On the Cryopreservation of Individual Cells in Volumes Less than Nano Liter

B. Galmidi[1], Y. Shafran[1], R. Orvieto[2], N. Zurgil[1] and M. Deutsch[1]
[1]*The Biophysical Interdisciplinary Schottenstein Center for the Research and Technology of the Cellome, Physics Department, Bar Ilan University, Ramat Gan, Israel*
[2]*Infertility & IVF Unit, Dept. Obstetrics & Gynecology, The Chaim Sheba Medical Center, Tel Hashomer, Ramat Gan, Israel*

1 STAGE OF THE RESEARCH

Micro-arrayed donut-shaped chambers (DSCs) are miniature vessels which were developed in the frame of this work. Each chamber is designed to act as an individual isolated reaction compartment, which creates an in-vitro assay, mimicking biology environments. Such a device enables individual live cell treatment and analysis, with the assistance of a designated image processing algorithm. In this work, we use DSCs for cryopreservation of individual sperm cells. Preliminary experiments show that fluid does not exit the donut during the freezing-thawing cycle. Figure 1 shows fluorescent images of the donut structure with fluorescein (5uM) drop, and following the freezing-thawing cycle after partial removal with blotting paper.

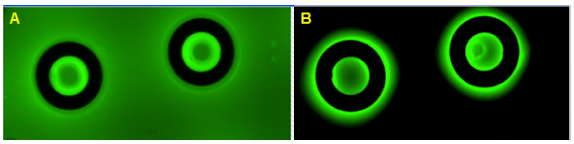

Figure 1: Fluorescein based preliminary experiment: freezing-thawing cycle. A - Fluorescent image of donut structure with fluorescein (5μM) drop, B – following the freezing-thawing cycle after partial removal with blotting paper.

Next, Molt 4 cells were used for viability test after freezing-thawing cycle. FDA and PI fluorescence dyes were added to the cell suspension. Then, suspension was loaded onto the donut array and into a standard cryo-tube for the control experiment. Donut array was carefully washed using a pipette with a washing medium, and a bright field image of the same region was taken before (Figure 2A) and after (Figure 2B) washing. It is clearly seen that cells in between donut structures can be easily washed out, while cells within donuts retain their position. A fluorescent image (Figure 2C), which was taken after thawing, demonstrate that most cells inside the donut structures are alive. The green fluorescing spots are FDA positive cells (live cells), and the red spots are PI positive cells (dead cells). Similar experiments were performed using donuts of differing diameters, with volumes between nl and μl, all showing similar results.

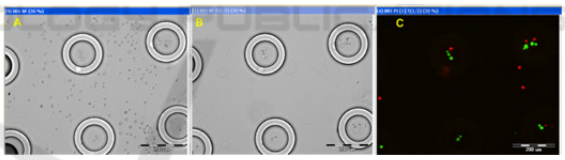

Figure 2: Cryo-preservation of individual Molt 4 cells within donut structures. A - a bright field image was taken after suspension was loaded onto the donut array **B-** the same region after donut array was carefully washed. C- Viability test of the cells following the freezing-thawing cycle. The green spots are FDA positive cells (live cells) and the red spots are PI positive cells (dead cells).

Finally, cryo-preservation of individual sperm cells within donut structures was examined. Sperm cells were collected using a micro-manipulator pipette. The pipette, via the micromanipulator, was brought into the proximity of the sperm cells (suspended in a drop of medium, covered by oil layer, (Figure 3A) after which cells were collected by gentle pumping. Then, the collected sperm cells were released into cryo-preservation medium inside donuts with varying diameters, covered by oil (Figure 3B,C). Sperm cell suspension was also loaded into a standard cryo-tube for the control experiment. Results with sperm cells show that (a) following freezing-thawing procedure most of the cells (>90%) remained in the original donuts; (b) the percentage of motile cells in the µl volume donuts was about 80%, which is even greater than that of the control experiment and (c) opposed to (b), sperm cells in the ≤ lnL volume donuts, did not survive at all.

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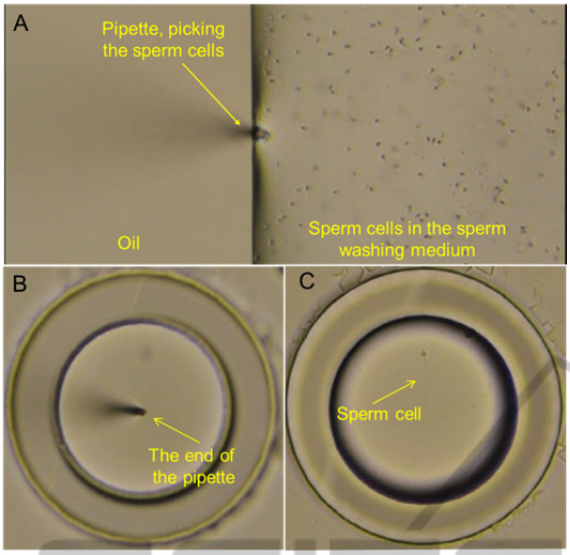

Figure 3: Cryo-preservation of individual sperm cells within donut structures – micro-manipulation technology. (A) Sperm cell collection using a pipette which reaches the sperm washing medium drop through a covering oil drop. (B) Release of the collected sperm cell into cryo-preservation medium inside the donut covered by oil (with air bubble trapped inside). (C) Single sperm cell inside a single donut in cryo-preservation medium.

The fact that vitality of sperm cells following freezing-thawing cycle in relatively large volumes was found to be extremely larger than that obtained within the nL volumes, urges us to more directly examine the dependency of vitality of sperm cells upon the freezing-thawing volume. Cryo-preservation medium drops containing sperm cells, was injected to oil inside a petri dish with varying diameter (volume) between 50-800µm, (Figure 4).

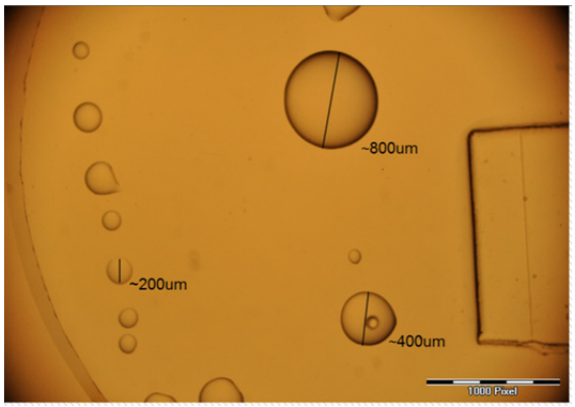

Figure 4: 4x microscopic image of cryopreservation medium drops containing sperm cells, placed on a Petri dish under oil.

Before freezing, about 80% of the sperm cells were motile. Following the freezing-thawing cycle, more than 99% of the sperm cells lost their motility. Only the 800µm drop contained about 10% of the motile cells. As a control experiment, a drop of 5µl with sperm cells was frozen. Following thawing, about 50-60% of the cells remained motile.

To date, we have promising results with sperm cells that have undergone the freezing-thawing cycle in suspension volume of about 1-2 µl. Under the same freezing-thawing procedure, the motility of thawed sperm cells in a nl volume was unsatisfactory. Results suggest that freezing sperm cells in suspension volume less than 1 nl damages the motility of thawed sperm cells.

2 OUTLINE OF OBJECTIVES

- Design and production of cellular chip array of nl containers of chosen size.
- Examination of spermatozoa freezing-thawing protocols on a nl volume scale.
- Investigating the chemo-physical aspect of freezing-thawing cycle of suspension in nanoliter volume.
- Finding an ideal method for sealing the small volume container in order to avoid evaporation (e.g. oil layer or hard cover).
- Connecting a micromanipulator and a micro-pump to the microscope system which will enable control of minute (nl) volumes required to fill individual DSCs.
- Development of an efficient protocol for sperm cryopreservation within a cellular chip array at a single cell resolution.

3 RESEARCH PROBLEM

Initial attempts to cryopreserve and thaw spermatozoa in nano liter containers yielded exceptionally high mortality of cells, in comparison with that obtained when cryopreservation and thawing of sperm cells was performed within microliters containers. Furthermore, with Molt4 cells, cryopreservation and thawing in nano liter containers was quite successful. The conjecture that the overcrowding of the nl containers may damages the tennis racket like shape sperm cells, was immediately rebutted when spermatozoa incubated both in nl containers and in a standard cryotube, yielded the same cell motility. Furthermore,

spermatozoa have shown satisfactory motility after thawing when fluid within the donuts was physically connected with the fluid in between donuts.

These findings, i.e. the dependency of a successful freezing-thawing cycle of sperm cells upon freezing-thawing volume, shape of cell and upon fluid contact lead us to suspect that a unique physical mechanism might be the cause for the explored phenomena.

Therefore, an analytical-physical research is under way, to understand the microscopic changes in the freezing-thawing process of water, solution and cell suspension (with different shapes), at very small volumes, together with a biology research aimed to understand the effect of these changes on spermatozoa. These will be followed by medical research for the development of applied clinical device and protocol.

4 STATE OF THE ART

Sperm freezing is one of the most explored fields of cryobiology. The first freezing experiments in the 1930s were done with frogs. Cryopreservation of human spermatozoa was introduced in the 1960s and has been recognized as an efficient procedure for management of male fertility. A great number of medical conditions, as well as many biological and environmental factors can cause low sperm count temporarily or permanently, and require the freezing and retrieval of very few sperm cells. However, the conventional methods for sperm cryopreservation are not suitable for cryopreservation of small numbers of sperm cells, such as epididymal and testicular spermatozoa. When individual cells are cryopreserved, they are virtually undetectable after thawing, in the large volume of a standard cryo-tube. Efficient cryopreservation of surgically retrieved spermatozoa reduces the number of surgical interventions and evades the logistic problems associated with coordinating oocyte retrieval with spermatozoa retrieval. Although novel cryopreservation approaches have been designed for limited numbers of motile sperm in very small volumes (Table 1), all with limitations and disadvantages, to date, no trials have been conducted to demonstrate that any single carrier is superior to another. No fully established technique for cryopreservation of a single human spermatozoon is being used today by the majority of IVF laboratories.

Furthermore, to date there is limited use of these technologies in the majority of IVF programs. This suggests that novel cryopreservation technology designed to handle small numbers of sperm needs to be further explored.

Table 1: List of biological and non-biological carriers proposed for the cryopreservation of small numbers of spermatozoa.

Cryopreservation techniques	Main disadvantages
Microdroplets (M. Gil-Salom, 2000)(C. Quintans, 2003)(E. Sereni, 2008) (N. Bouamama, 2003)	Risk of cross-contamination; shape and size of dishes make it difficult to handle and store in conventional freezers and liquid nitrogen tanks
ICSI pipette (Adamson, 2001) (J. O. Sohn, 2003)	Not practical for long-term storage; fragility of ICSI pipettes; risk of cross-contamination
Empty zona pellucida (M. Montag, 1999) (Y. Y. Hsieh, 2000) (J. Liu, 2000) (P. E. Levi-Setti, 2003) (A. Cesana, 2003)	Risk of biological contamination
Volvox globator spheres (A. Just, 2004)	Exposure to genetic material from the algae; constant source of algae
Alginate beads (A. Herrler, 2006)	Decrease sperm motility with encapsulation
Agarose microspheres (D. A. Isaev, 2007)	Clinical value of this approach not evaluated
Cryoloop (F. Nawroth, 2002) (T. G. Schuster, 2003) (N. Desai C. C., 2004)	Open system: risk of cross-contamination
Straws (N. Desai D. G., 1998) (V. Isachenko, 2005) (I. Koscinski, 2007)	Not ideal for severely impaired specimens; sperm loss due to adherence to the vessel

5 METHODOLOGY

5.1 Measurement System

Images are acquired using a motorized Olympus inverted IX81 microscope (Tokyo, Japan). The microscope is equipped with a sub-micron Marzhauser-Wetzlar motorized stage type SCAN-IM, with an Lstep controller; (Wetzlar-Steindorf, Germany) and a filter wheel including fluorescence cube (excitation filters, dichroic mirrors, and emission filters, respectively) for fluorescein: 470-

490 nm, 505 nm long pass and 510-530 nm, for DSC auto-fluorescence: 355-405 nm, 410 nm long pass and 420-450 nm. All filters were obtained from Chroma Technology Corporation (Brattleboro, VT, USA). Objectives of X4/X10, X20 and X60 were used for the nL, pL and fL DSCs, respectively. A cooled, highly sensitive 14-bit, ORCA II C4742-98 camera (Hamamatsu, Japan) was used for imaging. Olympus Cell^P software was used for image analysis (Tokyo, Japan). TransferMan®NK2 Eppendorf micromanipulator and P625 Peristaltic Pump are used to accurately control the minute nl and smaller volumes, required for this work.

5.2 Original Developed Device

DSCs are micro-arrayed, miniature vessels, in which each chamber acts as an individual isolated reaction compartment (Figure 5). Individual live cells can settle in the pL and nL DSCs, share the same space and be monitored under the microscope in a noninvasive, time-resolved manner. theDSCs was designed and constructed to accommodatethe requirements of cryopreservation, namely thefreezing and thawing conditions. It is made of materials having appropriate durability for cryopreservation conditions and to adjust to cryopreservationequipment generally and to cryo-microscopyin particular.

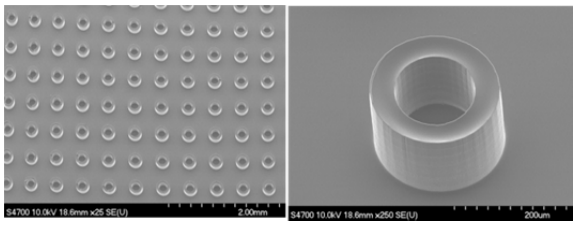

Figure 5: Micro-arrayed donut-shaped chambers (DSCs).

The DSC arrays were fabricated using a Photo Lithographic Patterning technique. A 175 µm thick BSG glass type D263 was spin coated at 3500 rpm with SU8-5 photoresist, to a thickness of 2-2.5µm. The DSC array was patterned on the photoresist by illuminating it through a prefabricated Chromium-mask. This was followed by thermal annealing at 175˚C for 60 min, resulting in stiff and smoothed surfaces of the structured SU8-5 donuts. Finally, the arrayed glass was sawed into 5x5 mm^2 chips, cleaned (mainly from glass debris) by water jetting, dried with clean compressed air and kept in antistatic bags until fabrication. Then, DSC arrays were glued onto a standard microscope slide with a small droplet of NOA81 cured by UV light for 25 sec. Donut structure device was suited for conventional cryo-preservation by gluing it to a spoon-like carrier (Figure 6) and storing in a Standard cryo-tube.

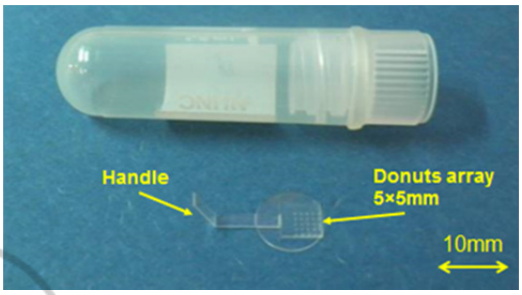

Figure 6: A spoon-like carrier and a standard cryo- tube for device storing.

5.3 Analytical-physical Research

For a theoretical understanding of the microscopic processes involved in freezing and thawing of minute volumes particularly, it is necessary to develop the heat transfer equation for our specific case of nL scale of cylindrical shape suspension. The flow rate of heat energy (Q) through a surface at distance d from the object core, is given by Fourier's law and is proportional to the temperature gradient across the surface:

$$\frac{dQ}{dt} = \frac{KA}{d}(T - T_e) \quad (1)$$

where K is the thermal conductivity, A is the surface area from where the heat energy is transferred, T is the time-dependent temperature within the object, and T_e is the time-dependent object edge temperature. Heat flows from a body to the liquid or gaseous environment around it by convection, following Newton's law of cooling:

$$\frac{dQ}{dt} = hA(T_e - T_\infty) \quad (2)$$

where T_∞ is the environment temperature (e), and h is the heat transfer coefficient. When dividing Equation 2 by Equation 1, we get:

$$\frac{T - T_e}{T_e - T_\infty} = \frac{hd}{K} \quad (3)$$

The ratio hd/K is known as "the Biot number" (Bi). It is clearly seen that when Bi is very small, one can overlook the temperature difference along the cooling body, hence the conductivity. (accepted criterion is $hd/K < 0.1$ (DeWitt, 2007)).

Loss of heat through radiation is negligible due to low temperature, and loss of heat through conduction is negligible either due to the minuscule

dimensions of the body. As the dimensions of a material decrease, the melting temperature scales with the material dimensions (Figure 7). Nanoparticles have a much greater surface to volume ratio than bulk materials. The increased surface to volume ratio means surface atoms have a much greater effect on chemical and physical properties of a nanoparticle. Surface atoms bind in the solid phase with less cohesive energy because they have fewer neighboring atoms in close proximity compared to atoms in the bulk of the solid. Each chemical bond an atom shares with a neighboring atom provides cohesive energy, so atoms with fewer bonds and neighboring atoms have lower cohesive energy. The decrease in melting temperature can be on the order of tens to hundreds of degrees compared to the standard melting temperature of a bulk (T_{MB}). The theoretical size-dependent melting point of a material ($T_m(d)$) can be calculated through classical thermodynamic analysis. The result is the Gibbs-Thomson equation (Haas, 1936) below:

$$T_m(d) = T_{MB}(1 - \frac{4\sigma_{sl}}{H_f \rho_s d}) \quad (4)$$

Where σ_{sl} is the solid-liquid interface energy, H_f is the bulk heat of fusion, ρ_s is density of solid and d is the particle diameter. Although suspension volume in this work is larger than nanometer scale, pre-melting is initiated at the corners and edges of the crystals (Pan D, 2011), and must be considered in this work.

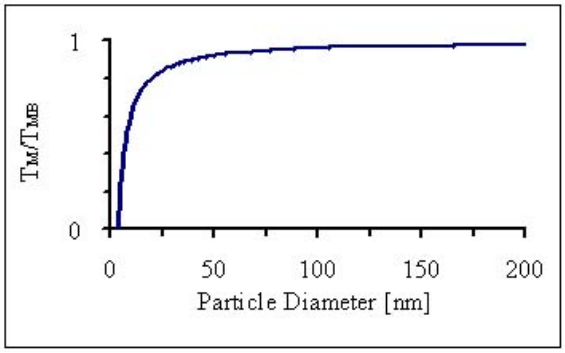

Figure 7: A normalized melting curve for gold as a function of nanoparticle diameter. Experimental melting curves for near spherical metal nanoparticles exhibit a similarly shaped curve.

6 EXPECTED OUTCOME

The aim of this work is to understand the unique physical phenomenon of freezing in very small volumes, and its effect on lodged live sperm cells. This knowledge will make it possible to develop a device, and protocols for freezing and retrieving small numbers of sperm cells at a pre-selected location, on a novel cryo-preservation chip. Such an innovation will improve treatment of male infertility for those who suffer from Oligo-Terato-Asthenozoospermia (OTA) syndrome, as well as those with azoospermia after Testicular Sperm Extraction (TESE) procedure. Worldwide, there are about 20,000 IVF labs. At the average, each laboratory treats about 50 cases of low sperm count related diseases a year, with unsatisfactory, unsuitable cryopreservation procedures and means. In this respects, we strongly believe that overcoming the above mentioned temporary problems of cryopreservation of individual sperm cells within volumes less than nano liter, using our novel device for cryo-preservation, will ensure there is no loss of spermatozoa during the freezing-thawing process.

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