched spermatozoa, the top layer of the device needs to be punctured which leaves the device useless after only a single sorting run.

Process of wire assisted technique utilized to fabricate our device is highly simplified and costs-effective as compared to the microfluidic sperm selection device reported by Nosrati *et al.* (2014) fabricated by using soft lithography technique. Though, the soft lithography technique provides great accuracy and repeatability, but it is time consuming, requires cleanroom environment that adds up to the cost of the devices. Another unique microfluidic sperm sorter (MFSS) reported by Shirota *et al.* (2016) uses a single microfluidic sorting channel and requires sample and media to be injected from separate input ports. The sorting technique does not fully utilize the swim-up potential of the motile spermatozoa, also the device has been reported to work only with human sperm cells, not with samples from livestock. The capability of our device to perform real time enrichment in a continuous flow sample makes it a unique

device. The device demonstrated in this work does not cause sperm damage at any stage, thus making the technique completely non-invasive and novel. The microchannel walls fabricated using wire assisted technique are smooth and do not cause any turbulence in the flow compared to the poly(methyl methacrylate) (PMMA) based devices fabricated using laser micromachining fabrication process (Dorranian *et al.* 2009). The optimization of semen flow rate, reservoir height, internal diameter of the reservoir and the positions of the inlet and outlets have played a key role in achieving the reported enrichment efficiency. Multi-level reservoirs could be added in future to further improve efficiency. We have developed a reservoir based microfluidic approach for sperm separation as depicted in Fig. 1. The microreactor consists of one inlet and two outlets. The inlet, abbreviated as (I), is placed exactly at the middle of the cylindrical reservoir (R). Two outlets, top outlet (O_T) and bottom outlet (O_B) are designed in order to achieve maximum separation efficiency. The dimensions of microfluidic device comprising of inlet, reservoir and outlets are given in Fig. 2.

Fig. 3A, represents live (black) and dead (white) cells infused through inlet (I). Because of the swim up property of live cells, motile cells were collected from the top outlet (O_T) and low and non-motile sperm cells were discarded from the bottom outlet (O_B). Experimental set up is shown in Fig. 3B. Fig. 4A and 4B represents the side and top views of the microfluidic device and depicts the inlet, reservoir and the two outlets. The capability of device to perform real time enrichment in a continuous flow sample makes it a unique device. The optimization of semen flow rate, reservoir height, internal diameter of the reservoir and the positions of the inlet and outlets have played a key role in achieving the reported enrichment efficiency. Control and sorted semen samples were evaluated for kinetic parameters by CASA respectively. The results are summarized in Table 1.

Motility is contemplated as an important parameter for the evaluation of frozen-thawed semen quality in livestock breeding industry. Motility estimation is a crucial step in evaluating male fertility, and can be considered a functional test because of its relationship with the energy status of mammalian sperm (Lauria *et al.* 1998, Quintero-Moreno *et al.* 2004). Although motility and kinetic parameters alone cannot be considered as reliable markers for the fertilizing ability of a given ejaculate (Holt *et al.* 1985, Marshburn *et al.* 1992, Barratt *et al.* 1993, Krause 1995, Macleod and Irvine 1995, Holt *et al.* 1997), spermatozoa with low movement are unlikely to reach the oviduct, studies have proved that more is the progressive motility of spermatozoa, higher is the chance of pregnancy (Muñoz *et al.* 2008). Over the last few decades, computer assisted semen analysis (CASA) has emerged to make an objective and repeatable analysis of sperm motion as a measure of fertility assessment and quality assurance of semen for artificial insemination (Mortimer 1997). In this study, we

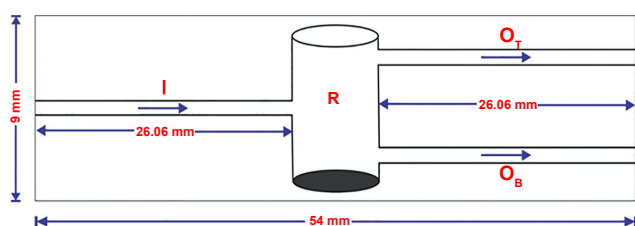


Fig. 2. Dimensions of the fabricated microfluidic device having a reservoir (R), an inlet microchannel (I) positioned at the middle of the reservoir, and two outlet microchannels positioned at the top (O_T) and bottom (O_B) of the reservoir.

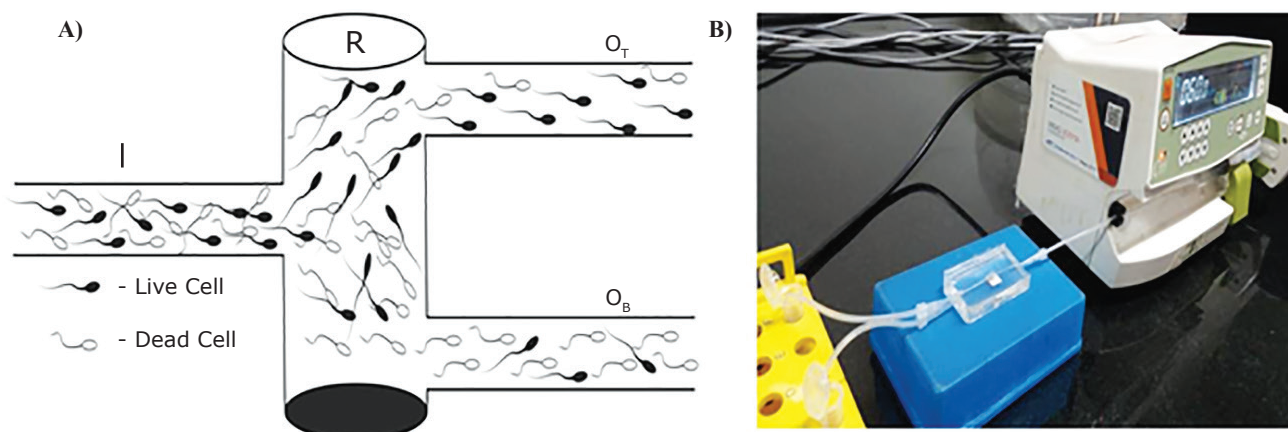


Fig. 3. (A) Microfluidic device consisting an inlet (I), reservoir (R), top outlet (O_T) and bottom outlet (O_B), illustrating live and dead cells separation in a reservoir, and (B) Digital image of the experimental setup.

aimed to separate highly motile and live cells from low motile and dead cells in cattle semen by using simple and inexpensive microfluidic device followed by assessment of various quality parameters using CASA. Sorting motile spermatozoa from semen samples using microfluidics offers several advantages over other isolation techniques. Sperm processing techniques, like density gradient separation and semen washing require centrifugation, which has been reported to cause sub-lethal damage to the spermatozoa (Alvarez *et al.* 1993, Smith *et al.* 1995). Using microfluidics for motile sperm isolation eliminates the need for centrifugation. Unfortunately, with swim-up or density gradient separation, the ability to recover motile spermatozoa lost with processing is limited. However, with microfluidic sperm sorting, if there are insufficient motile spermatozoa after sorting, more motile spermatozoa could be recovered from fluid in the unsorted outlet by placing it back into the inlet well and reprocessing the sample. The efficiency of isolating motile spermatozoa from a semen sample using microfluidics is dependent on several variables such as the rate of flow of the fluid streams, the duration of contact between parallel streams, sperm motility parameters. Also, the width of the fluid streams can impact

efficiency, since the chance of swimming into the parallel stream prior to stream divergence is random. Ideally, semen samples would flow through the thinner stream, allowing a greater likelihood that motile spermatozoa could swim into the wider stream and be sorted. Total motility refers to the percentage of sperm making any sort of movement. This can include non-progressive movement also. The average total motility of semen samples collected from top outlet was enriched up to 82% from 53% of average total motility of control samples. Progressive motility refers to sperm that are swimming in almost straight line or in very large circles. Progressive motility is needed in order for the sperm to swim their way up the female reproductive tract. The average progressive motility and rapid motility were improved up to 42% and 61% whereas control sample values are 24% and 31% respectively. The values for VSL and VAP of semen samples collected from outer outlet were improved to 121.7 $\mu\text{m/s}$ and 145 $\mu\text{m/s}$ from 73 $\mu\text{m/s}$ and 102 $\mu\text{m/s}$, respectively. We observed more than 100% improvement in VCL of semen samples collected from outer outlet. We observed a small difference in the ALH of the semen samples collected from the top and bottom outlets. We observed significant improvement of the semen

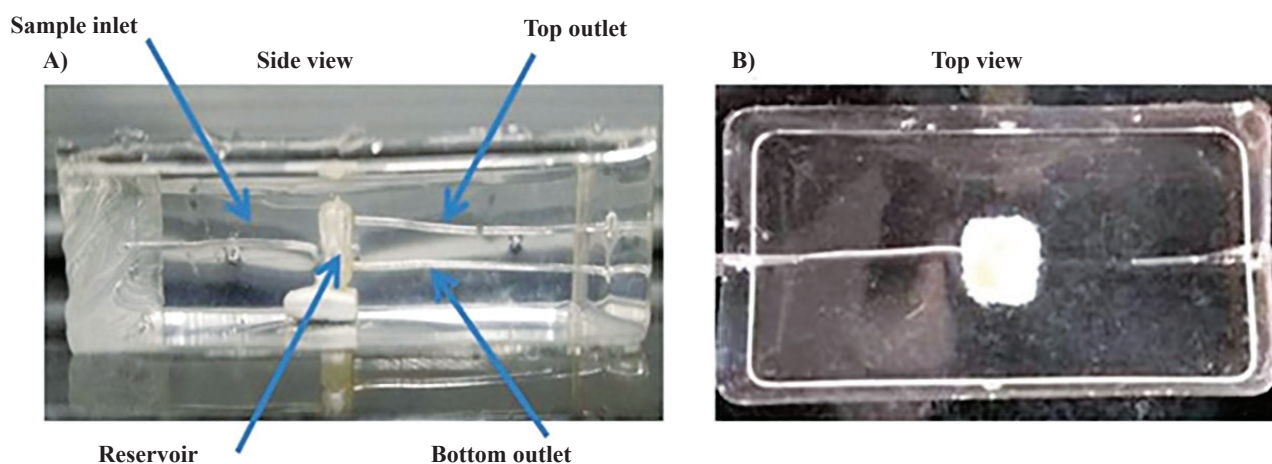


Fig. 4. (A) Side view, and (B) top view of the microfluidic device with inlet positioned between top and bottom outlet channels.

Table 1. Values (mean±standard error) of semen sample variables (n=15)

Variable	Control	Top outlet	Bottom outlet
Total motility	53.7 ^a ±5.8%	82.1 ^b ±7.1%	32.5 ^c ±6.9%
Progressive motility	24.9 ^a ±11.3%	42.7 ^b ±15.8%	13.6 ^c ±6.6%
Rapid motility	31.1 ^a ±15.4%	61.4 ^b ±17.6%	12.1 ^c ±6.13%
Straight linear velocity (VSL)	79.3 ^a ±16.2 µm/s	121.7 ^b ±21.3 µm/s	46.3 ^c ±19.5 µm/s
Average path velocity (VAP)	102.5 ^a ±20.5 µm/s	145.6 ^b ±30.1 µm/s	76.7 ^c ±25.8 µm/s
Curvilinear velocity (VCL)	162.6 ^a ±23.4 µm/s	281.8 ^b ±28.6 µm/s	97.4 ^c ±21.57 µm/s
Average lateral head displacement (ALH)	9.1 ^a ±1.2 µm	11.4 ^b ±1.9 µm	8.4 ^a ±2.1 µm
Beat cross frequency (BCF)	24.1 ^a ±1.97 Hz	37.7 ^b ±2.8 Hz	21.5 ^a ±3.1 Hz

Means bearing different lowercase superscript letters in the same column differ significantly (P<0.05).

samples collected from top outlet and it was found to be 37.7 Hz whereas control samples exhibited BCF of 24 Hz.

In our study, we found that microfluidic technology is a simple and cost-effective method to separate motile sperm cells from low and non-motile sperm cells. The microfluidic test, motility was compared for sperm swimming in buffer for both top and bottom outlet. The fabricated microfluidic device was used to create a highly controlled platform with which these tests were performed. The functionality of the device was achieved by self-propelled sperm swimming through the low viscosity medium in the main channel. The data suggests that motile and non-motile sperms can be differentiated through microfluidic chamber (Table 1). Sperms with higher progressive motility rate travel far longer distance and can be collected through the top outlet while the non-motile sperm cells can be collected through bottom outlet. These findings clearly indicate a strong correlation between rapid progressive sperm motility and vitality since sperm with highly progressive motility are able to swim in a shorter amount of time and subsequently exhibits a higher vitality than sperm with less rapid progressive motility. The trend also indicates that the act of swimming further through the device did not have a negative impact on sperm vitality, or, at minimum, any potential negative effect was more than compensated by the higher vitality of the faster swimming population.

The motility of sperms is critical for a successful fertilization. In this study, we demonstrated a microfluidic device that can separate motile and immotile sperms from the cattle semen samples. The design of this microfluidic system for sperm separation relies upon the swim up behaviour of motile spermatozoa in the fluid flow direction. We experimentally measured the sperm cell kinematics for the evaluation of microfluidics device and achieved 82% enrichment of motility of sperm cells in cattle semen samples. Our device provides a portable, inexpensive, and

reusable platform for the separation of motile cells which can further be used for field applications. This is a simple and inexpensive method which could be helpful for dairy industry to improve the fertility rate of the livestock.

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