Microfluidic assessment of swimming media for motility-based sperm selection

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Selection medium is important in sperm isolation for assisted reproductive technologies. Contrary to the naturally occurring human cervical mucus which has a high viscosity, most current practices for motility based sperm selection use a low viscosity medium. In this study, we used a microfluidic device to assess the effects of high viscosity media made with hyaluronic acid (HA) and methyl cellulose (MC) on bovine and human sperm motility and viability (sperm transferred directly from cryoprotectant). The microfluidic penetration test, viability, and motility were compared for sperm swimming in both HA and MC media with about 20cp viscosity (measured at 20 °C). Our resulted indicate that MC medium resulted in a significantly higher number of viable bovine sperm penetrating the medium as compared to HA. Furthermore, MC resulted in the selection of a sperm subpopulation with a 274% increase in sperm viability in comparison to the raw semen, while HA increased viability by only 133%. In addition to viability, bovine sperm motility parameters were significantly higher in the MC medium as compared with HA. Experiments with human sperm swimming in MC indicate that sperm swim slower and straighter at higher viscosities. In conclusion, the results indicate that in a micro-confined environment representative of the *in vivo* environment, MC is a preferred high viscosity medium to ensure the highest concentration of motile and viable sperm. © *2015 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4928129]

I. INTRODUCTION

Infertility affects more than 70 million couples worldwide with male infertility accounting for about half of the cases.[1,2] For successful fertilization, sperm must swim long distances through the highly confined and viscous environment of the female reproductive tract to reach the ovum. Throughout the journey through the female tract, sperm encounter a variety of spatio-temporal changes in the rheology that play a key role in sperm guidance[3,4] and coordinate the timing of insemination.[5] For example, the cervical mucus and oviductal fluids, non-Newtonian viscoelastic media present in the female reproductive tract, naturally select sperm for fertilization by creating viscous barriers through which sperm must penetrate.[6,7] Improved understanding of the response of sperm motility to these rheological changes can bring new insight into infertility treatment and the biology of reproduction.

Assisted reproductive technologies (ARTs) such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have been developed to overcome infertility.[8] Sperm selection is a key step in ARTs, influencing both the treatment success rate and offspring health.[9] The current sperm selection approaches of swim-up and density gradient centrifugation, however, bypass almost all the natural barriers *in vivo* and include a centrifugation step which are both

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known to cause iatrogenic transmission of genetic defects.[10,11] Recently, microfluidics emerged as a high-throughput, automated, and sensitive platform for studying gamete function,[12,13] sperm selection,[14,15] and embryo development.[16,17] Several factors urge the application of microfluidics for ARTs including: (1) providing a geometrically confined environment that closely mimics the *in vivo* environment,[18] (2) possibility of highly multiplexed sample analysis,[14] (3) operating with small sample volumes relevant for hypospermia (i.e. low semen volume),[19] and (4) possibility of on-chip visualization due to transparency of the microfluidic devices.[20,21]

In the context of sperm selection, microfluidic approaches based on sperm motility have shown promise for rapid selection of sperm with high DNA integrity.[22–24] Most recently, a microfluidic device that selects sperm based on their progressive motility within a viscous medium, mimicking the *in vivo* environment, achieved more than 80% improvement in human DNA integrity, significantly out-performing current practices.[25] Since one of the benefits of using microfluidics is that it resembles the *in vivo* environment, the medium used in the devices for motility-based sperm selection should have a viscosity similar to cervical mucus, without causing a large impact on the motility of the sperm. Most microfluidic devices, however, use low viscosity media. Hyaluronic acid (HA)[26] and methyl cellulose (MC)[27] have been studied as alternative viscous media to cervical mucus for laboratory tests. Neuwinger *et al.*[26] showed that using HA at a concentration of 2.75 mg/ml, sperm motility, migration rates, and 24-h survival rates were comparable to those obtained with human cervical mucus. It has also been found that normal sperm have a higher incidence of binding to HA[28] which is used as a method for selecting sperm for ICSI.[29,30] Ivic *et al.*[27] showed that MC at 10 mg/ml resulted in comparable mucus penetration and motility as human cervical mucus. The use of the MC medium was found to serve as a suitable environment to discriminate swimming behaviour of normal and oligozoospermic spermatozoa.[27] Despite the established importance of the medium characteristics on *in vitro* sperm motility assessment, there are no studies directly comparing the motility and viability of sperm in MC and HA media, particularly within the highly confined geometry of microfluidic devices.

In this paper, we investigate the effects of MC and HA media on bovine and human sperm motility and viability for motility-based sperm selection. An electric circuit analogy and a COMSOL Multiphysics simulation were used to design a highly controlled microfluidic platform. Bovine sperm motility parameters and viability are compared for MC and HA media using this microfluidic device. Our results indicate that sperm swim faster in MC as compared with HA, while MC results in the selection of a sperm subpopulation with a significant increase in sperm viability as compared to the raw semen. Furthermore, we report the motility parameters for human sperm in MC—as the preferred medium for motility-based sperm selection—with viscosities ranging from 20 cp to 250 cp.

II. MATERIALS AND METHODS

A. Microfluidic device design

A microfluidic device was designed and fabricated to assess the effects of HA and MC media on sperm motility and viability, as shown in Fig. 1. In addition to the microchannel, the microfluidic chip contains a temporary seal layer (Fig. 1(a)), buffer (medium) reservoirs, and an extraction device for controlled collection of the sperm from the microfluidic chip (Fig. 1(b)). The temporary seal layer ensures that there is no flow in the device when the sample is added to the inlet. The absence of flow makes it possible for the live and motile sperm to swim out of the cryoprotectant and semen, and away from dead cells, into the main channel. Removing the temporary seal layer and exposing the buffer reservoirs ensure that the extraction device evenly draws fluid from all the outlets such that sperm in each of the channel junctions, illustrated with dashed lines in Fig. 1(c), are collected via the corresponding outlet channels.

The main channel was 500 μm wide and 12 mm long. The distances along the main channel corresponding to each outlet channel were 4.5 mm, 7 mm, 9.5 mm, and 12 mm for outlet channels 1, 2, 3, and 4, respectively. The side channels for the buffer and outlets were comparatively

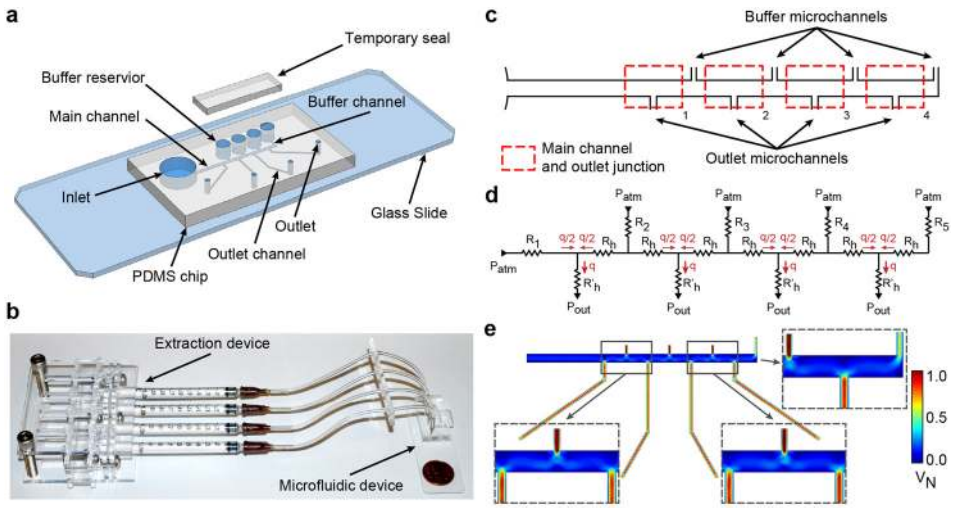

FIG. 1. Microfluidic device for assessment of swimming medium on sperm motility. (a) Schematic view of the device showing the microfluidic chip and the temporary seal layer. (b) Extraction device with connection to the outlets of the microfluidic device for sample collection. (c) A schematic of the main channel of microfluidic device showing the junctions of the main channel with the outlet channels and buffer channels. The areas marked with the squares indicate the junctions where the images were taken for the microfluidic sperm penetration test and where the videos were taken for the motility assessment. (d) Equivalent fluidic circuit of the device with flow rate and pressure conditions used for calculation. (e) COMSOL Multiphysics simulation results indicating normalized velocity field (normalized with min and max of range) and streamlines in the device during the sample extraction. Normalized velocity field and flow streamlines for regions containing outlet channels 1 and 2, outlet channels 3 and 4, and buffer channels 3 and 4 are shown as inset.

narrow, 150 μm and 200 μm wide, respectively. By considering equal lengths for the outlet channels, an electric circuit analogy was used to calculate the required length of the buffer channels for equal and simultaneous extraction of the sperm from the main channel.[31] The equivalent fluidic circuit of the microchannel network is shown in Fig. 1(d). In this analogy, pressure, P, flowrate, q, hydraulic resistance, R_H, and channel length, L, are similar to voltage, current, resistance, and conductor length. By assuming that the pressure at the channel inlets are atmospheric pressure, P_{atm}, and each outlet channel extract flow equally from the left- and right-side of the corresponding junction in the main channel, the hydraulic resistance for the buffer channels can be defined as

$$R_2 = R_3 = R_4 = 0.5\,R_5 = 0.5\,(R_1 - R_h), \tag{1}$$

where R_1 is the hydraulic resistance of the main channel between the inlet and first outlet channel, R_h is the hydraulic resistance of the main channel between each two consecutive outlet channels, and R_2–R_5 are the hydraulic resistances of the buffer channels 1–4. The hydraulic resistance for a microchannel can be approximated as

$$R_H = \frac{12\mu L}{wh^3}, \tag{2}$$

where μ is fluid viscosity, w is the channel with, and h is the channel height (h $\ll$ w). Using Eqs. (1) and (2), the buffer channel lengths can be calculated as $L_2 = L_3 = L_4 = 0.49$ mm and $L_5 = 0.98$ mm where L_i is the channel length corresponding to hydraulic resistance R_i.

To study the flow behavior in the designed microchannel network, a COMSOL Multiphysics simulation for a stationery laminar flow was carried out. The normalized velocity field (with respect to the maximum and minimum range) and streamlines are shown in Fig. 1(e). The results indicate that upon applying a negative pressure at the outlet channels via the extraction device, an even and symmetric flow with respect to the outlet channel is drawn from the corresponding junction area in the main channel.

B. Microfluidic device fabrication

The desired microfluidic channels were designed in AutoCAD and printed on a photomask (Pacific Arts & Designs Inc., Markham, Canada). Standard soft lithography techniques were employed to create the master mold from SU-8 2075 negative photoresist (MicroChem, Newton, MA, USA) with channels 65 μm in height.[32] The microfluidic chip was fabricated to a 3 mm thickness using Poly-dimethylsiloxane (PDMS) (Sylgard 184 Elastomer Kit, Dow Corning, MI, USA) substrate with a 1:10 mixing ratio. Miltex Dermal Biopsy punches were used to punch 5 mm, 2 mm, and 1 mm diameter holes in the PDMS chip for the inlet, buffer reservoir, and outlet ports, respectively. The PDMS chip was bonded to a glass slide using a plasma cleaner. A PDMS square 4 mm $\times$ 12 mm was used as a temporary seal layer to close the buffer reservoirs during the experiments. The entire device was placed on a 65 °C hot plate for 5 min to complete the material bonding. An extraction device was fabricated to ensure equal and simultaneous extraction of sperm from the outlets, as shown in Fig. 1(b). The extraction device was composed of four 1 ml plastic BD-syringes held together by a polymethyl methacrylate (PMMA) (Plastic World, Toronto, Canada) structure. A 19 gauge needle was attached to each syringe and inserted into tygon tubing (McMaster-Carr, IL, USA) with an internal diameter of 1/32 in. The other end of the tubing had a 19 gauge cylindrical metal tip, which connected to the outlets of the microfluidic device.

C. Semen sample preparation

For the bovine semen, frozen bovine semen cryoprotected in a low concentration of glycerol was purchased in 500 μl straws (ABS Global Inc., Canada) and stored in liquid nitrogen. Before use, the bovine specimen were thawed in a 37 °C water bath for 2 min and removed from the straw using an artificial insemination syringe. The semen was then incubated at 37 °C for 2 min prior to use in the experiments detailed below. For the human semen, cryogenically frozen human semen cryoprotected in a 3:1 solution of seminal fluid to Sperm Maintenance Medium with Glycerol (SMMG) was purchased in 1 ml vials from ReproMed Ltd. (Toronto, Canada) and stored in liquid nitrogen. All donors provided consent for research participation in accordance with regulations of the Assisted Human Reproduction Act. Human semen vials were thawed for 5 min in a 37 °C water bath and used immediately in the experiments. Human semen with 50×10^6 sperm per milliliter concentration and 40% motility was used in the experiments. All semen samples were kept at 37 °C at all times.

To stain the sperm for viability, a Live/Dead Viability Kit (Molecular Probe®, Canada) was used. A working solution of the SYBR 14 dye (component A—live) was prepared by diluting 1 μl of component A in 49 μl of dimethyl sulfoxide (DMSO) (Fisher Scientific Company L.L.C, Canada). The propidium iodide (component B—dead) was prepared by diluting 1 μl of component B in 10 μl of the medium being evaluated. Live and dead sperm were labeled with green and red fluorescence, respectively, by adding 3 μl of each of the working solutions of components A and B per semen straw. The semen was incubated at 37 °C for 5 min to allow the stain to set.

D. Buffer preparation

Medium preparation materials were purchased from Bioshop Canada unless otherwise indicated. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered saline (HBS) (135 mM NaCl, 5 mM KCl, 12 mM D-glucose, 25 mM HEPES, 0.75 mM $Na_2HPO_4{\cdot}2H_2O$) supplemented with 1 mg/ml Poly(vinyl Alcohol) (PVA) was used as the base solution to prepare the high viscosity MC and HA media. Note that the stated viscosity is the viscosity at 20 °C unless otherwise indicated.

1. Methyl cellulose

Solutions of 0.5%, 0.875%, and 1.125% MC (M0512; Sigma-Aldrich Corp, MO) were prepared in the base buffer to have final viscosities of 20 cp, 100 cp, and 250 cp, respectively, at

20 °C. A 1 M solution of NaOH (VWR, West Chester, PA) was used to adjust the buffer to pH 7.4 at room temperature. The MC medium was stored at 4 °C until used for experiments at 37 °C and used within one week of preparation. Prior to using the MC medium it was inspected by microscope to confirm the absence of bacterial and fungal contamination. The viscosity of the 0.5% MC medium was measured using a Brookfield DV-E digital viscometer (Brookfield Engineering Laboratories, Inc., MA, USA) to have a viscosity of 21.9 cp at 20 °C and 18.8 cp at 37 °C. In all the experiments, 0.5% MC medium was used unless otherwise stated.

2. Hyaluronic acid

The HA was made from sodium salt from *Streptococcus pyrogenes* with a molecular weight of 731 000 (Calbiochem, USA). A concentration of 1 mg/ml of HA was prepared in the base buffer for use in the experiments such that the final viscosity of the medium was measured to be 20.9 cp at 20 °C. A 1 M solution of NaOH (VWR, West Chester, PA) was used to adjust the buffer to pH 7.4 at room temperature. The HA medium was stored at 4 °C until used for experiments at 37 °C and used within one week of preparation. Prior to using the HA medium it was inspected by microscope to confirm the absence of bacterial and fungal contamination. The viscosity of the 1 mg/ml HA medium was measured to have a viscosity of 20.9 cp at 20 °C and 17.4 cp at 37 °C.

3. Media toxicity test

Once the media were prepared a toxicity test was performed. To conduct the test, a 350 μl aliquot of semen was added to 350 μl of each HA and MC. The samples were incubated at 37 °C and an aliquot removed every 10 min for viability assessment using the live/dead stain as described above. The toxicity test was performed for a total of 40 min as the experimental time did not exceed 30 min. An initial viability assessment was also conducted on the raw semen at the start of the test. The toxicity test confirmed that neither medium has a toxic effect on the sperm since the initial percentage of live cells in the raw semen sample was $46.80 \pm 3.69\%$, and after exposure to both the HA and the MC for 20 min the percentage of live cells was $44.45 \pm 6.00\%$ and $43.70 \pm 7.84\%$, respectively. After an additional 10 min in the media, the percentage of live cells remained at $42.14 \pm 7.06\%$ in the HA and $41.99 \pm 5.89\%$ in the MC. These results confirm that neither media are particularly toxic to the sperm and sperm show similar lifetimes in both media in bulk experiments.

E. Experimental procedure

To fill the microfluidic device with the medium, it was submerged in the desired medium and placed in a vacuum chamber for 2 h at −30 psi to replace the air inside the microfluidic channels with the medium. Prepared devices were removed from the excess medium and placed on a 37 °C hot plate. The pins of the extraction device were pressed into the outlets of the microfluidic device. The assembly was left on the hot plate for at least 10 min to ensure no flow, and to ensure the temperature of the device reached 37 °C. For all experiments, a maximum exposure time of the sperm to the PDMS was set to 30 min in keeping with established PDMS biocompatibility to have no negative effect on sperm motility after 30 min exposure to PDMS.[33] All the data are presented as means ± standard error of the means (s.e.m.). The statistical significance of the results was determined by conducting a two-way analysis of variance (ANOVA) between the outlets and the different media. A P-value of 0.05 was used in all of the statistical analyses to determine statistical significance.

1. Microfluidic penetration and motility test

A volume of 20 μl of the fluorescently labeled semen was pipetted into the inlet of the device, as illustrated in Fig. 2(a). Images of the main channel at the junction with each outlet channel (as illustrated with 1–4 in Fig. 1(c)) were captured at 10× magnification every 5 min for 25 min (Fig. 2(b)). Images were taken using a Leica L5 filter for the live cells (component

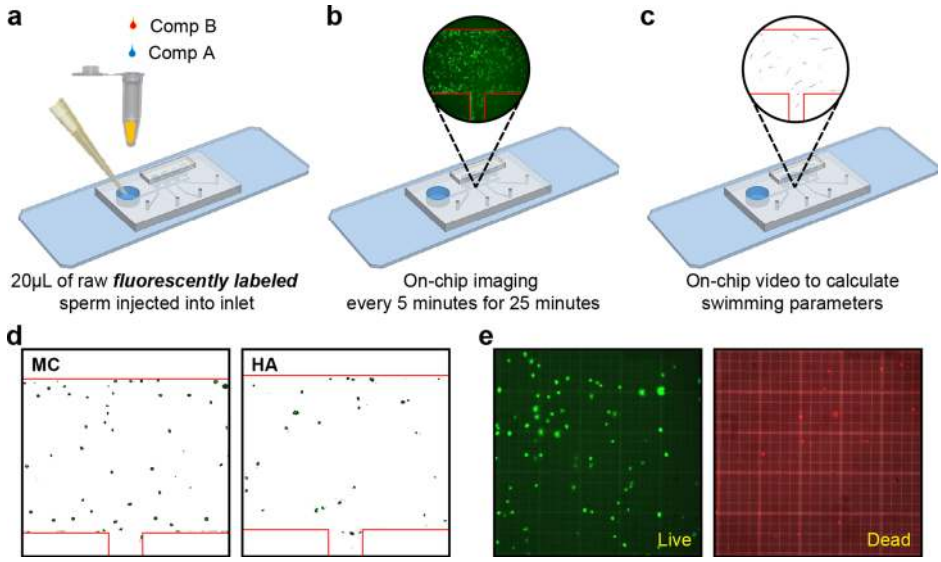

FIG. 2. Experimental procedure for the microfluidic penetration test, sperm motility test, and sperm viability test. (a) 20 μl of stained specimen (unstained specimen in case of viability test) were pipetted into the inlet of the device. (b) Images were taken at each of the channel junctions every 5 min for 25 min to track sperm penetration. (c) On-chip videos were used to assess the motility parameters of sperm at each of the channel junctions along the microfluidic device. (d) Sample images of sperm penetration in the microfluidic devices filled with MC and HA. (e) Viability test: unstained sperm extracted from the device are stained with the Live/Dead viability kit and loaded into a haemocytometer. Fluorescence images were taken in order to determine the concentration of live (green fluorescence) and dead (red fluorescence) sperm.

A—green) and a Leica TX2 filter for the dead cells (component B—red). A Java image processing software, ImageJ (National Institutes of Health, USA), was used to count the number of live cells along the main microfluidic channel. The total number of sperm for each junction was used to determine how far the sperm were able to travel within the media (Fig. 2(d)).

Following the microfluidic penetration test, 30 s videos ($f_{frame} = 35$ Hz for bovine) were taken at 10× magnification with the Leica L5 filter for live cells (green) at each of the outlet channel junctions. In addition, motility parameters for the human sperm were collected by taking 30 s videos ($f_{frame} = 50$ Hz for human experiments) of sperm swimming in bulk medium, and not using the microfluidic device. The image sequences were analyzed using the computer-aided sperm analysis (CASA) plugin for ImageJ to obtain the motility parameters (Fig. 2(c)). Motility parameters were calculated to be compatible with the current standards used by CASA systems.[34,35] Similar to the usage of CASA, upon obtaining the motility data from the CASA plugin for ImageJ, the sperm trajectories were visually verified to ensure the accuracy of the data collected. The motility parameters calculated were defined for the motile sperm in the field of view as follows: (i) *Curvilinear velocity* (VCL): the sum of incremental distance between each two consecutive sperm position in the trajectory/the associated time difference (μm/s); (ii) *Straight line velocity* (VSL): the distance between the first and the last tracking point of the sperm in each trajectory/total duration of the track segment (μm/s); (iii) *Average path velocity* (VAP): time-average velocity of the sperm along its average path (μm/s); (iv) *Linearity* (LIN): VSL/VCL (%); (v) *Amplitude of lateral head displacement* (ALH): time-average deviation of the sperm head from the average path (μm); and (vi) C*urvature* (CUR): time-average curvature of sperm along its actual trajectory (1/μm).

2. Sperm viability test

New devices with no flow at 37 °C were used for the viability experiments. A pipette was used to inject 20 μl of raw, unstained, semen into the inlet of each device. The sperm were allowed 30 min to swim through the microfluidic device. To extract the sperm, the temporary seal layer was removed and the syringe array, from the extraction device, was immediately

pulled back to create a negative pressure and draw 10 μl of fluid from the microfluidic channels into the tygon tubing. The separated sperm were then transferred into vials for analysis. To stain the separated sperm and the raw sample, 1 μl of each of the working solutions for components A and B was added to each of the vials. After 5 min of incubation, 10 μl of the sample were collected and placed in a haemocytometer. The haemocytometer was mounted on the inverted microscope (Leica microscope) which was used to image the live (green fluorescence) and dead (red fluorescence) cells, as shown in Fig. 2(e). The live and dead sperms were then manually counted to determine the concentration and viability of the cells collected. Similarly, to measure the viability of the raw semen, 3 μl of each of the working solutions for components A and B were added to the raw semen at the start of the experiment. After the required 5 min incubation period for the stain to take effect, the concentration of live and dead cells were evaluated as per the protocol described above.

III. RESULTS AND DISCUSSION

The microfluidic penetration test, viability, and motility were compared for sperm swimming in both HA and MC media with about 20 cp viscosity (measured at 20 °C), mimicking the in vivo environment which can have viscosities reaching values above 200 cp[4]. A microfluidic device was used to create a highly controlled platform with which to perform these tests. The functionality of the device was achieved by self-propelled sperm swimming through the stagnant high viscosity medium in the main channel.

A. Microfluidic penetration and viability tests

The microfluidic penetration test was used to determine how far the sperm were able to travel within the media in order to determine whether the environment would impede sperm motility ($n \geq 8$). The final penetration of the bovine sperm after 25 min had elapsed is shown in Fig. 3(a). The results indicate that, for the same migration length, the average number of sperm in the MC medium is constantly higher than with the HA medium. As the penetration distances increase, there is a notable decrease in the number of sperm migrating those distances and an increase in the difference between MC and HA. For example, the number of sperm that migrated 4.5 mm in MC is 1.4 time higher than in the HA medium. This ratio increases to 3.6 for a migration length of 9.5 mm. Figure 3(b) shows the effects of time on sperm penetration in MC and HA media for a migration length of 7 mm. Results indicate that for both media, the number of sperm migrating 7 mm in the main channel increases with experimental time. More specifically, the number of sperm migrating this distance is consistently higher in the MC medium as compared with the HA medium.

The finding that a lower number of sperm were able to migrate the same distances over the same time in HA than in MC indicate that MC is preferable for motility-based sperm selection within the microfluidic device. It should be noted that as HA is a substance that is naturally present in the human body, such as fluids in eyes and joints, the results from the HA test might have physiological relevance. Our microfluidic penetration test used the principle of the Kremer test (human cervical mucus penetration test)[27] on the microfluidic scale, whereby sperm swim in the high viscous medium of their own volition. Although the test performed used a 12 mm microchannel, small semen volumes ($\sim$20 μl of semen), and a run time of 25 min, the observed distributions of the sperm along the microchannel were similar to previously published results using the Kremer test in MC medium with a comparatively longer channel and larger semen volume.[27] The similarities in the sperm penetration between the two studies validated the microfluidic penetration test developed here.

Figure 3(c) shows the concentration of sperm collected from each outlet after 30 min in comparison to the raw semen sample for the viability experiments with unstained bovine sperm ($n \geq 11$). Analogous to the penetration test results, the concentration of sperm collected from each outlet is higher for devices tested with MC as compared with HA. For both the MC and HA media, the sorted sperm concentration decreases with migration distances corresponding to each outlet channel. The longer migration lengths associated with each outlet, decrease the

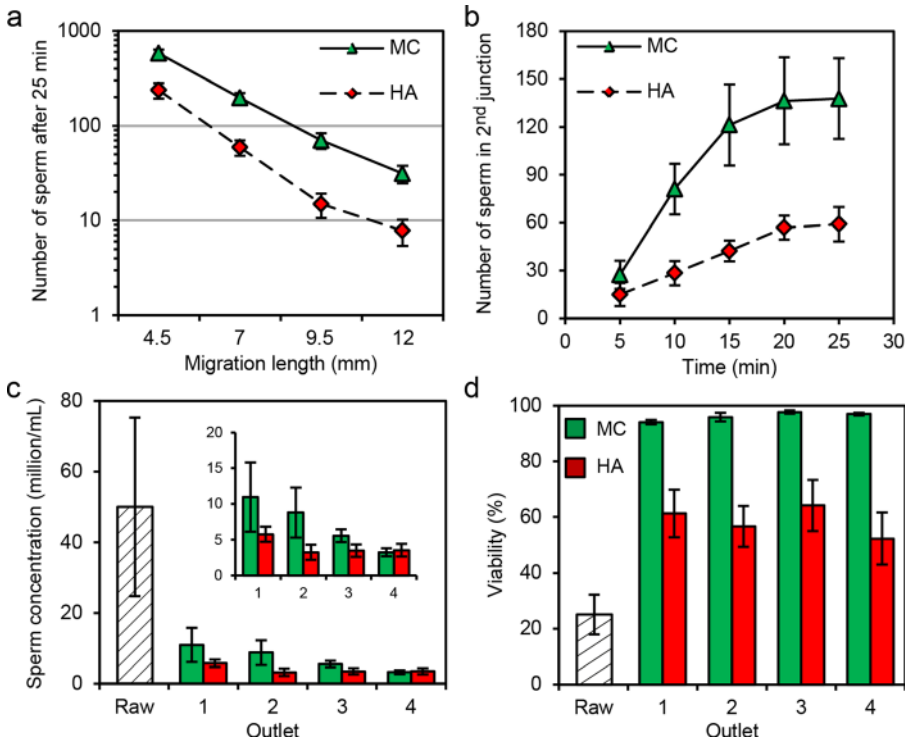

FIG. 3. Sperm penetration and viability test in the MC and HA media. (a) Sperm penetration in the MC and HA media after 25 min have elapsed. (b) Effect of time on sperm penetration in MC and HA media for 2nd junction. (c) Concentrations of the sperm collected after 30 min from devices filled with MC and HA media for experiments with unstained samples. Sperm concentration collected form outlets shown as inset. (d) Comparison of the percentage of live sperm collected after 30 min from devices filled with the MC and HA media. A two-way analysis of variance was conducted, yielding a P-value less than 0.005 ($n \geq 8$).

variation between selected sperm concentration from each outlet in MC and HA media. Specifically, the total concentration of sperm collected from the MC medium was higher than the concentration collected from the HA medium for outlets 1–3. The concentrations collected at outlet 4 were approximately the same for each medium.

Figure 3(d) shows the viability of sperm collected from each outlet after 30 min of swim time in the microfluidic device, in comparison with the raw semen sample. It can be seen that the MC medium yields a minimum of 94.1% ($\pm$0.8%) viability, where the HA medium yields only 52.3 ($\pm$9.3%) to 64.2% ($\pm$9.1%) viability ($n \geq 13$, P-value < 0.001). When comparing the percentage of live sperm selected using the microfluidic device with the HA and MC separately, the HA yielded a 108%–133% improvement in sperm viability, and the MC yielded considerably more improvement in sperm viability, 274%–289%. It is important to consider that the viability results are reported for sperm collected from different outlets (corresponding to four different migration lengths) at the same time (after 30 min swim time). Taken together, the data suggest that for shorter migration lengths greater number of sperm with lower progressive motility reach the outlet; however, these sperm will die faster than the lower number of sperm that migrated longer distances. Therefore, while there is a higher number of sperm at shorter distances, there are higher number of dead cells at the same time, and as a result the viability remains almost constant along the channel. When comparing both the HA and MC results to the raw semen, it is evident that selecting sperm using the microfluidic sorter and either of the media will yield a significantly higher percentage of live sperm than the raw semen which only has 25.1% ($\pm$7.1%) live sperm. These results indicate that selecting sperm with a microfluidic device and high viscous medium will in fact result in a subpopulation of sperm with higher viability than the raw semen.

In all the cases, a certain number of initially live sperm will die with time, making the percentage of live sperm collected less than 100%. Since it was ensured that no flow was present during the swimming time of the experiment, the results show that the rate of death of the sorted sperm is significantly lower in the MC medium than the HA medium. In the context of sperm viability, it should be noted that since migration length and experiment duration were similar for experiments with both media (and the viscosities the same), the observed increase in death rates for HA cannot be attributed to longer swimming times or longer total swimming lengths, but more likely that HA reduces sperm swimming velocity[36] and makes it more difficult to swim, causing sperm to expire at a higher rate as they migrate in the microfluidic device. The slower progressive motility of sperm in HA as compared with MC is attributed to the effects of HA on sperm which encourages hyperactivation and negatively affects progressive motility. In the context of the migration lengths, the number of sperm that migrated 7, 9.5, and 12 mm decreased by 75%, 74%, and 48%, respectively, as compared to the number of sperm at the previous migration length. Considering the viability rates and the numbers collected at each length, it is critical to note that sperm are collected at *different* lengths, at the *same* time. Thus the population of sperm collected at length 3 is not the same as the (relatively slower) population collected at length 2. The significant decline in the number of sperm at each migration length is attributed to a combination of (a) inherent differences in swimming speed, (b) a decrease in viability with swimming distance, and (c) the fact that not all of the viable sperm are motile. It is also of particular interest that although the total concentrations of sperm were equal at outlet 4, the concentration of live sperm was substantially higher with the MC medium than with the HA medium. These results further suggest that MC is a preferred medium over HA for selection of viable sperm based on their motility.

B. Microfluidic sperm motility test

Figure 4 details the motility parameters of bovine sperm in MC and HA media after 25 min have elapsed ($n \geq 6$). Results indicate that the MC medium yields a significantly higher percentage of motile sperm than the HA medium, for all migration lengths (Fig. 4(a); P-value less than 0.005). Specifically, the respective motility for sperm migrating 4.5, 7, 9.5, and 12 mm in MC medium were higher than sperm migrating the same lengths in the HA medium by 44% (84.1% to 58.1%), 63% (75.0% to 45.9%), 151% (59.1% to 23.5%), and 194% (47.7% to 16.2%), respectively. The non-motile sperm seen in the device were motile at the beginning of the test, however, as they migrated along the channel some sperm tire and become non-motile. The lower motility of sperm in HA as compared to MC is attributed to the slowing effect of HA on sperm swimming velocity.[36] The higher sperm motility in MC as compared with HA indicates that MC may be preferable for motility-based sperm selection. In addition, for both media, the percentage motility linearly decreases with increasing distance from the inlet ($R^2 = 0.97$), indicating that sperm with highly progressive motility that swim longer distances over a fixed time will substantially lose their motility.

Sperm motility analysis in HA and MC media revealed higher values of VCL, VAP, and VSL in MC as compared with HA, as shown in Figs. 4(b)–4(d). Specifically, the respective VCL values for sperm migrating 4.5, 7, 9.5, and 12 mm in MC were 1.5, 1.4, 1.13, and 1.05 times higher than sperm migrating the same distance in HA. The results indicate that the variation in sperm curvilinear velocity caused by the medium decreases as sperm migrate longer distances in the main channel. In contrast to VCL, VAP and VSL values are always significantly higher in MC as compared with HA by 39%–80% and 25%–98%, respectively (P-value < 0.05), while VSL for sperm migrating in HA slightly decreases for longer distances. These observations indicate that sperm swim with highly linear progressive motility in MC as compared with HA. When considering assisted reproduction, a sperm's ability to exhibit progressive motility is essential to ensuring it can complete the trajectory and fertilize the ovum. The fact that the sperm migrating longer distances in HA exhibit both a faster swimming speed and a lower net progress as compared with the ones migrating shorter distances indicates that HA encourages hyperactivated swimming which negatively affects progressive movement. Taken together, the

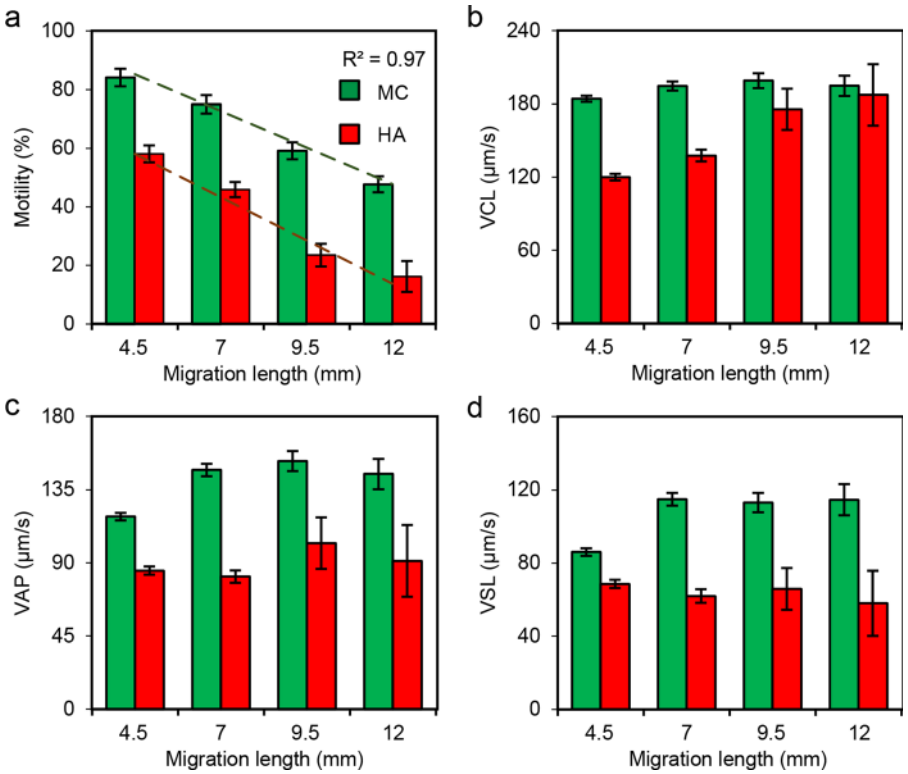

FIG. 4. Sperm motility parameters in MC and HA media within the device. (a) Motility, (b) curvilinear velocity (VCL), (c) average path velocity (VAP), and (d) straight line velocity (VSL) of bovine sperm as a function of migration length in the main channel after 25 min have elapsed ($n \geq 8$).

higher vitality and linear progressive motility of sperm in MC as compared with HA, further indicate that MC is the preferred medium for motility-based sperm selection. The microfluidic device used herein was solely for the purpose of evaluating the media for motility-based sperm selection and more optimized microfluidic designs are possible for high-throughput sperm selection.

C. Human sperm motility in MC medium

As the preferred medium, motility parameters were tested for human sperm in bulk MC to quantify the potential impact of the medium on human sperm. Figure 5 shows the motility parameters of human sperm in the MC medium with viscosity ranging from 20 cp to 250 cp ($n \geq 15$), mimicking the natural range of viscosity *in vivo*.[6] The velocity values reported for human sperm are lower than the velocities for bovine sperm due to the inherent nature of human sperm to swim slower than bovine.[37,38] The results indicate that increased viscosity reduces VCL, VAP, and VSL more than 43%, 48%, and 54%, respectively (Figs. 5(a)–5(c)). The decrease in sperm swimming velocities observed is attributed to the increase in the viscosity-induced drag acting on the cell bodies at higher viscosities. We observed that LIN is strongly correlated with viscosity (Fig. 5(d)), indicating that sperm swim straighter at higher viscosities. This can be explained by the increase in viscosity of the swimming medium dampening the transverse component of the flagellar wave and restricting the yaw in swimming trajectory, resulting in higher LIN. The results also demonstrate a strong negative correlation between ALH and viscosity (Fig. 5(e)), suggesting lower flagellar wave amplitude at higher viscosities. This decrease in ALH is attributed to higher drag forces acting normal to the flagellum by increasing the viscosity. Finally, the CUR of the swimming trajectory also decreases at higher viscosities (Fig. 5(f)), which indicates an increase in symmetry of the flagellar wave.

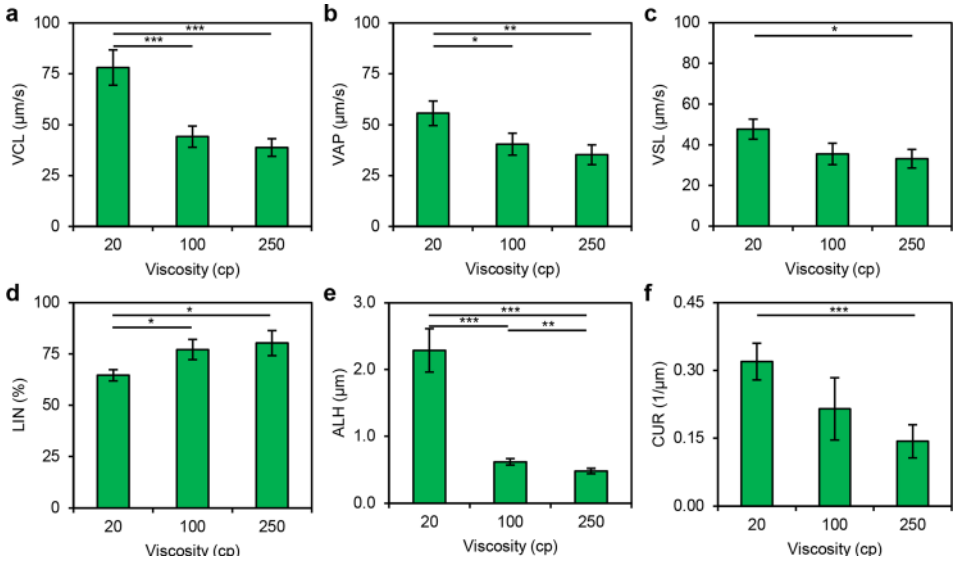

FIG. 5. Human sperm motility parameters in MC medium as a function of viscosity. (a) curvilinear velocity (VCL), (b) average path velocity (VAP), (c) straight line velocity (VSL), (d) linearity (LIN), (e) amplitude of lateral head displacement (ALH), and (f) curvature (CUR) for human sperm swimming in MC medium with viscosity ranging from 20 cp† to 250 cp†. P-values were determined by the two-tailed t-test, * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ ($n \geq 15$). † all viscosity values specified at 20 °C.

Taken together, these results show that human sperm swim slower and straighter as the viscosity of the swimming medium increases.

IV. CONCLUSION

We used a microfluidic device to assess the effects of high viscosity media made with HA and MC on bovine and human sperm motility and viability (sperm transferred directly from cryoprotectant). The results reveal that an MC medium yields a higher number of sperm penetrating the medium than those penetrating the HA. Analogous to the penetration test results, MC medium results in a higher concentration of sperm collected from each outlet as compared with HA. With respect to the viability of the sperm collected from the device, the MC medium yielded a sperm subpopulation with more than a 274% increase in the sperm viability as compared to the raw semen, while the HA medium resulted in less than 133% increase in the sperm viability. When comparing both the MC and HA results to the raw semen, selecting sperm using the microfluidic sorter and either of the media yields a significantly higher percentage of live sperm than the raw semen. In addition to viability, our results indicated significantly higher motility parameters for sperm in MC medium as compared with HA medium, while the percentage motility was negatively correlated with the distance that sperm migrated in the main channel. The higher vitality and progressive motility of sperm in MC as compared with HA indicate that MC is a preferred medium for motility-based sperm selection. Experiments with human sperm in MC, indicated that an increase in the viscosity of the swimming medium results in a slower swimming velocity but a straighter swimming trajectory. Taken together, the results obtained indicate that MC is preferred to HA when implementing a high viscosity environment *in vitro* for sperm assessment or selection based on motility.

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