Applications

In recent years, the techniques applied to help infertile couples having children of their own have improved considerably. Intracytoplasmic sperm injection (ICSI) has become a powerful means of overcoming male infertility in patients. With the development of ICSI, the modern infertility lab typically includes micromanipulation equipment attached to inverted microscopes. A sophisticated advancement to ICSI is IMSI, intracytoplasmic morphologically selected sperm injection, a method described by Bartoov et al. [1]. Prior to sperm injection the morphology of the sperm cell is evaluated with high magnification DIC microscopy. Preceding studies have already demonstrated the advantage of IMSI over the conventional IVF-ICSI procedure in terms of the pregnancy rate.

Introduction

It has been estimated that approximately 40 % of sterility in couples can be attributed to male subfertility. ICSI (Intracytoplasmic sperm injection) has raised hopes that these couples can have children of their own. This method of treating predominantly male-factor patients has achieved a breakthrough [2], and it has established itself as the preferred method of treatment in the field of assisted reproduction. When ICSI is used in the routine way, one sperm is taken from the sperm pool after routine selection under a regular microscope that magnifies 200 to 400 times. The birth rate resulting after ICSI treatment ranges between 10-30 % [3]. Sperm morphology has been recognized to be a crucial factor for the efficiency rate of fertilization, pregnancy and birth rate [1;4]. Examination of spermatozoa with the light microscopy can provide only limited information on their internal structure. Using conventional magnification, only sperm morphology can be visualized, but not the sperm nuclear abnormality. To overcome this drawback, Bartoov et al. introduced a modified ICSI procedure: IMSI, or ‘intracytoplasmic morphologically selected sperm injection’.

Figure 1: Motile sperms with morphologically normal nuclear shape but large nuclear vacuole.

* Bartoov Fertility system
which is based on microinjection into retrieved oocytes of spermatozoa with strictly defined morphologically normal nuclei. These sperm are selected by MSOME, a method of unstained, realtime, high magnification ‘motile sperm organellar morphology examination’ [1], see Figure 1. The optimized IMSI treatment does, in fact, result in significantly higher pregnancy rates of 66% versus 30%, compared with conventional IVF-ICSI [5]. A great advantage of the Eppendorf manipulators for this special procedure lies within the small step resolution of 40 nm and the storage of three working positions, thus enabling a sophisticated working under high magnification with sure instinct.

Materials and Methods

Devices (a typical workstation is shown in Figure 2)

- Inverted microscope Olympus IX71/81 equipped with high power DIC (Nomarski/DIC) optics and Uplan Apo ×100 and ×40 oil/1.35 objective lens (Olympus, Germany)
- 2 Micromanipulators TransferMan NK 2 (Eppendorf AG, Germany)
- Adapter for inverted microscope (Eppendorf AG, Germany)
- 2 manual microinjectors CellTram Air and Oil (Eppendorf AG, Germany)
- Video system, either analog or digital system
  - 1. Analog system: ½ inch 3 × CCD color video camera equipped with high resolution monitor, zoom ability of 45×.
  - 2. Digital system: high quality digital camera + computer with digital monitor as described above.

Consumables and media

- WillCo dish (Willco wells BV, Amsterdam, The Netherlands)
- IVF 4 Well Dish (Nunc, Hereford, UK)
- Sterile paraffin oil (OVOIL-100; Vitrolife, Kungsbacka, Sweden)
- Universal IVF or ISM1 medium (MediCult, Jyllinge, Denmark)
- Sperm preparation Medium (MediCult, Jyllinge, Denmark)
- Mineral oil (MediCult, Jyllinge, Denmark)
- PVP solution (ICSI-100, Vitrolife, Kungsbacka, Sweden)
- Sil-Select Plus (FertiPro N.V., Beernem, Belgium)
- EBSS (Ghent, Belgium)

Sperm preparation

The pre-selection procedure of motile high density spermatozoa for IMSI is performed on the basis of a two-layer Sil Select density gradient system which consists of 1 ml upper (low density) and 1 ml lower (high density) layers of saline-coated colloidal silica particles suspended in HEPES-buffered Earle’s balanced salt solution (EBSS). The obtained motile high density sperm fractions are used for further MSOME preparation [5;6].

MSOME

The examination is performed in real time using high power Nomarski optics enhanced by digital imaging to achieve a magnification exceeding 6000 x. It has to be stressed that preferably motile sperm are used for this examination, who under low light-microscopic magnification have a high potential to be selected by routinely performed ICSI (for the IMSI procedure sperm with poor motility can also be selected, provided that they have a normal nucleus). About 4000 motile high density sperm cells, obtained from the above fraction, are transferred to a 4 µl observation microdroplet of sperm preparation medium containing PVP solution. In order to estimate the morphological state of the sperm nucleus, one has to follow the motile sperm cell by moving the microscopic stage in the x, y and z directions for about 20 seconds. To reduce PVP toxicity, which has to be used to slow down highly motile sperm from disappearing from the monitor screen, the concentration of the PVP is adjusted to a minimum (range 0 – 8 %). The observation microdroplet is placed in a sterile, glass-bottomed dish under sterile paraffin oil. The sperm cells, suspended in the observation microdroplet, were used for individual retrieval by MSOME.
The criteria for a normally shaped nucleus by MSOME are smooth, symmetric and oval configurations (previously defined by scanning electron microscopy [1,5,7]). For MSOME, the average length and width of this configuration were estimated in 100 spermatozoa with an obviously normal nuclear shape [8]. During sperm selection, a fixed, celluloid form of a sperm nucleus representing the normal criteria is superimposed on the examined cell; any sperm cell with a nuclear shape has to be excluded from selection if it varies in length or width by 2 SDs from the normal mean axes values. Also, all sperm with more than one vacuole or with vacuoles occupying more than 4% of nuclear area have to be discarded [7].

**Sperm retrieval after MSOME**

MSOME selected sperm cells are retrieved from the observation droplet and placed into a recipient selection droplet, containing 4 µl of sperm preparation medium in the same petri dish. This procedure is performed using the Eppendorf TransferMan NK 2 Micromanipulation System, which is equipped with a sterilized, non-angled, glass microcapillary with a 12 µm inner diameter and 45° bevelled, non-spiked tip. Prior to the experiment the microcapillary has to be fitted, aligned and equilibrated before starting the MSOME procedure. It is also necessary to prime micropipettes with medium before use so that the selected sperm and later on the retrieved oocytes never come into contact with air or oil. The glass microcapillary was inserted from above in a z-axis movement into the observation droplet. Since the TransferMan NK 2 can store up to 3 positions, one position is set as a parking position above the droplet and another one serves as the actual working position inside the droplet in the focal plane of the sperms next to the bottom of the petri dish. By pressing the joystick button twice (double-click) the capillary can be returned to a preset position. The capillary can be moved easily in 40 nm resolution steps in any direction (x/y/z) by means of a single joystick. This feature enables the fast and precise retrieval of the highly morphologically qualified sperm cells visualized on the monitor.

**Microinjection**

The retrieved, cumulus-free ova were placed into drops of sperm preparation medium prepared in the same glass dish with the recipient droplet. The latter contained the sperm cells morphologically selected for ICSI. A schematic overview of the prepared petri dish is shown in Figure 3.

Figure 3: Petri dish prepared for IMSI procedure. Red circle: droplet with unselected sperm, retrieved via pre-selection procedure of motile high density spermatozoa on the basis of a two-layer Sil Select density gradient system; pink circle: recipient droplet for selected spermatozoa; blue circles: droplets with retrieved oocytes for sperm injection; purple droplet: additional PVP.

The microinjection procedure is performed according to standard procedures at the usual magnification of 200 – 400x [9]. A typical injection is demonstrated in Figure 4. Each microinjected oocyte is immediately transferred to a 4-well dish, incubated in 0.5 ml of universal IVF or ISM1 medium and covered with 0.5 ml of mineral oil at 37 °C with an atmosphere of 5% CO₂.

Figure 4: The spermatozoa is deposited towards the center of the aspirated oocyte.
Results and Discussion

In our studies [1;8;10], we came to the conclusion that the MSOME criteria for normally shaped nuclei are smooth, symmetric and oval configuration, with average length and width limits and a homogeneity of the nuclear chromatin mass, with no regional nuclear disorders, and containing no more than one vacuole, which occupies no more than 4% of nuclear area. During selection, a fixed, transparent, celluloid form of a sperm nucleus fitting the normal nuclear shape is superimposed on each examined cell. Spermatozoa which vary in length or width by two standard deviations from the normal mean axes values have to be considered as abnormally shaped cells. In order to investigate the homogeneity of the chromatin mass, we are using the front and side views of the differential interference contrast (DIC) optics, thus being able to distinguish between the topography of the chromatin vacuoles, which are always surrounded by a smooth chromatin mass, and the topography of the regional disorders of the nucleus, which look like craters or extrusions. No other observation methods, including electron microscopy, can do so, since only MSOME is performed in real time on motile spermatozoa, which, during observation, change position (see Figure 5).

Thus, sperm motility is a must at least for the precise detection of nuclear content. In order to immobilize and transfer the selected motile sperm, fast, but ultra-fine resolution movement of the transfer capillary and the possibility to store working and storage position is a prerequisite, especially when working under thus high magnification condition. We try to collect two highly morphologically selected sperm cells for each ova expected to be retrieved. It could be that in the male partner’s semen we will not be able to find morphologically qualified sperm cells, and then we select ‘second best’. We could show [8;10] that in comparison to ICSI performed with spermatozoa selected by conventional techniques (microscope magnification of 200–400 times), our new method exhibits a higher pregnancy rate per cycle (66 % versus 30 %) and a significantly lower abortion rate per pregnancy (9 % abortion rate versus 33 %).

In conclusion, microinjection of sperm with abnormal shape or nuclear vacuoles appears to reduce pregnancy outcome. This drawback can be prevented by morphological sperm selection based on MSOME. To this date, some 500 babies have been born in Israel after IMSI treatment, some 200 more in Europe.
Literature


More information (including how to become a BFS* certified specialist) can also be found on http://www.bartoov-sperma.com/eng/eng_home.html and www.eppendorf.com/imsi

* Bartoov Fertility system
## Ordering Information

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WillCo dish is a registered trademark of Willco wells BV, Amsterdam, The Netherlands.
ISM1 medium is a trademark of MediCult, Jyllinge, Denmark.
ICSI-100 is a trademark of Vitrolife, Kungsbacka, Sweden.

* This product is registered in Europe as a medical device (according to Medical Device Directive MDD/93/42/EDD). This product is not registered in the U.S. as a medical device and does not have a 510(k) registration. For research use only. Not for use in human medical applications.

** Proven non-cytotoxicity by the mouse embryo development test