Screening of Sperm Velocity by Fluid Mechanical Characteristics of a Cycloolefin Polymer Microfluidic Sperm Sorting Device

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Abstract

The microfluidic sperm sorting (MFSS) device is a promising advancement for assisted reproductive technology (ART). Previously, poly(dimethylsiloxiane) and quartz MFSS devices were developed and used for intracytoplasmic sperm injection (ICSI). However, these disposable devices were not clinically suitable for ART. To potentiate the clinical application of ART, a cycloolefin polymer MFSS (COP-MFSS) device was developed. By micromachining, two microfluidic channels with different heights and widths (chip A: 0.3 × 0.5 mm; chip B: 0.1 × 0.6 mm) were prepared. The sorted sperm concentrations were similar in both microfluidic channels. Linear velocity (LV) distribution using the microfluidic channel of chip B was higher than that of chip A. Using confocal fluorescence microscopy, it was found that the highest number of motile sperm swam across the laminar flow at the bottom of the microfluidic channel. The time required to swim across the laminar flow was longer at the bottom and top of the microfluidic channels than at the middle of the channels because of the low fluid velocity. These results experimentally demonstrated that the width of microfluidic channels should be increased in the region of laminar flow from the semen inlet to the outlet for unsorted sperm to selectively recover sperm with high LV.

Key Words: microfluidic sperm sorting, laminar flow, motility, linear velocity

Introduction

Microfluidic sperm sorting (MFSS) devices are chip devices used for selecting motile sperm during assisted reproductive technology (ART) (Schuster et al., 2003; Cho et al., 2003; Wu et al., 2006). As shown in Fig. 1A, two gravity-driven laminar flows within the microfluidic channel are important for sperm selection. The fluid flowing through the semen inlet (A) and the medium inlet (B) should move parallel to each other and then exit through their respective outlets (A→C and B→D). Sperm are sorted on the basis of their ability to swim across the streamline into the medium stream, and hence only motile sperm are recovered in the outlet D. Some procedures may takes up to 2 h for semen processing by conventional protocols, such as density gradient centrifugation and following swim-up (Jeyendran et al., 2003). However, with MFSS, embryologists can perform a 1-step sorting protocol without centrifugation and complete processing within 30 min (Hughes et al., 1998). Reducing the treatment time and eliminating the centrifugation step minimizes the exposure of sperm to concentrated reactive oxygen species (ROS) and prevents DNA fragmentation (Mortimer, 1991). Schulte et al. previously reported that DNA fragmentation was significantly decreased in MFSS-treated sperms (Schulte et al., 2007). On the basis of these results, MFSS can be used in clinical semen processing protocols for efficient intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF).

The first MFSS device was fabricated from poly(dimethylsiloxiane) (PDMS), which is a silicone-elastomer. However, this material is not suitable for clinical use because its safety is not guaranteed (Schuster et al., 2003; Cho et al., 2003; Wu et al., 2003). Subsequently, a quartz MFSS device was developed for an ICSI clinical study (Shibata et al., 2007). In these studies, the effect of MFSS treatment to conventional semen processing and the impact on fertilization in the same patient

was assessed. It was found that the fertilization rates after sperm washing processes (conventional centrifugation only) with and without the quartz MFSS device were 59.6% (59/99) and 46.8% (51/109), respectively (Shibata et al., 2007). However, as the quartz device is not disposable because of its high production cost, development of a device made of plastic for disposable use is desired.

To overcome this problem, a cycloolefin polymer (COP)-MFSS device was produced (Fig. 1B). Because COP is an approved material for clinical use, it is believed that COP-MFSS would also be suitable for clinical use. The microfluidic channel dimensions can be modified by micromachining the plastic chip device. Two microfluidic channels with different dimensions by micromachining were fabricated, following which their relationship with computer-assisted sperm-motility analyses were investigated. To obtain information about sperm sorting at different focus depths of the microfluidic channels, the number of sorted sperm in various recording planes was surveyed using confocal fluorescence microscopy. On the basis of these experimental results and previous numerical simulations, the efficiency and characteristics of sperm separation in the microfluidic channels of the MFSS device were discussed.

Materials and methods

Microchannel Dimensions

The COP-MFSS device (Strex Inc., Osaka, Japan) as shown in Fig. 1B was used. Top and side views of the MFSS device are shown in Fig.s 1C and 1D, respectively. The circles labeled A–D in Fig. 1C are the liquid reservoirs. D_A, D_B, D_H, and h_{MC} are the widths of the channels (from A to C and from B to D, respectively), the difference of head-water, and the height of the microchannels, respectively. Fig. 1E shows the difference in channel dimensions between the two MFSS devices. The other dimensions of the two MFSS devices, such as the dimension of the reservoirs, are the

same. Confocal imaging of the microfluidic channel filled with approximately 0.1 mM fluorescein isothiocyanate (FITC) solution in distilled water was performed using a FluoView 1000 (Olympus Co. Ltd., Tokyo, Japan). Consequently, the fluorescent, cross-sectional images in Fig. 1E were constructed.

Semen and sperms

Human semen was supplied by a healthy fertile male who was aged 32-35 years. Ejaculates of semen were obtained before these experiments. Samples after ejaculation were immediately incubated at a room temperature or 37 °C for 0.5-1 hour to liquefy. Each ejaculated semen was carefully diluted with HTF medium (Irvine Scientific, Santa Ana, CA) to 5 times before the use in MFSS. The motile sperm concentrations after the dilution were approximately 1.0×10^7 cells/ml. We used 5 ejaculated samples for the experiments over a three year period.

Sperm Sorting Protocol

The experimental protocol of sperm sorting by MFSS devices used was as follows. To make the streamline in the center of the microfluidic channel, HTF medium of 200, 200, and 1200 μl was dispensed into inlets D, C, and B, respectively, and 200-800 μl of diluted semen was dispensed into inlet A. After confirmation that the laminar flow from inlet A did not reach inlet D, sperm motion was tracked by a sperm-motility analysis system (SMAS) (Kaga Electronics Co. Ltd., Tokyo, Japan), using a frame rate of 60 per second.

Fast Fluorescent Scanning in MFSS Device

For fluorescent live-imaging of motile sperm in the COP-MFSS device, human sperm were

stained with 10 μM of Fluo-4 AM (Invitrogen, Carlsbad, CA). The intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) was measured in stained sperm from COP-MFSS devices using a fluorescence microscope (Olympus, IX70) with 40× (Olympus, Fluo APO 40) objectives attached to a CSU10 confocal scanner (Yokogawa Electric Co., Tokyo, Japan) (Cho et al., 2003). For confocal fluorescence microscopy, the fluorescence intensity was correlated to $[Ca^{2+}]_i$, the excitation wavelength was 488 nm and the emission was detected at 510 nm. The time resolution of each frame was approximately 30 ms. In these experiments, the confocal plane was fixed during recording. The COP-MFSS device with a 0.2-mm height microfluidic channel was used for the measurement.

Statistical Analyses

Student's *t* test was used to determine the difference of the velocity distributions between the two groups, with P values <0.05 considered statistically significant. Pearson's product–moment correlation coefficients were used in observed motile sperm velocities, with P values <0.001 considered statistically significant (Zderic et al., 2002).

Results

Sperm Concentration and Motility

The unprocessed semen samples used in this experiment had a mean sperm motility of 53.0%. Table 1 includes a summary of the motility and concentration of motile sperm both 10 min before and after the MFSS experiments. It was found that more than 90% of sperm were motile in both chips. Recovery rates using the COP-MFSS devices were 0.2~0.3% (Table 1).

Assessment of Linearity and Linear Velocity

Subsequently, the relationship between the LV distribution and the microchannel dimensions were investigated. As shown in Fig. 2A and 2B, the LVs of sperm separated by the COP-MFSS device increased compared to untreated sperm, which is consistent with the previous results for the quartz MFSS device (Shibata et al., 2007). These trajectories showed that the linearity of the selected sperms has improved. Specifically, the average LV significantly ($P < 0.01$) increased from 21.0 μm/s (n = 145, SEM 1.7) in the unsorted outlet to 51.7 μm/s (n = 172, SEM 2.1) and 59.5 μm/s (n = 79, SEM 1.6) in the sorted outlets of chips A and B, respectively (Fig. 2C). The LV of the sorted sperm in chip B was determined to be significantly higher than the LV in chip A ($P < 0.01$). These results suggest that sperm with high LV can be selectively sorted using the COP-MFSS device.

Confocal Fluorescent Images of Motile Sperm in the Microfluidic Channel of the COP-MFSS Device

It is important to demonstrate the difference of sperm sorting efficiencies for different heights of microfluidic channels due to the laminar flow velocity distribution. The fluorescent images that revealed a zigzag motion in the COP-MFSS microfluidic channels under the 40× magnification were considered heads of motile sperm. Twenty minutes after loading the semen sample, the number of sperm that swam across field of view and the maximum velocities of the fluid during the 1-min recording of the bottom, middle, and top of the channel (Table 2) were determined. The highest number of motile sperm was present at the bottom of the adjacent inlet. At both the top and bottom of the COP-MFSS channel ($z = 0$ and h_{MC}), the fluid velocity had decreased and sperm readily swam across the interface. These results suggest that the number of sorted motile sperm obtained depends both on the flow velocity and the concentration of motile sperm at each height in

the COP-MFSS microfluidic channel.

Discussion

Comparison of Motilities and Recoveries using PDMS Channel, Quartz Channel, and the COP-MFSS Device

The results of recovered motilities using the COP-MFSS device were similar to the results obtained from PDMS (Cho et al., 2003) and quartz channels (Shibata et al., 2007). Because the number of sorted motile sperm for both chips was similar, these results suggest that the COP-MFSS device can be applied to ICSI and micro-scale IVF techniques, which require 10^3 motile sperm in 10 μl (Smith et al., 2007). However, the number of motile sperm obtained in this experiment is insufficient for conventional IVF, which requires 10^5 motile sperm in 1 ml (Smith et al., 2007). Recovery using PDMS-MFSS device was approximately 40 % (Cho et al., 2003), while that using the COP-MFSS device was 0.2 %. Possible reasons for the decrease in the recovery were the increased fluid velocity in the microfluidic channels and the larger volume of the inlet reservoir (~1 ml) in the COP-MFSS device. When we performed the sorting experiments of the COP-MFSS device for 10 min, and 40 % of diluted semen in the inlet was treated. Because sperm concentration before sorting and treatment times were not reported in the printed matter (Cho et al., 2003), we cannot directly compare the number of sorted motile sperms. The number of sorted motile sperm to the reservoir D without centrifugation treatment would increase to approximately 10 times of that observed in cases of the PDMS and quartz MFSS devices due to the larger volume of the inlet reservoir of COP-MFSS.

Possible Reason for Efficient Separation of Motile Sperm at the Bottom

Motile sperm could be separated most efficiently at the bottom of the COP-MFSS channel because the fluid velocity was slow enough to allow sperm to swim across the interface, and the motile sperm were concentrated by gravity and sperm/geometry interactions. Because the density of mature human sperm (1.10 g/cm^3) is greater than that of the buffer, human sperm swim down to the bottom of devices (Kaneko et al., 1986). Because the observed velocity in the z-direction was approximately 1 μm/sec, it is difficult for sperm to swim up once they have swum down. Furthermore, Lopez-Garcia et al. reported that bull sperm tended to preferentially swim along the walls, including bottom and ceiling, of their microfluidic device (Lopez-Garcia et al., 2008). A similar trend was observed in human sperm. In the COP-MFSS device, the fluid velocity at the wall is almost zero. Based on the density of human sperm, their velocity in the z-direction, and their tendency to swim along the walls of devices, the study concluded that human sperm concentrates at the bottom of microfluidic channels with decreased fluid velocity. This characteristic is an important consideration when attempting to increase the number of sorted motile sperm obtained.

Relationship between Fluid Velocity and LV of Sorted Sperm

The correlation between the LV of sorted sperm motion (v_s) and that of the fluid (v_f) for the sorted motile sperm was examined. The relationship of these parameters and observed velocities of the sorted sperm (v_{xobs} and v_{yobs}) using microscopy are shown in Fig. 3A, where ϕ is the angle between v_f and v_s. It was unable to numerically calculate v_s and v_f because they are defined by the following equations (1) and (2). When there are three parameters (v_s, v_f, and ϕ), it is impossible to solve the two equations.

$$v_{yobs} = v_s \sin\phi \quad (1)$$

$$v_{xobs} = v_f + v_s\cos\phi \quad (2)$$

The trajectories of the motile sperm in the laminar flow from B to D can be approximated as a line, because as mentioned above the linearity of the sorted sperm motion is higher than that of unsorted sperm. The motion in the z-direction (height) was not considered in this discussion because the sperm velocity in the z-direction was significantly lesser than those in the x- and y-directions (Corkidi et al., 2008).

Fig. 3B shows the plot of the observed velocities of the sorted sperm (v_{xobs} and v_{yobs}) from the confocal microscopy (red triangles) and bright-field (black squares) images to examine the correlation between v_s and v_f, although these parameters cannot be numerically determined. This graph suggests significant correlation between $\log(v_{yobs})$ and $\log(v_{xobs})$ ($P < 0.001$). Based on the result of the velocity of sorted sperm shown in Fig. 2C, the maximum v_s is approximately 100 μm/s. The value of v_f is between the values of v_{xobs} and v_{xobs}–100 μm/s. To sort sperms with higher v_{yobs} more than 20 μm/s, v_{xobs} more than 200 μm/s is required. A fluid velocity (v_f) dependence of sperm sorting was observed and consistent with the previous simulation results of the separation dependence on fluid velocity (Hyakutake et al., 2009). The data shown in Fig. 3B also indicate that higher v_s was sorted when v_f is larger, and that human motile sperm were unable to swim across the fluid when the velocity (v_f) was over ~1 mm/s, and that the maximum v_f was 1 mm/s.

Suitable Microchannel Dimensions to Sort Sperm with High LV

The aim of this study is to optimize the dimensions of the microfluidic channels in the COP-MFSS device by characterizing the fluid velocities and motility of sorted sperm. After COP-MFSS treatment, when h_{MC} decreased and D_A increased, the LV distribution of sorted human sperm

increased. These analyses of the trajectories of sorted motile sperm after COP-MFSS treatment indicated a positive correlation between the velocity of the sperm and the velocity of the fluid (Fig. 3B). These results demonstrated that motile sperm cannot swim across a fast fluid velocity of over 1 mm/s. The findings of this study can be used to develop a microfluidic channel that can sort sperm with high LV and/or a higher concentration of motile sperm.

Using confocal, fluorescent live-imaging, it was demonstrated that motile sperm cannot swim across the interface in the middle of the COP-MFSS microfluidic channel in the yz plane. Furthermore, it was also found that when the h_{MC} increases the maximum velocity of the fluid at the center also increases. However, the average LV of sorted motile sperm from chip A (h_{MC} = 0.3 mm) was determined to be lower than that from chip B (h_{MC} = 0.1 mm). On the basis of these results, it was concluded that increasing h_{MC} is not effective for sorting motile sperm with higher LVs. One explanation for this may be that the region of the flow velocity below 1 mm/s in the microfluidic channel with an h_{MC} of 0.1 mm is similar to the channel with an h_{MC} of 0.3 mm. Another possible reason for the decrease in the chip A average LV in is the smaller width of its microfluidic channel (D_A = 0.25 mm) compared to that of chip B (D_A = 0.28 mm). Therefore, it is suggested that D_A should be increased to recover sperm with a high LV, and that the result can explain these experimental results of the LV distributions.

The method was explored to increase the number of sorted sperm generated by the MFSS device. In this study, D_A:D_B was set to 1:1 in the COP-MFSS device. The numerical simulation suggested that the number of motile sperm increased when $D_A/(D_A + D_B)$ decreased (Hyakutake et al., 2009). Currently, a minimum of 5×10^5 sorted motile sperm are required for conventional IVF (Smith et al., 2007). Therefore, the design of microfluidic channels for COP-MFSS devices should aim to satisfy this motile sperm concentration (10^5 cells/ml) criterion. It is technically difficult to

increase the length of the channel portion where the flow runs parallel (L) because the two laminar flows are not stabilized using a microfluidic channel with a large L. Thus, to effectively increase the number of sorted motile sperm, the focus should be on decreasing $D_A/(D_A + D_B)$ and the height of the inlets and outlets. An additional method to improve the number of sorted sperm in a time efficient manner would be to run parallel microfluidic sperm sorting channels with a common collection chamber for the sorted motile sperm (chamber D Fig 1) which would increase their yield closer to 5×10^5 needed for conventional IVF. However, to control laminar flows in the parallel microfluidic sperm sorting channels without instrument such as pumps, the structure of the channel connecting the sorting channels and collection chambers should be optimized.

Physiological and Clinical Importance using the MFSS Device

Physiological and clinical importance using the MFSS device was discussed. The results suggest that the MFSS device can selectively recover sperm with higher LV based on the fluid mechanical character of the device. Because it is reported that LV were significantly correlated with *in vitro* fertilization rate (Liu et al. 1991), the MFSS device could sort sperm that can fertilize under both physiological and non-physiological conditions. Sperm motility is strongly related with intracellular calcium concentration ($[Ca^{2+}]_i$) in the cell, and capacitation and acrosome reaction (AR) are regulated by $[Ca^{2+}]_i$ (Costello et al. 2009). It should be demonstrated in future that sperm with higher LV sorted using the MFSS device are sperm to effect fertilization under physiological condition by analyzing $[Ca^{2+}]_i$ and AR of the sorted sperms.

The PDMS-MFSS device produced a decrease in DNA fragmentation compared to conventional semen processing techniques such as swim-up, with the sperm isolation having the highest motility and the lowest level of DNA fragmentation (2007 Schulte et al.). The COP-MFSS

device could also decrease in DNA fragmentation and increase in fertility rates as well as the PDMS- and quartz-PDMS devices, because the sorting mechanism of the COP-MFSS device is same as that of PDMS-MFSS device. Clinical multi-center studies of DNA fragmentation assays are required to demonstrate the clinical importance of the MFSS device by comparison between conventional semen processing techniques as much as possible and MFSS.

Finally, benefits of the MFSS devices in clinical use were mentioned. Regardless of sperm concentration in semen, the MFSS devices can sort sperm with a clean highly motile sperm population. MFSS can be used for both motile oligozoospermic and normospermic samples. However, semen with concentration lower than approximately 10^4 cells/ml could not be sorted according to 0.2% of the recovery. Although it is difficult at present to apply the COP-MFSS for conventional IVF based on the number of the collected sperms, one of the benefits using the MFSS is non-laboring selection of motile sperms for injection to oocytes in the clean highly motile sperm population for ICSI protocol. Furthermore, in porcine IVF, the rate of monospermic fertilization using MFSS-IVF system significantly increased than that using standard IVF, resulting in improved efficiency of embryos developing to the blastocyst stage (Sano et al. 2010). Reduction of polyspermic fertilization in IVF would be a one of benefits of the MFSS devices in clinical use.

Conclusion

It was experimentally demonstrated that D_A, and not h_{MC}, should be increased to recover sperm with higher LV selectively, whereas decreases in $D_A/(D_A + D_B)$ and the height of the inlets and outlets would effectively increase the concentration of motile sperm. Because the fluid velocity is low at the top and bottom of the microfluidic channels, the time to swim across the laminar flow also increases, and the highest number of motile sperm swam across the laminar flow at the bottom

of the microfluidic channels.

FIGURE CAPTIONS

Fig. 1. (A) Schematic presentation of the MFSS principle. An illustration of two laminar flows (A→C and B→D) in the MFSS channel. (B) A photograph of a COP-MFSS chip used in this study. (C, D) Definition of the microfluidic channels parameters. Axes x, y, and z are length, width, and height, respectively. Top and side views are shown in (C) and (D), respectively. (E) Reconstructed, fluorescent, cross-sectional images of the microfluidic channels in the chips A (left) and B (right).

Fig. 2. Images of motile sperm tracking (A) before MFSS treatment and (B) in reservoir D of the COP-MFSS microfluidic channel. (C) LV distributions analyzed from the images. Yellow bars represent the percentages of sperm with the indicated velocity distribution without COP-MFSS treatment. Red and blue bars represent the percentages of sperm with the indicated velocity distribution in reservoir D of chips A and B, respectively.

Fig. 3. (A) The definition of the parameters (v_{xobs}, v_{yobs}, ϕ, v_s, and v_f) in this study. (B) The correlation of v_{xobs} (horizontal axis) and v_{yobs} (longitudinal axis) from confocal (red triangles) and bright-field (black squares) microscopic observations. The value of v_f is between the values of v_{xobs} and v_{xobs}–100 μm/s.

Table 1. Sperm concentration and motility before and after COP-MFSS treatments

	Diluted semen	Chip A (Reservoir D)	Chip B (Reservoir D)
Motility (%)	53	90	100
Concentration (cells/ml)	1.1×10^7	2.0×10^4	1.3×10^4
Recovery (%)[a]	-	0.3	0.2

[a] Recovery was defined as the ratio of the number of motile sperm in the motile sperm outlet. Reservoir D to the total number of motile sperm in the sperm sample inlet reservoir A.

Table 2. Average number of motile sperm and maximum fluid velocities observed in confocal planes (n =5)

Focus Position	Average number of motile sperm swimming across the interface	Average of observed maximum fluid velocity (mm/s)	Average number of motile sperm in the inlet A
Top	8.2 (SEM 2.1)	0.38 (SEM 0.11)	5.2 (SEM 1.0)
Center	0 (SEM 0)	1.39 (SEM 0.48)	5.8 (SEM 2.1)
Bottom	12.3 (SEM 2.5)	0.56 (SEM 0.13)	14.6 (SEM 4.2)

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