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Hydrophobic Silicone Elastomer Chamber for Recording Trajectories of Motile Porcine Sperms without Adsorption

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Abstract. Motile porcine sperms adhere to hydrophilic materials such as glass and plastics. The adsorption of sperms to a hydrophobic poly(dimethylsiloxane) (PDMS) membrane is less compared with that to glass. We investigated the linear velocity (LV) and amplitude of lateral head displacement (ALHD) of motile porcine sperm on glass and PDMS preparations using computer-assisted sperm analysis (CASA). Significant decreases were observed in the 15-min LV ($P<0.05$) and ALHD ($P<0.05$) in motile porcine sperm on glass preparations compared with those on PDMS preparations. These differences were due to adsorption of the head and/or neck to hydrophilic substrates. Because of the elasticity of PDMS, we propose that a PDMS membrane should be used for CASA. To investigate the dynamics of motile porcine sperms with microfluidics, we do not recommend plasma treatment to bond PDMS and glass in the microchannel preparation; instead, we suggest that a PDMS molding process without plasma treatment be used for preparation of microfluidic channels.

Key words: Adsorption, Porcine sperm motility, Silicone elastomer, Trajectories

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Sperm motility analysis is a representative method for evaluation of male fertility, since motility correlates with viability [1–4]. The conventional method, commonly referred to as Computer-Assisted (Aided) Sperm Analysis (CASA), to records motility and linear velocity (LV) utilizing a microscope with a charge coupled device [1–3]. The advantage of CASA over manual observation is the absence of subjective calibration [1]. It is difficult to record trajectories of motile porcine sperms and investigate LV related to fertility because sperms often adsorb to glass and plastic, which remarkably decreases their motility. To record the trajectories of motile sperms and investigate their velocity distribution quantitatively under a microscope, the use of transparent materials that do not promote adsorption of motile sperms is necessary.

For observation of motile sperms, diluted semen is usually sandwiched between hydrophilic glass slides [5]. The trajectories of human and bull sperms can be recorded using this glass preparation; however, it is difficult to record the trajectory of motile porcine sperms, because they adsorb to glass and hydrophilic plastics such as poly(methylmethacrylate) (PMMA) [6]. We hypothesized that it may be possible to record the trajectory of these sperms using transparent materials with high hydrophobicity represented by a high contact angle (>90 degrees) to water droplets (Fig. 1).

Hydrophobic silicone elastomer poly(dimethylsiloxane) (PDMS) having a contact angle of 110 degrees is a key material capable of extending device applications for reproductive technology because it is nontoxic, transparent, inexpensive, and easy to handle [7–11]. PDMS microfluidic devices prepared by molding the microstructure and bonding the cured structure with a cover or slide glass can be used for manipulation and culture of cells to investigate their physiological functions [7–9]. Microfluidic channels are used for *in vitro* fertilization in the case of low sperm number (>10^5 cells) and for *in vitro* culture to mimic the oviduct environment [7, 11, 12]. Lopez-Garcia *et al*. observed bull sperm motions without adsorption to glass substrates in glass-bottom PDMS microchannels [12]. Despite previous documented applications, there are few practical applications for PDMS membranes combined with CASA in routine analysis.

In this study, using a PDMS preparation, we were able to record the trajectories of motile sperms without adsorption and compare the sperm motility parameters. Furthermore, we found that to observe motile porcine sperm dynamics using microchannels, the PDMS chamber should be prepared without oxygen (O_2) plasma treatment. This technology can be applied to recording live imaging and the mechanics of motile porcine sperms that tend to adhere to hydrophilic materials [13].

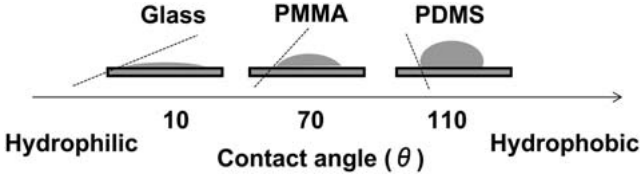

Fig. 1. Definition of contact angles to water droplets on different materials. Materials with smaller contact angles are hydrophilic, while those with greater contact angles are hydrophobic.

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Materials and Methods

Preparation of fresh boar spermatozoa

Semen-rich fractions (about 50 ml) were collected from four Berkshire boars (aged 25–31 months) at a local experimental station using the glove–hand method. The samples were diluted five times with modified Modena solution [14]. The diluted semen samples were then transported to the laboratory at 26–32 C within 2 h of collection. Semen samples were diluted to a concentration of 1×10^8 cells/ml with modified Modena solution containing 5 mM cysteine and 20% (v/v) boar seminal plasma. We used fresh sperm for the following experiments within a week of ejaculation. This preparation follows that outlined in a previous report [15].

Preparation of the silicone elastomer chamber and microfluidic channels

A PMMA mold was fabricated for the recording chamber using a conventional mechanical microdrilling process (MDX-40; Roland, Osaka, Japan). PDMS slabs and membranes with microstructures were prepared by casting prepolymer (TSE 3032; Momentive Performance Materials, Tokyo, Japan) at a 1:10 curing agent-to-base ratio against positive relief features [10, 16]. After degassing liquid PDMS with a vacuum pump for at least 30 min, the prepolymer was cured at 70 C for 1 h under normal atmospheric pressure. Cured PDMS has a highly cross-linked 3D structure. The microstructure of the PMMA mold was transferred to the cured PDMS.

To prevent overlap of motile sperm images, we designed the preparation to decrease the focal depth. Semen was sandwiched with two PDMS sheets (Figs. 2A and B). Due to the elastic properties of the PDMS, the lower membrane was deflected by the weight of the semen. The flat surface of the upper membrane was turned up and faced across it (Figs. 2C and D). The thickness of the semen was approximately 0.1 mm (Fig. 2D), and we confirmed no overlap of sperm images (Fig. 2E). With this preparation, we were able to record trajectories and analyze the distribution of sperm motility parameters. The PDMS membrane can be reused after washing until it breaks. However, we recommend disposing of the sheets to avoid artifacts, such as dispensable particles, on recorded images and video. Cross-sectional images were recorded by a VHX1000 microscope (Keyence, Osaka, Japan) with a tilt angle of 90°.

Experiment 1: comparing the absorption of motile porcine sperm to different materials

A 2 μl aliquot of fresh semen was sandwiched between glasses or PDMS membranes without and with O_2 plasma treatment. Using a BM ×10 lens (Nikon, Tokyo, Japan), sperm and particle motions were tracked with a sperm motility analysis system (SMAS; Kaga Electronics, Tokyo, Japan). This system comprised a high-resolution digital scanning camera, a personal computer with a digital frame grabber and image processing software, and a computer monitor [17]. The frame rate for sperm tracking using the SMAS was 60 frames per second. The experiments were continued until the semen dried. The number of replications of SMAS imaging was 3 and enough to explain the results.

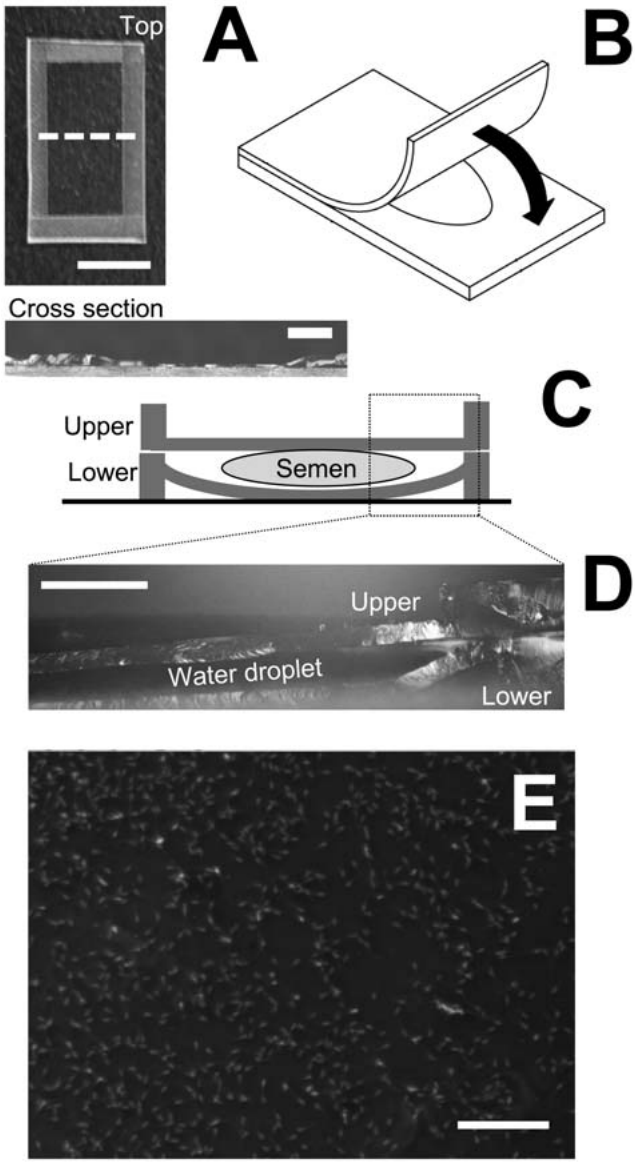

Fig. 2. A: PDMS membrane for preparation, with an area of 0.5 × 1 mm^2. The scale bars in the top and cross-sectional images represent 5 and 0.5 mm, respectively. B: Method of sandwiching semen between the two membranes with a thickness of 0.1 mm. C: Cross-sectional image for recording the trajectories of motile sperms to decrease the focal depth to approximately 0.1 mm. Dark and light gray objects represent the PDMS membrane and semen, respectively. D: Cross-sectional image of the preparation. A water droplet is sandwiched with two PDMS membranes, as indicated in Fig. 2A. The scale bar represents 0.5 mm. E: Sperm in this preparation is displayed by CASA. Overlap of motile sperm is not observed in this frame.

Experiment 2: performance of optimized chambers and sperm motility parameters

The preparation method to investigate sperm motility parameters was the same as that in Experiment 1. Based on the recorded

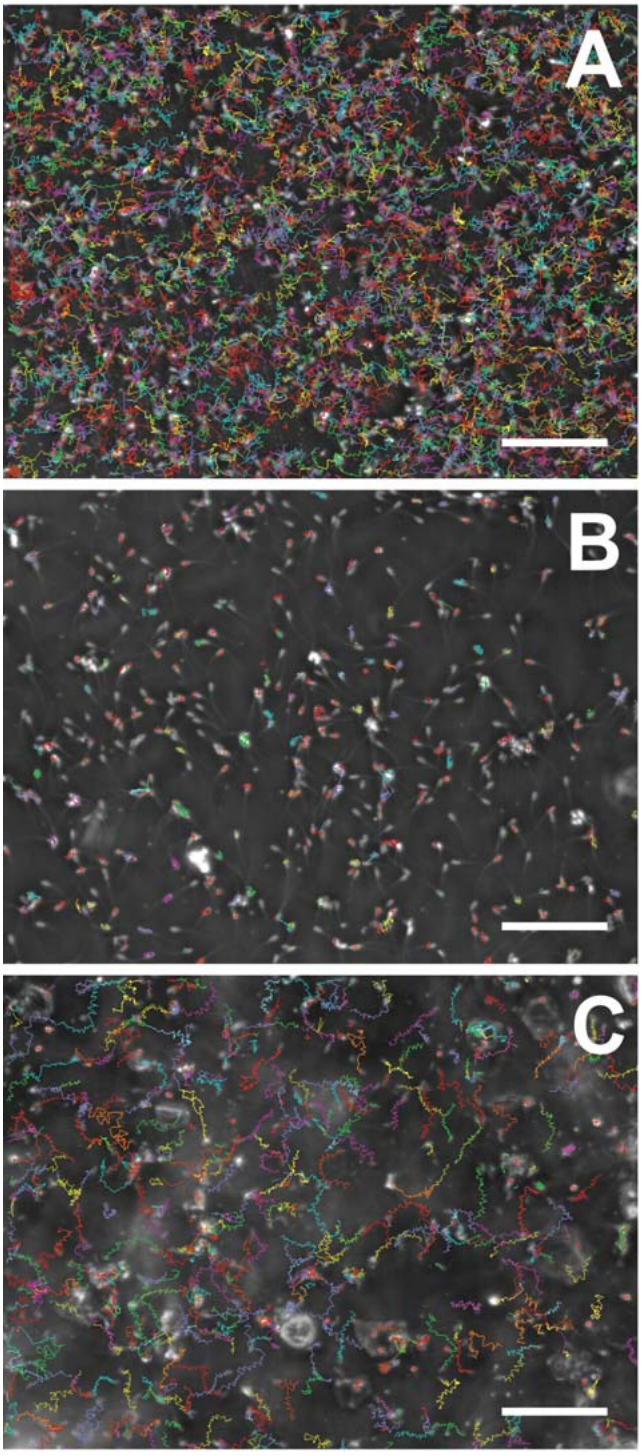

Fig. 3. Computer-assisted sperm analysis images indicating differences in trajectories of fresh porcine sperms on (A) glass, (B) glass after 15 min and (C) a PDMS membrane after 15 min. The different colors represent different trajectories of individual sperms. Image B clearly suggests adsorption of the porcine sperm to the glass substrate. Using the PDMS membrane (C), the adsorption onto the substrate decreased based on the trajectories of the motile sperms. The scale bars represent 0.1 mm.

trajectories of motile porcine sperm, LV and amplitude of lateral head displacement (ALHD) were estimated using the SMAS imaging software. The number of replications was 3.

Experiment 3: live imaging application in PDMS microchannels

The PDMS microchannel substrate was cured as described above, and access holes (2 mm diameter) were punched through the elastomer for fluid connection to the microchannels. A PDMS microchannel and glass slide were joined after being exposed to O_2 plasma (PDC-32G; Harrick Plasma, Ithaca, NY, USA) for 30 sec prior to contact [18]. A full-molded PDMS microchannel not subjected to O_2 plasma treatment was also prepared using one-step curing. The height and width of the channels were 0.1 and 2 mm, respectively. The number of replications of the comparison was 5.

We injected boar semen into the PDMS microchannel inlet using a micropipette and incubated the sample for 10 min. After incubation, we washed the microchannel with diluted water and dried it. We observed the bottom of the washed microchannel and counted the number of adhered porcine sperm. All experiments were performed under static conditions in this study.

Statistical analysis

The Student's *t*-test was used to determine differences in LV and ALHD between groups. $P<0.05$ was considered significant. Numeric data are presented as means ± SEM.

Results and Discussion

Experiment 1: comparing the absorption of motile porcine sperm to different materials

Almost all the sperms adsorbed to slide glass 15 min after preparation, while the number of sperms adsorbed to the PDMS membrane was significantly lower (Fig. 3). We found that more than half of the motile sperms adsorbed to the hydrophilic PDMS substrate treated with O_2 plasma 15 min after preparation. The adsorption properties of porcine sperms to transparent materials are summarized in Table 1 [19, 20]. We have confirmed that the hydrophobicity of the substrate materials is important for adsorption. To prevent adherence, materials allowing >80° contact angle with water should be used for preparation. Furthermore, the materials should not be prepared using hydrophilic treatments, such as O_2 plasma, to decrease the contact angle.

Table 1. Comparison of contact angle and adsorption of motile sperms to transparent materials

	Contact angle of water (deg)	Adsorption	References
Glass	30	Yes	[5]
PMMA	70	Yes	[21]
PDMS	110	No	[19]
PDMS after O_2 plasma treatment	50	Yes	[19]

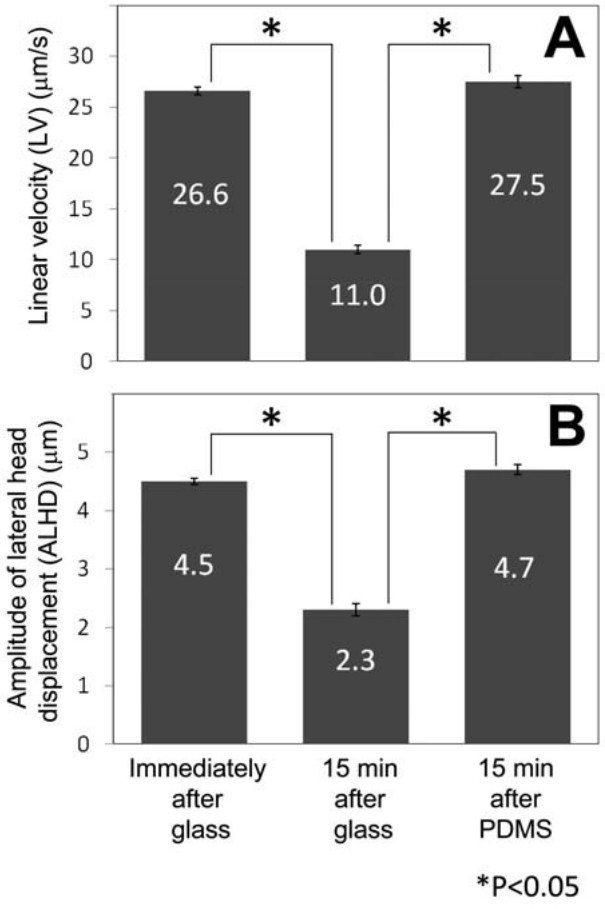

Fig. 4. (A) LV and (B) ALHD distributions recorded on glass and PDMS preparations. No significant difference was observed in the LV and ALHD between the distribution immediately after glass preparation and 15 min after PDMS preparation (P>0.05). These data are presented as means ± SEM.

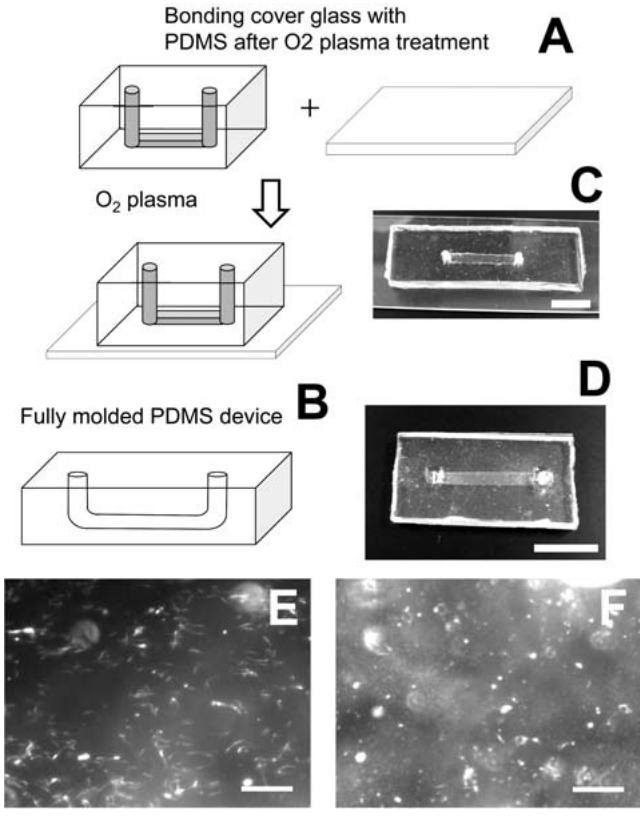

Fig. 5. The structure and adsorption of sperm to PDMS microchannels prepared by different methods. PDMS microchannels were either prepared by bonding with glass (A) or molding (B). Pictures C and D depict microfluidic devices that are formed by bonding with glass and one-step molding, respectively. The scale bars represent 1 mm. Micrographs E and F indicate sperm adsorption to glass-based and full-molded PDMS devices, respectively. The scale bars represent 0.1 mm. Semen was injected into the microchannels and incubated for 10 min under static conditions. After removing the semen and washing the microchannel with diluted water, we observed the bottoms of the microchannels. The number of adhered porcine sperm on the bottom of channel B was less than that of channel A, suggesting that hydrophilic treatment and the glass-based device were not suitable for analyzing motile porcine sperm.

Experiment 2: performance of optimized chambers and sperm motility parameters

We compared the LV distributions of motile porcine sperms inside chambers to quantitatively investigate motility changes in relation to adsorption to hydrophilic substrates. The average LVs immediately after, 15 min after glass preparation and 15 min after PDMS preparation were 26.6 ± 0.4 (n=1260, ± SEM), 11.0 ± 0.4 (n=196) and 27.5 ± 0.6 μm/sec (n=589), respectively (Fig. 4A). The average ALHD amplitudes immediately after, 15 min after glass preparation, and 15 min after PDMS preparation were 4.5 ± 0.05 (n=1260), 2.3 ± 0.10 (n=196), and 4.7 ± 0.08 μm (n=589), respectively (Fig. 4B). No significant differences in the LV and ALHD were observed between the distribution immediately after glass preparation and 15 min after PDMS preparation. We suggest that the significant decreases in the LV and ALHD were due to adsorption of the head and/or neck to the hydrophilic substrate (Figs. 3 and 4).

Experiment 3: live imaging application in PDMS microchannels

Microchannels for sperm motility analysis can be easily prepared by PDMS soft lithography; however, there is a problem with microchannel preparation after O_2 plasma treatment since hydrophilicity of PDMS increases [19]. We compared sperm adsorption to a PDMS microchannel with a cover glass on the bottom bonded with O_2 plasma treatment (Channel A) to a PDMS microchannel without O_2 plasma treatment (Channel B) (Figs. 5A, B, C and D). After washing the microfluidic channels of the chambers with diluted water, the number of adhered porcine sperms on the bottom of Channels A and B were approximately 1080 ± 430 (n=5) and 170 ± 20 sperms/mm^2 (n=5), respectively (Figs. 5E and F; P<0.05). This result is consistent with the LV distributions (Fig. 3A). When

preparing the microchannel, the standard bonding method for PDMS and glass by O_2 plasma or UV light should not be used due to increases in hydrophilicity of the materials [19]. To prevent the adsorption of motile porcine sperms, it is important to not use hydrophilic materials such as glass and to not perform O_2 plasma treatment as this treatment increases hydrophilicity of surfaces.

Furthermore, the reason why motile porcine sperms adsorb to hydrophilic surfaces is discussed below. After O_2 plasma treatment, the contact angle of PDMS with water changes from 110 degrees to 50 degrees (Table 1), and the PDMS surface becomes hydrophilic [5, 19]. As demonstrated in Experiment 1, some porcine sperms adsorbed to PDMS substrates after O_2 plasma treatment. This treatment increases the hydrophilicity of substrate surfaces by converting the C-H groups to more hydrophilic C-OH groups at the substrate surfaces [20]. These results suggest that porcine sperm heads and necks tend to adsorb to hydrophilic materials whose contact angles are less than 70 degrees. Thus, we investigated the reason for hydrophilic molecules at the surface of the porcine sperm head interacting more strongly with and getting adsorbed to hydrophilic surfaces compared with hydrophilic substrates.

Microchannels are important in sperm motility analysis because they allow the trajectories of motile bull and human sperms to be evaluated [12, 13]. Interestingly, it has been reported that bull sperms tend to preferentially swim along the walls and that this phenomenon occurs in both flow and non-flow systems [12]. Koyama *et al*. designed a microfluidic device for sperm chemotaxis with three inlets and three outlets to make a gradient in the chemotaxis chamber [18]. The PDMS substrate and a glass cover plate were bonded by exposure to air plasma, which would decrease the hydrophobicity of PDMS; a treatment based on our present results would not be suitable for analysis of porcine sperm chemotaxis. Our results suggest that a PDMS-bottom microchannel without hydrophilic treatments, such as O_2 and air plasma, can be used to investigate the chemotaxis and fluid mechanics of motile porcine sperms.

Motile porcine sperms adhere to hydrophilic materials such as glass and PMMA. The adsorption of sperms to the hydrophobic PDMS membrane was less than that to glass. Because of the elasticity of PDMS, we propose the use of this preparation for conventional CASA to reduce overlap of motile sperm images, which are artifacts of CASA. Because of the potential sperm adhesion, we do not recommend O_2 plasma treatment for bonding PDMS and glass during investigation of the dynamics and chemotaxis of motile porcine sperms using microfluidics. We suggest that the one-step PDMS molding process is suitable for preparation of microfluidic channels to be used with motile porcine sperms.

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