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Microfluidic Applications for Andrology

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Perhaps one of the most exciting and revolutionary scientific discoveries of the past 3 decades has been the development of in vitro fertilization (IVF) to treat human infertility. It is impossible to quantify its effect on numerous families since the first IVF birth in 1978 in Old-ham, England (Steptoe and Edwards, 1978). With increasing clinical utilization of assisted reproductive technologies (ART), scientists and clinicians gain insights into basic gamete and embryo biology and translate that knowledge into improving the process of IVF. Critical analyses of individual steps have improved outcomes. Attention to sperm processing and isolation to increase recovery of motile sperm and reduce sperm damage has improved fertilization rates and embryo development (Mortimer, 1994). Use of intracytoplasmic sperm injection (ICSI) has allowed fertilization even in severe cases of compromised sperm quality or number (Bonduelle et al, 1999). Finally, refinement of embryo culture has led to improved in vitro embryo development and implantation rates (Gardner and Lane, 1998; Pool, 2002). Most scientific attention, however, has focused on methodologies rather than technology development and equipment. Semen is still processed in test tubes regardless of technique, sperm are physically placed with oocytes after processing, and fertilization and embryo culture occur in culture dishes, test tubes, or both with relatively large volumes (Trounson and Gardner, 2000). With the exception of gamete and embryo micromanipulation, no technologic advancements in IVF have reached widespread use. Nevertheless, it is precisely those technologic advancements, rather than procedural or methodology changes, that have had the greatest effect on assisted reproduction.

A promising new technology, microfluidics, exists and is becoming increasingly studied. This technology shows promise as an alternative for each step in the IVF process. Microfluidics, based on physical principles of fluid behavior in a microenvironment, has been used widely in chemistry and molecular biology applications (Tomlinson et al, 1995). Currently, microfluidics is gaining interest in studies of cellular behavior and interactions (Shim et al, 2003). In this article, we introduce basics of fluid behavior at the microscale and highlight previous uses of this technology outside of the reproductive sciences. We then describe fabrication of devices and review initial studies that

used microfluidics in sperm sorting and microinsemination. Last, we point out some limitations of this new technology and provide speculation on future directions and application of microfluidics in ART.

Microenvironment Fluid Behavior

Fluid mechanics is a complex physical and mathematical science; therefore, an extensive technical description and review of fluid physics is beyond the scope and intent of this review. Instead, basic principles will be discussed that govern fluid behavior in a microenvironment, especially those aspects with a specific link to devices and technology currently being developed for IVF. We have purposely avoided including mathematical details, choosing instead to convey a general conceptual sense of fluid mechanics present within microchannels. A comprehensive technical and mathematical description of microfluidic physics can be found in excellent reviews from Beebe et al (2002a) and Brody et al (1996).

Fluids at the microscale are subject to forces typically not important at scales present in our everyday lives. Fluid at the scale of our normal environment is turbulent; particles within a stream of fluid move in an unpredictable pattern. Turbulent flow depends on certain fluid characteristics (viscosity, density, and velocity) and the geometry and size of the channel, leading to calculation of a value known as the Reynold's number. As the scale of the channel reaches micrometer levels, the Reynold's number decreases and becomes increasingly dependent on fluid characteristics. Decrease of the Reynold's number below a threshold value leads to fluid flow in a laminar fashion. Simply put, flow within microchannels becomes streamlined and predictable (Figure 1). At the microscale, fluid behavior becomes increasingly governed by viscous forces and surface tension, which can be described as the cohesiveness of the liquid's molecules.

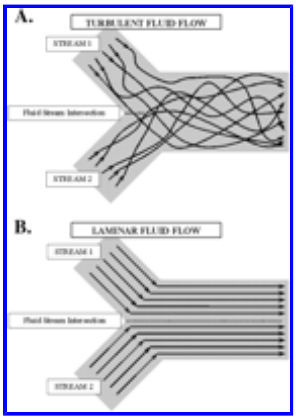

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Figure 1. Schematic representation illustrating fluid mixing in turbulent **(A)** and laminar **(B)** flow patterns.

This dominance by viscous forces results in several interesting phenomena. Flows with a low Reynold's number possess little to no momentum; thus, fluids within a microchannel respond quickly and reliably to changes in external forces. In addition, at the microscale, 2 or more streams of laminar flow in contact with each other do not mix, except by diffusion of molecules across the interface of the streams. The rate of diffusion between the contacting surfaces at the microscale can be very quick, partially because of the relatively short distances needed to cross fluid volumes.

Many of these fluid characteristics at the microscale form the principles driving the interest in the use of microchannels for gamete and embryo manipulation. In general, a microenvironment more closely resembles the in vivo conditions of fertilization and development when compared with a culture dish or drop of media. Below, we discuss the theory behind investigating the use of microfluidics in andrology, its testing, limitation, and potential future influence.

Microfluidics and Nonreproductive Cell Biology

Interest in microfluidics began with attempts to miniaturize chemical and biological analysis devices in the laboratory (Kricka, 1998). Current designs are often referred to as "laboratory-on-a-chip" or micrototal analysis systems (μTAS) and function by allowing a variety of chemical processes and interactions to occur as fluid flows within their miniature channels and chambers (Weigl and Yager, 1999). Such devices perform all the analytical functions necessary for their purpose, including sample handling, mixing, incubation, sorting, transport, interaction, and detection or signaling within an integrated microfluidic "chip." Examples include, but are not limited to, immunoassays for antibodies present in serum (Linder et al, 2002) and assays determining enzyme reaction kinetics (Xue et al, 2001; Yakovleva et al, 2002).

Additional applications in cellular biology have emerged, such as integrated cell sorting devices working at the microscale (Fu et al, 2002) and microfluidic devices that allow for the study of cellular interactions with substrates or other cells (Shim et al, 2003). Advances in cell biology have been demonstrated with the use of microfluidics and the principle of laminar flow, allowing for selective exposure of subcellular areas of interest to membrane-permeable molecules (Takayama et al, 2001). Such precise delivery of molecules to cellular subdomains illustrates the precision with which microfluidic regulation of fluid flow is capable.

Advantages of such laboratory-on-a-chip technology are multiple. First, once designed and tested, the manufacture of such devices is straightforward and inexpensive, allowing them to be disposable (McDonald et al, 2000). Microfluidic analysis devices use very low volumes of samples and reagents and provide for faster reactions and response times (Weigl and Yager, 1999). Miniaturization very importantly allows for integration of multiple processes within a small, self-contained unit (Kricka, 1998). This can be translated into either multiple parallel analyses, consecutive serial processes, or both.

The brief overview given here is only intended to familiarize readers with the variety of capabilities of microfluidic technology and is by no means a comprehensive listing of microfluidic applications in sciences. Readers are encouraged to consult more thorough reviews (Khandurina and Guttman, 2002; Verpoorte, 2002).

Fabrication of Microfluidic Devices

Microfluidics systems were initially fabricated with the use of materials and techniques common in the industry that inspired them—microelectronics (McDonald et al, 2000). Photolithography and etching of silicon and glass was a highly developed technology also readily available to researchers interested in miniaturizing analytical systems, yet costs were a significant barrier. In search of a suitable alternative, polymers have quickly emerged as a material for microfluidic biological device fabrication (McDonald et al, 2000). Compounds such as poly(methyl)methacrylate (Martynova et al, 1997), fluorinated ethylene propylene (Sahlin et al, 2002), and poly(dimethylsiloxane) (PDMS; McDonald and Whitesides, 2002) are cheaper and easier to manipulate than silicon-glass alternatives (Martynova et al, 1997). PDMS in particular has become one of the most actively explored and promising materials thus far, possessing numerous characteristics specifically suitable for

biological use. It is nontoxic, transparent, insulating, and permeable to gases (McDonald and Whitesides, 2002). From a fabrication standpoint, PDMS permits submicron fidelity with molding, cures at low temperatures, and can easily seal reversibly to itself and a host of other materials (McDonald et al, 2000).

Although PDMS is generally regarded as nontoxic, special consideration must be given to its use with gametes and embryos, which can be very sensitive to their environment compared with transformed cell lines. Before the use of microfluidic devices with sperm, testing confirmed that no negative effects resulted from prolonged exposure to the materials used in their fabrication. Schuster et al (2003) reported that 30 minutes of exposure to PDMS did not alter sperm survival. In addition, Glasgow et al (2001) found that development of 2-cell mouse embryos to the blastocyst stage was unchanged by continuous exposure to numerous photolithography compounds compared with controls. Thus it appears that PDMS-composed microchannels or the materials used in their construction do not confer deleterious effects to gametes or embryos.

Microfluidics and Sperm Isolation

Numerous efforts have improved methods of semen processing and sperm isolation. Currently, swim-up techniques or density gradient separation are methods of choice (Trounson and Gardner, 2000). Both methods result in adequate recovery of motile sperm, although additional steps might be necessary in poor-quality semen samples (Bourne et al, 1995a,b). However, some researchers have stated concern that these methods could contribute to sperm morphological damage, DNA damage, production of oxygen-free radicals, or multiple injuries (Aitken and Clarkson, 1988; Zini et al, 1999). In addition, these techniques can be labor and time intensive. Ideal sperm isolation would involve a simple, rapid, and atraumatic method to obtain sufficient motile sperm for use in either IVF or ICSI, depending on need and the quality of the original semen sample.

Attempts have been made to develop devices for such a purpose. The Wang tube (Wang et al, 1992), a uniquely configured glass tube, allows motile sperm to progress to an upper arm that is then separated for sperm use in intrauterine insemination or IVF. Comparison testing with swim-up and density gradient separation for normozoospermic samples revealed greater motility and morphology with the device (Wang, 1995). Lih et al (1996) have developed and tested a Lucite microchamber consisting of a central loading well surrounded by slightly depressed sidewells that was conceived from the observation that motile sperm migrate to the periphery of microdrops. This device concentrated motile sperm up to 13-fold in the sidewells, yielding a sufficient number for use in ICSI.

A microfluidic device has been explored for sperm diagnostic purposes. Kricka et al (1993) designed and fabricated silicon and glass devices for sperm motility evaluation. They evaluated sperm progression along the length of a microchannel (80 µm wide by 20 µm deep) and navigation through a network of branching channels. In initial studies, they demonstrated feasibility and hypothesized that this device could replace conventional methods of motility assessment and semen analysis. Subsequently, they demonstrated that sperm movement within microchannels, judged by the time needed to reach the end of the channel, correlated with forward progression scores (Kricka et al, 1997). However, the design of the device did not give reliable information regarding sperm concentration or percent motility and therefore could only serve as an adjunctive test of motility and forward progression rather than a comprehensive semen analysis tool.

Schuster et al (2003) developed a microfluidic device taking advantage of parallel laminar flow streams present at the microscale. In this device, a flowing stream of semen was placed in parallel with a flowing stream of media within a microchannel. Flow within microchannels was maintained by a

novel gravity-driven, horizontally oriented pumping system developed specifically for the device (Cho et al, 2003). As discussed, these 2 parallel laminar flow streams mix only by diffusion. Motile sperm demonstrated the ability to actively propel themselves across contacting surface areas and deviated from the initial streamline into the media stream for collection, whereas nonmotile sperm and cellular debris remained in the initial stream and exited the device (Figure 2).

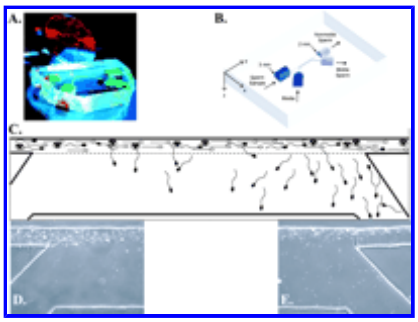

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Figure 2. **(A)** Picture of microfluidic sperm sorter. **(B)** Three-dimensional view of microfluidic sperm sorter. **(C)** Theoretical vision for microfluidic sperm sorter. Media flows from left to right. **(C, D)** Semen sample is loaded into the upper stream inlet, and fresh media is placed in the lower stream inlet. **(C, E)** Motile sperm are able to deviate from the initial streamline and cross the interface of the laminar flow streams, exiting into the lower stream outlet for recovery. Debris, nonmotile sperm, and some motile sperm are collected in the upper stream outlet.

Testing of this laminar flow sorting system was performed with 40 µL of unprocessed human semen, followed by semen samples artificially filled with debris from a stock solution of round immature germ and white blood cells to simulate poor-quality samples. For unprocessed semen, the device consistently produced a sorted fraction with increased motility (mean 98% motile) and improved Kruger strict sperm morphology (mean 22% normal forms) compared with the initial specimen (mean 44% and 10%, respectively). For debris-filled samples, the device not only concentrated motile sperm (mean 98% motile) within the collected fraction, but was also able to produce a round cell:sperm ratio of 1:33 compared with a 10:1 ratio in the starting specimen (Schuster et al, 2003).

Microinsemination

Microfluidics might be particularly suitable for IVF for a number of reasons (Suh et al, 2003). The microenvironment of a microchannel more closely resembles in vivo fertilization conditions than a culture dish or microdrop. Microfluidic channels allow for nonturbulent bathing of gametes with fresh media throughout insemination and coincubation. Sperm-oocyte interactions occur in an active environment, rather than the static conditions present in a culture dish or droplet. In addition, sperm can be predictably delivered via laminar flow to each oocyte within the microchannel, eliminating the randomness of sperm-oocyte interaction. In a culture dish, sperm can travel randomly in any direction, thereby relying on random sperm movement toward the oocytes; however, in a microchannel environment, sperm movement is limited by the direction of flow, allowing for active transport to the oocytes. Finally, microchannel environments use extremely small volumes of media, theoretically requiring fewer sperm to achieve insemination concentrations equal to standard IVF with larger volumes.

Previous investigators have attempted, with some success, to reduce the volume of insemination medium with various low-volume vessels, although none have gained widespread acceptance. Van der Ven et al (1989) tested the use of sterile, nonheparinized hematocrit capillary tubes (75 mm length, 0.9 mm inner diameter) for IVF in humans. Normospermic samples were used with standard culture tubes as controls. Volumes of 5-10 µL containing a range of 500-4000 sperm per oocyte were used in these

capillary tubes. Overall fertilization rates between controls and capillary tubes was similar (78% and 66%, respectively), although slightly lower for sperm totals of 500-1000 (56%) compared with 2000-4000 total sperm (79%). Ranoux and Seibel (1990) used embryo cryopreservation straws in volumes up to 200 µL (Ranoux et al, 1988) with 2000-4000 motile sperm. Results compared favorably with controls, with 167 of 322 oocytes (51.8%) fertilized by the straw technique.

We have recently demonstrated that mouse IVF can be conducted successfully within microfluidic channels (unpublished data). Not only are lower total numbers of sperm required because of the use of reduced media volumes, we have also demonstrated murine fertilization within microchannels with lower insemination concentrations, further decreasing sperm requirements. We continue to develop design improvements that will result in increased efficiency and ease of use. Such microfluidic devices should ultimately be useful in clinical IVF, not only for oligospermic patients but potentially as a replacement for standard insemination.

Limitations and Future Applications

This microfluidic sperm sorting device provided a simple, atraumatic method of obtaining motile sperm of normal morphology from both unprocessed normal semen and poor-quality specimens containing significant debris. A limitation of the device regards the flow rate, estimated at ~20-40 µL/h. In its current form, it is not capable of processing an entire semen specimen; however, it does provide a means of quickly and easily isolating a small sample of motile sperm of normal morphology for ICSI, insemination in microdrops under oil, or microinsemination in an integrated microfluidic device (Clark et al, 2003; unpublished data). In addition, modifications and improvements in the design are in progress that might allow for large-scale processing in parallel and increased efficiency of flow and sorting (Schuster et al, 2003).

As with any new technology, design plays an important role. Improvements in loading methods, which can allow for visualization under magnification without adjustment of the microscope focus, should improve outcomes. Addition of oocytes and sperm to the device under a microscope, which requires time outside of the humidified 5% CO_2 environment, has significant deleterious effects on gamete health and survival. Reduction of this time currently requires a significant learning curve. Last, the implementation of more sophisticated but less operator-reliant mechanisms for fluid flow should improve efficiency. Current studies are focused on a variation of the gravity-driven, horizontally oriented reservoir pumping system (Zhu et al, 2004) from the microfluidic sperm sorter developed by Cho et al (2003) and its application to microfluidic insemination.

Although much of the work with microfluidics in IVF has been performed in a stepwise fashion, the ultimate goal of process miniaturization and microfluidic technology is integration. Use of microfluidic technology for sperm processing ultimately results in a small volume and fraction of motile sperm. Such volumes are difficult to subsequently use and translate into a macroscale environment. However, laminar flow-sorted sperm have been used for subsequent fertilization within a microenvironment (unpublished data). Integration of a microfluidic channel for the oocyte and the collection stream of sorted sperm would result in automatic coincubation of the oocyte with these motile sperm. Following insemination, the oocyte can be directed to a secondary site for cumulus removal, evaluation for fertilization, and embryo culture (see review in Beebe et al, 2002b). Sequential media can be provided for ideal embryo development. Each step logically follows the other, with no cell manipulation other than directing flow along a variety of channels. Miniaturization allows the entire system to be small and self-contained. Decreased intervention by laboratory personnel not only decreases gamete and embryo manipulation but also provides for greater consistency of incubation conditions.

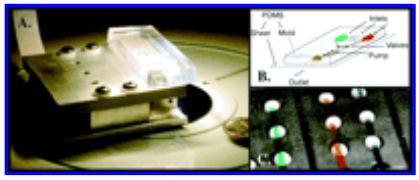

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Figure 3. Braille displays. **(A)** A handheld, battery-operated refreshable Braille display that can be used in combination with elastomeric microfluidic chips **(B)** for valving and actuation. An array of Braille pins **(C)** that have been positioned in sequence under a microfluidic chip with media of various colors. Where a microchannel of media is interrupted (white Braille pinhead is completely visible) the pin is depressed up onto the PDMS sheet, which subsequently depresses into the channel to interrupt the colored media. This can be used to open or close valves, or when pin depresses into microchannels in sequence, this generates media pumping within the microchannel.

Obviously, multiple hurdles exist in engineering integration of these processes. The development of reliable methods for directing flow through a network of channels is necessary. Recently, numerous active and passive valves (Beebe et al, 2002a) or switches (McDonald et al, 2000) have been designed for microfluidic devices, but the applicability to gamete and embryo manipulation must be demonstrated. Automated delivery of fluid at precise rates is important for sperm sorting, culture media exchange, and embryo manipulation. A passive gravity-driven fluid pump has been employed for microfluidic sperm sorting (Cho et al, 2003), and active hands-on regulation of fluid flow with syringes has been used for insemination and embryo handling (Davis et al, 2000). Recently, we developed a new computer-controlled, integrated, microfluidic control system with up to hundreds of on-chip pumps and valves powered by individually actuated Braille pins on a portable, refreshable Braille display (Figure 3; Gu et al, 2004). Typically these displays are used as a reading tool for the blind. The display can convert electronic signals (from a computer) to vertical translations of 8 small individual pinheads on each of the many Braille cells (typically 8-80 cells for a total of 64-640 individually controlled and actuated pinheads). A line of these cells would typically represent a line of text displayed by a computer. We took advantage of the fast refresh rate and minuscule size of the Braille pinhead by aligning the pinhead's localized pressure onto microchannels, squeezing them shut at high refresh rates. The system takes advantage of the resilient yet elastic nature of PDMS microchannels fabricated with soft lithography and the movement of Braille pins to "squeeze" fluid through channels. Each stroke of a Braille pin can be used to generate a forward or backward flow of liquid through the microchannel when synchronized to various valving patterns. The volume of flow generated per stroke can be controlled by adjusting the volume of liquid displaced by the pin. This method of fluidic control is portable, versatile, and cost effective. Braille displays are commercially available, can be battery powered, and have embedded computerized control in devices the size of a person's hand. With this new concept, we have demonstrated 3 key functions necessary for future application of microfluidics for microinsemination and embryo culture and biochemical analysis system: 1) valve actuation (opening and closing), 2) pumping, and 3) mixing. Finally, methods of fabrication and packaging of microfluidic devices must be refined before widespread acceptance of this technology for human applications. Current devices have been for research purposes. However, development of an IVF laboratory-on-a-chip is a realistic and exciting goal.

Footnotes

* *Andrology Lab Corner* welcomes the submission of unsolicited manuscripts, requested reviews, and articles in a debate format. Manuscripts will be reviewed and edited by the Section Editor. All submissions should be sent to the *Journal of Andrology* Editorial Office. Letters to the editor in response to articles as well as suggested topics for future issues are encouraged.

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