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Design and fabrication of a microfluidic chip for oocyte denudation.

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Abstract
Intracytoplasmic sperm injection (ICSI) is one of the most successful techniques of Assisted Reproductive Technology (ART) and is mostly in use for the treatment of infertility with male factors. In this method, before injecting sperm into the intracytoplasmic of the oocyte, cumulus cells around the oocyte must be stripped to facilitate the injection process. To achieve this, both enzymatic and mechanical methods are used in embryological laboratories for denudation, which has major deficiencies, including the possibility of damaging the oocyte prior to the injection process. In this research, a microfluidic-based device is introduced for the separation of cumulus cells around the oocyte with minimum manual operations. The results prove high efficiency, and non-destructive denudation of the oocyte with the reduced amount of culture medium leads to the low-cost preparation process of oocytes. The process can also be integrated with ICSI chips under development and will be reported shortly.

Keywords
Assisted Reproductive Technology (ART), Denudation, In Vitro Maturation (IVM), Microfluidics, lab-on-chip.

Introduction

Despite significant advances in medical science, infertility remains a prevalent global condition. Infertility is estimated to affect between 8 and 12% of reproductive-aged couples worldwide. However, in some regions, such as South Asia, sub-Saharan Africa, the Middle East, North Africa, Central and Eastern Europe, and Central Asia, the rates of infertility are much higher, reaching 30% in some populations [1]. The first IVF baby was born in 1978 as the breakthrough in ART technology [2] and since then, despite the significant advances in ART, most ART processes require manual operations, and therefore massive reliance on operator skills. In addition, the high cost of process materials normally used in high volumes is a major barrier to the popular availability of these methods. Microfluidic technology opens innovative opportunities to simplify ART procedures and enable the transition from manual processes to autonomous and cost-effective systems. ART procedures generally need to be performed by highly skilled personnel, the whole process is still invasive and may potentially cause some complications such as ovarian hyper-stimulation syndrome and so on. Current tools and environment of IVF for gamete manipulation and embryo culture are still far from the required conditions for in- vivo [3].
Many patients around the world still have no or at least limited access to ART treatments in infertility centers. Therefore, reducing the cost of infertility treatment processes and public availability are the main missions of researchers in ART. IVF and ICSI are the two successive ART methods accepted globally to treat infertile couples [2]. Each one follows specific procedures for inoculation between sperm and oocyte and embryo formation.
Microfluidic technologies have demonstrated great potential in restructuring the ART procedures [3,5] including achievements in sperm selection [6] and analysis [7], sperm motility and chemotaxis screening [8], oocyte analysis [9], oocyte trapping [10] embryo cumulus removal techniques [11], insemination [12], IVF micro-device [13], IVM [14].
ICSI is a dominant treatment for almost all forms of male infertility and has been increasingly adopted to overcome fertilization failure [15]. In 2012, ICSI was used in 93.3% of fresh IVF procedures (embryos transferred without being frozen) with male factor infertility and was used in 76.2% of all fresh IVF procedures with and without male factor infertility [16]. An oocyte is surrounded by cumulus cells, the specialized granulosa cells, forming an organized structure known as the Cumulus–Oocyte-Complex(COC). The cumulus cells that are in close contact with the oocyte, also known as corona cells, develop cytoplasmic projections which cross the zona pellucida and form gap junctions with the oolemma (Fig. 1).
Naturally, oocytes are required to be denuded from the cumulus before the ICSI to facilitate the injection of sperm and to allow the evaluation of oocyte morphology,

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in particular, the nuclear maturation stage [17]. To achieve this, ART clinics usually use the enzymatic action of hyaluronidase and mechanical pipetting, which are highly inefficient and completely depend on operator experience. Exploiting microfluidic chips has been studied for oocyte denudation and oocyte IVM [18,19]. Clinical IVM has increasing interest to provide an alternative source of competent oocytes without consuming significant levels of exogenous Follicle Stimulating Hormone (FSH), enabling the patient to undergo routine IVF (although fertilization is normally achieved via ICSI) and embryo transfer procedures. In ICSI, oocyte preparation is essential. Few studies have been done so far on the use of microfluidic chips in the denudation and the IVM field. In the current practice, we developed a microfluidic device to denude oocytes from the surrounding cumulus–corona cell mass, dragging in a continuous fluid flow. In this structure, by optimizing the geometry of the flow channel the oocyte streams through different parts of the microfluidic chip, and during this travel, the removal of cumulus cells around the oocyte is performed as a result of mild engagements, preparing the oocyte for ICSI or IVF. Consequently, excessive mechanical stress has been avoided on the oocyte in comparison to the manual stripping method. It is also possible to monitor and study the oocyte development stages during denudation.

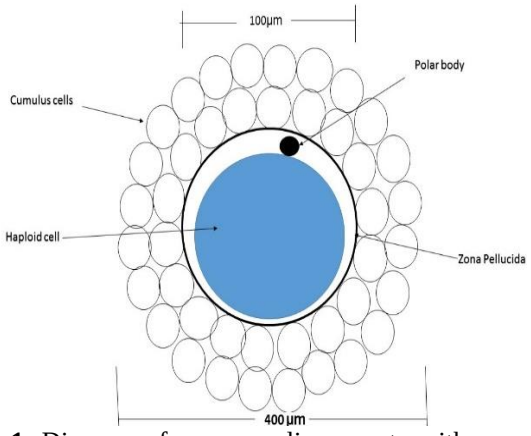

Fig. 1. Diagram of a mammalian oocyte with cumulus cells.

2.Materials and methods

2.1. Background

The fluidic model for the proposed devices is based on the steady-state Naiver–Stokes equation for incompressible Newtonian fluid [20].

$$\frac{\partial \rho}{\partial t} + \nabla.(\rho u) = 0 \quad (1)$$

$$\rho \frac{\partial u}{\partial t} + \rho(u \cdot \nabla)u + \nabla p - \mu \nabla^2 u - (\lambda + \mu)\nabla(\nabla \cdot u) = f \quad (2)$$

Where u is the normalized velocity vector, p the pressure, ρ the medium density, μ the dynamic viscosity, λ the molecular mean free path, and f the body force. The computational fluid dynamics was utilized to anticipate the wall shear stress as a function of the channel width and flow rate. Since the channel length was significantly large compared to the channel height, (L~4-6.5 mm, H~120-400 µm) the system could be effectively modeled using a 2D simulation. Using these conditions, the maximum shear stress applied to the cell (τs) could also be obtained using equation (3) [21].

$$\tau_s = (2 \times 2.95\mu Q)/H^2 \quad (3)$$

Where H is channel height, μ is the medium viscosity, and Q is the flow rate.

2.2. Computational model

COMSOL5.2 multiphysics software was employed to model and simulate the denudation microfluidic chip to optimize the design, improve the performance, and estimate flow profiles and shear stresses within the denudation device. Here, we built finite element models and used 2D simulations to study the flow behavior in a denudation microfluidic chip. The optimal design layout is shown in Figure 2. The channel width is decreasing from the inlet to the outlet of the design, and we have devised a corrugated channel profile to reconstruct the fallopian tubes to allow the removal of cumulus cells around the oocyte and facilitate the denudation process for IVF or ICSI process.

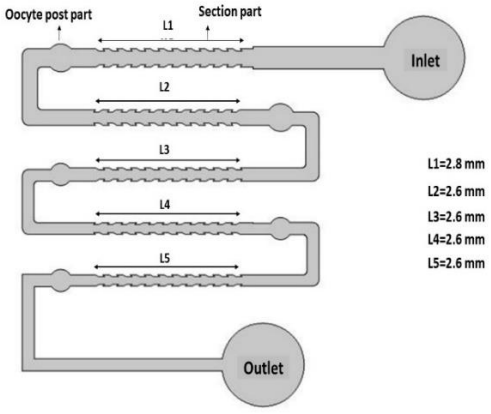

Fig. 2. Schematic diagrams of denudation chip.

At the end of each sub-channel, a small circular section is provided for visual evaluation of the oocyte at that stage. As already mentioned, to assist in the removal of the cumulus cells around the oocyte, jagged-wall micro-channels have been exploited.

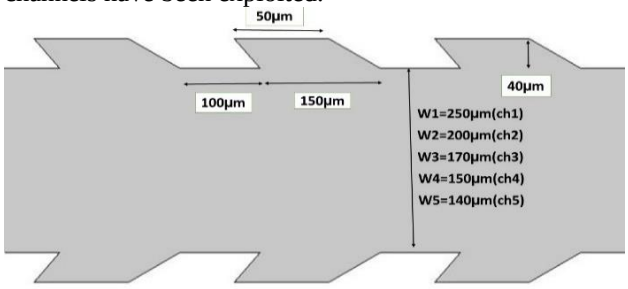

Fig. 3. Jagged-surface micro channels.

To avoid oocyte defection during the denudation, the shear stress should not exceed the maximum value allowed (1.2 dyne/cm2=0.12 Pa) [22]. Figure 4 shows the micro-channel shear stress line graph, and figure 5 and

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table 1 show the maximum and minimum shear stress points, which are calculated using Comsol 5.2 software. Calculation of shear stress for the oocyte moving in the channel is done by the particle tracing tool of Comsol software. The dimensions of the oocyte change due to the destruction of cumulus cells around it, so it is not possible to study oocyte shear stress in channels. Here, only the shear stress has been investigated for the positions of the channels.

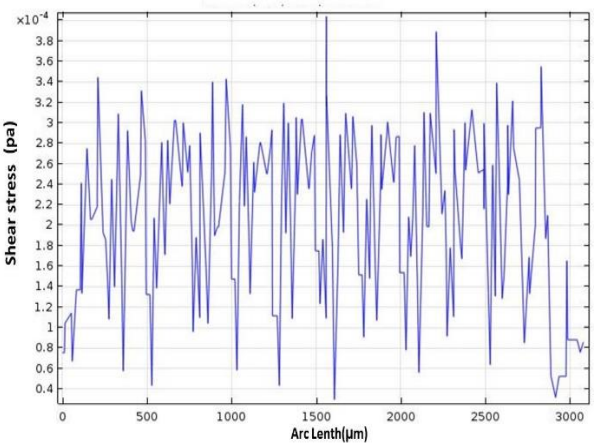

Fig. 4. Line Graph of shear stress (pa) changes along the microchannel (Section 5).

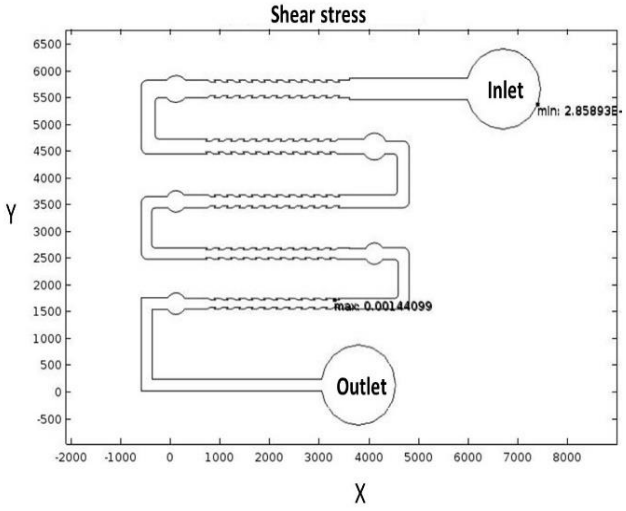

Fig. 5. Maximum and minimum shear stress points.

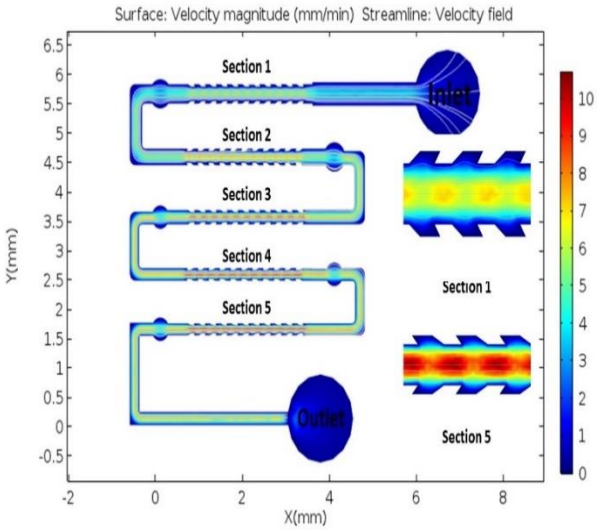

Fig. 6. Velocity magnitude(mm/min) of fluid in the denudation chip.

Table I. Maximum and minimum shear stress.

X (μm)	Y (μm)	Shear stress(Pa)
7248.2	5137.5	1.2679E-5 (Min)
3320.0	1730.0	0.0058691 (Max)

Figure 6 depicts variations of the velocity along and across the channels. The applied flow rate at the inlet is 1mL/min and the walls boundary conditions are no-slip and no-penetration. The flow of fluid from the inlet to the outlet increases its velocity and is one of the effective dragging forces for the oocyte inside the micro-channel. Figure 7 shows the velocity line graph in the substation area. Reducing the speed in this part makes it possible to stop the oocyte using controlling the liquid inlet pressure.

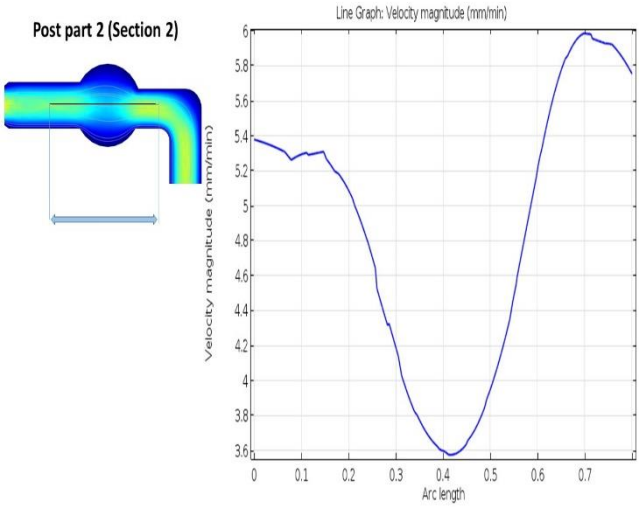

Fig. 7. Velocity magnitude(mm/min) of fluid in post part.

2.3. Fabrication

The standard soft lithography and injection molding process are exploited to fabricate the proposed device. The mold is prepared using 3D DLP (Digital Light Processing) printers, which is become mainstream technology for manufacturing microfluidic chips during the past recent years. However, in structures with dimensions smaller than 100 μm, the 3D printing process for the mold is challenging and requires proper design of process parameters including exposure time and slice layer thickness to achieve the best mold quality. 4K DLP 3D printer (Kavosh) is used to fabricate the mold (Figures 8 and 9) with standard UV-curable resin. The poor sealing of PDMS in 3D printing molds was a challenge that caused the lack of healthy molding of PDMS, which was solved by adding polyvinyl alcohol (PVA) as an interface layer. One of the most important challenges in this design is the use of a DLP 3D printer to make a micro-fluidic chip mold. Although the final chip has slightly different compared to the designed one, the results of tests in the embryology laboratory still show the viability of the device. For this design, the template was printed more than 50 times with a DLP 3D printer with different conditions, and the best-printed template was used. Although the cost of mold making is very low compared to other methods such as soft lithography, for structures

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with small dimensions (<150um), the expected quality will not be achieved.

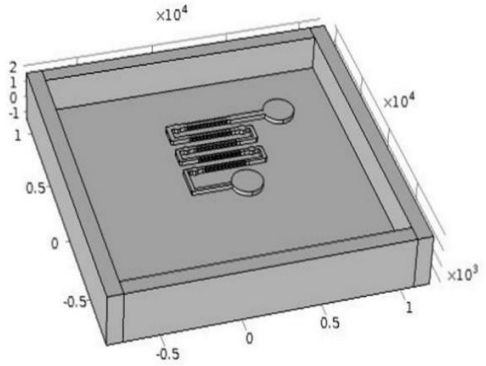

Fig. 8. Template design generated for 3D printing.

Fig. 9. Printed chip template using DLP 3D printer.

The substrate is then prepared using PDMS elastomer (Sylgard 184, Dow Corning). The prepolymer was mixed with the cross-linker at a ratio of 10:1 (in weight). The mixture was then poured onto the UV resin master mold, degassed, and cured at 75 °C overnight. The cured PDMS replica was then removed from the mold and inlets/outlets through-holes were punched out for fluidic connections. To achieve the best chip with no liquid leakage, the PDMS slab should be surface treated before compression bonding to the substrate glasses. The process is attained under the standard surface activation, using a lab-made plasma stylus for 3 minutes and then immediately bonded to a 40 mm × 25 mm × 0.13 mm glass slide to assemble the final oocyte denudation chip. To reach the best bonding chip, it was left in the oven at 70 °C for 12 hours. The final chip is shown in Figure 10.

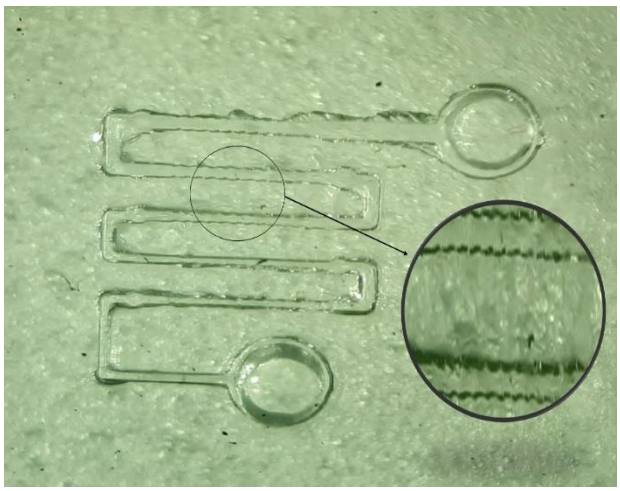

Fig. 10. Final microfluidic oocyte denudation chip.

3. Results and discussion

In the embryology labs of ART centers, it is necessary to prepare oocytes for micromanipulation before procedures such as ICSI. As mentioned already, denudation from the surrounding cumulus cell mass is the most important step and is usually accomplished using enzymatic or mechanical methods.

In the first technique, denudation is performed in the hyaluronidase enzyme, which digests the hyaluronic acid in the matrix between the cumulus cells. The mechanical method isolation is accomplished by pipetting. Both procedures are highly dependent on user skills and therefore are not efficient for mass ICSI operations. Furthermore, valued oocytes could be destructed during denudation procedures. We examined the feasibility of the jagged-wall micro-channels along a microfluidic device to replace the manual operations. As shown in Figure.2, there are 5 successive micro-channels whose internal width starts from 250μm and gradually decreased to 140μm. The oocyte is hydrostatically imposed to pass through the designated pathways of the surrounding cells. At the end of each stage, there is a circular post that can be used for observation of oocyte status. Final microfluidic denudation chips were sterilized by ethanol 70% w/v and one hour of UV light exposure. Then immature oocyte samples were then tested in the embryology lab of the ART center. Initially, Hyaluronidase 80 has been added to the oocyte and placed in the Ham's F10 medium to loosen the cells around the oocyte, the oocyte was then moved into the microfluidic channels by manual pressure to the syringe. The test results of this Microfluidic chip support the feasibility of using cheap and useful microfluidic technology. Figure 11(a) shows the position of the oocyte inside the micro channels.

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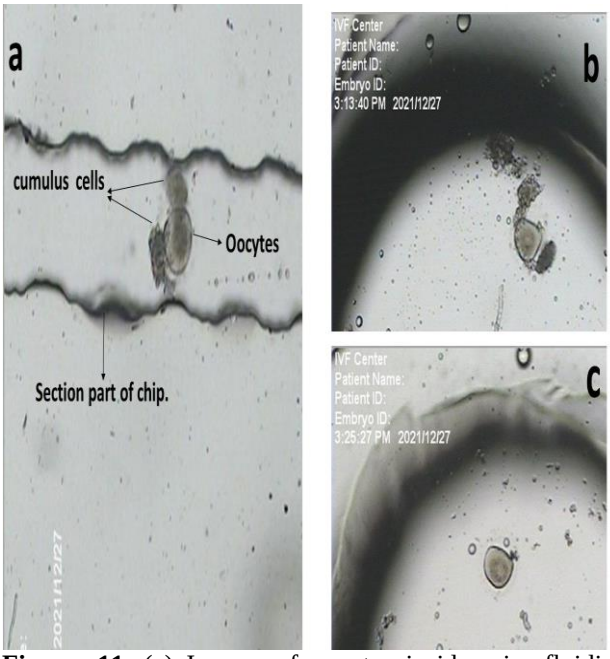

Figure. 11. (a) Images of oocytes inside microfluidic channels using a 40x-magnification microscope. (b) Before denudation oocyte with cumulus cells. (c) After denudation oocyte and without cumulus cells.

This chip was tested with 10 immature oocytes, and denudation resulted from 8 oocytes being successfully retrieved. The test results of this microfluidic chip support the feasibility of using cost-effective and useful microfluidic technology. Figures 11 (b) and 11 (c) show oocyte before and after denudation. Compared to the previous work [18], the introduced chip has advantages such as no need to control the suction of several outputs to perform denudation, the possibility of covering more around the oocyte for denudation, the possibility of better monitoring of the oocyte, and finally, the quality of observed denudation is better**.** In another study [23] the development of mammalian embryos was investigated using a microfluidic chip to remove cumulus cells. Like the previous study, this work also needs several vacuum pumps compared to our introduced chip, and it cannot cover the cumulus cells around the embryo completely.

4. conclusions

In this work, a microfluidic chip was introduced to remove cumulus cells around the oocyte. Unlike enzymatic and mechanical methods, this chip requires less skill and experience. It is also possible to study the state of the oocyte in the process. Due to the fact that the size of the chip is small, it uses less culture medium compared to the current conventional methods, and as a result, it will reduce the cost of infertility treatment.

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