# Efficiency of intracytoplasmic sperm injection and related micromanipulation techniques in the treatment of male factor infertility

Ph.D. thesis

Mátyás Szabolcs

Kaali Institute
Department of Embryology
Budapest

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**List of publications**

*Publications connected to the thesis*

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**Abbreviations**

| | |
|---|---|
| AH | assisted hatching |
| ART | assited reproduction technique |
| ELSI | elongated spermatid injection |
| ET | embryo transfer |
| HA | hyaluronic acid |
| HBA | hyaluronan binding assay |
| ICSI | intracytoplasmic sperm injection |
| IMSI | intracytoplasmic morphologycally selected sperm injection |
| IVF | in vitro fertilization |
| LAH | laser assisted hatching |
| MESA | microsurgical epididymal sperm aspiration |
| MSOME | motile sperm organellar morphology examination |
| OAT | oligozoo-asthenozoo-teratozoospermia |
| PESA | percutaneous epididymal sperm aspiration |
| PICSI | petri dish intracytoplasmic sperm injection |
| PZD | partial zona dissection |
| ROSI | round spermatid injection |
| SCOS | Sertoli cell only syndrome |
| TESA | testicular sperm aspiration |
| TESE | testicular sperm extraction |
| VEA | vaso-epididymo-anastomosis |

## Introduction

Data of the Hungarian Central Statistical Office (KSH) demonstrate that the population of Hungary is decreasing. One of the reasons is that the number of live birth has fallen back. On the other hand frequency of infertility in Hungary as well as in the Western European countries and in the USA has risen (15-20 % of the total population). Infertility was thought to be of female origin. In our days it is well known that generally the male partner is responsible for the infertility in 30 % of infertile couples. In 30 % of the cases exclusively the female and in 40 % both of them carry the fertility problems (16). Analysing the data of 1741 IVF cycles in 2007 (Kaali Institute, Budapest) we found that the distribution of the main diagnostic groups was as follows: male 33 %, female 36 %, mixed (male and female) 17 % and unexplained 14%.

Male factor infertility is a general term that describes couples where the male partner is responsible for the inabality to conceive. This problem may be associated with low sperm production (oligozoospermia), poor sperm motility (asthenozoospermia) or abnormal morphology (teratozoospermia). Male factor infertility also describes men with normal sperm production but conditions that prevent sperm transport (reproductive tract obstruction, ejaculatory dysfunction) or interaction with cervical mucus, zona pellucida or oolemma (48, 113). The cornerstones of evaluation of a subfertile man include a comprehensive history, physical examination, multiple semen analyses and an endocrine evaluation.

More than 30 years have been passed since the first successful IVF attempt was reported (95). In the early years of human IVF patients with azoospermia or severe oligozoospermia had almost no chance of treatment even though the use of microsurgical epididymal sperm aspiration (MESA) from a man who had received an irreversible vasectomy was reported in 1985 (97). Introduction of intracytoplasmic sperm injection (ICSI) opened new treatment possibilities in 1992 (77, 108). It was demonstrated later that spermatozoa derived from the testicle was capable of normal fertilization and pregnancy rates in cases of obstructive azoospermia (89). ICSI in conjuction with testicular sperm extraction (TESE) was extended further to treat men with non-obstructive azoospermia (109). Furthermore, alternative techniques like percutaneous epididymal sperm aspiration (PESA) and testicular sperm aspiration (TESA), were developed for surgical sperm retrieval (22, 90). Devroey (23) and Romero (87) demonstrated that frozen-thawed epididymal spermatozoa could be used as well as frozen-thawed testicular spermatozoa

for fertilization by ICSI. In case of incomplete spermatogenetic arrest TESE can be combined with ICSI (92) too. The feasibility of the use of round spermatids instead of spermatozoa has been confirmed by the first birth of a healthy child after round spermatid injection into human oocytes (100). In spite of these results, concerns has been raised about the potential drawbacks of intracytoplasmic sperm injection and related techniques. Certain steps of natural fertilization (e.g. binding to the zona pellucida, penetration) are missing if the spermatozoon is injected directly to the ooplasm. Traditionally a spermatozoon is selected based on its morphology and motility. It has been recently confirmed that hyaluronic acid (HA) binding capacity of spermatozoa is related to other important biochemical factors wich are involved in the process of fertilization (36). A newly developed method called PICSI (Petri dish ICSI) utilizes the HA binding ability of spermatozoa in sperm selection for ICSI. It is a new chance to refine the existed methods, since technical features of ICSI do influence subsequent embryonic development.

Micromanipulation of gametes – such as intracytoplasmic sperm injection (ICSI) and assisted hatching (AH) via partial zona dissection (PZD) - connected with human IVF in Hungary was introduced by the Kaali Institute in 1994 (13, 14, 15, 47, 52, 84). The first Hungarian testicular sperm extraction (TESE) in conjuction with intracytoplasmic sperm injection was done in the next year and the first „TESE-baby" was born in 1996 (85). The next improvement in the treatment of male infertility was the use of cryopreserved spermatozoa and round spermatids for fertilization and blastocyst culture (54, 55, 56, 57, 58, 59, 61, 63, 66). Later we started to use the above mentioned PICSI technique, for the first time in 2007 (69). With an eye to the future it is crucial to invastigate how the adopted techniques (ICSI, TESE+ICSI, ELSI, ROSI, PICSI) and other factors effect the efficiency of IVF cycles.

**Aim of the study**

The aim of the study was to evaluate and compare the efficiency of intracytoplasmic sperm injection (ICSI) and related techniques (PICSI, ROSI, ELSI) in the treatment of male factor infertility. The following aspects have been taken into account:

*Concerning the etiology*

- Does the type of male factor infertility have effect on the efficiency of intracytoplasmic sperm injection and related micromanipulation techniques?
- What is the effect of female factors in case of mixed (male and female) origin infertility?
- How effective is intracytoplasmic sperm injection (ICSI) in obstructive and non-obstructive azoospermia?

*Concerning the type and the quality of gametes used for fertilization*

- Do sperm concentration, motility and morphology effect the efficiency of ICSI cycles?
- How effective is intracytoplasmic sperm injection (ICSI) if fresh or cryopreserved spermatozoa are used for fertilization?
- How effective is intracytoplasmic sperm injection (ICSI) if testicular spermatozoa were used for fertilization?
- How effective is the applied micromanipulation technique if round (ROSI) or elongated spermatids (ELSI) are used for fertilization?

*Concerning the type of gamete micromanipulation*

- How effective is intracytoplasmic sperm injection (ICSI)?
- How effective is round spermatid (ROSI) and elongated spermatid (ELSI) injection?
- Perspectives of sperm selection based on hyaluronan binding capacity (PICSI)?
- Wich technical aspects should be taken into consideration in order to improve the efficiency of gamete micromanipulation?

**Materials and methods**

Retrospective (ICSI, ROSI, ELSI) and prospective (ICSI, ROSI, ELSI, PICSI) analysis of data of IVF cycles has been applied in the following groups:

1. ICSI using fresh ejaculated spermatozoa in mixed (male and female) factor infertility
2. ICSI using fresh ejaculated spermatozoa in exclusively male factor infertility (age of the women ≤ 37 years with normal basal FSH and LH levels, minimum 5 MII. oocytes at the time of fertilization)
3. ICSI using cryopreserved ejaculated spermatozoa
4. ICSI using fresh testicular spermatozoa in obstructive (4.a) and non-obstructive (4.b) azoospermia (TESE+ICSI)
5. ICSI using cryopreserved testicular spermatozoa (cryoTESE+ICSI)
6. ROSI/ELSI using round spermatids and elongated spermatids
7. PICSI using spermatozoa selected by hyaluronan binding

Following factors have been taken into consideration: paternal and maternal age, basal FSH of women, number of IVF-ET cycle, stimulation protocol, endometrial thickness, number of oocytes retrieved, fertilization rate of MII. oocytes, proportion of good quality embryos, number of total and good quality embryos, application of assisted hatching, number of transferred embryos, day of ET, clinical pregnancy rate, sperm parameters (ICSI and PICSI groups) and etiology of azoospermia (TESE+ICSI group). Following subgroups has been formed to examine the effect of sperm factors depending on the sperm concentration, motility and normal morphology in fresh semen (**Table 1,** ICSI in mixed and male factor infertility; PICSI), type of azoospermia and sperm count in prepared fresh testicular biopsies (**Table 2,3** TESE+ICSI) and post-thaw motility of cryopreserved testicular biopsies (**Table 4,** cryoTESE+ICSI).

**Table 1** Subgroups based on sperm concentration, motility and normal morphology in the Group 1, 2 and 7

| | |
|---|---|
| *Sperm concentration (sperm/ml)* | 1-100<br>100-100.000<br>100.000-1 $x10^6$<br>1 $x10^6$-4,9 $x10^6$<br>5 $x10^6$ -9,9$x10^6$<br>10 $x10^6$-14,9 $x10^6$<br>15 $x10^6$-19,9 $x10^6$<br>20 $x10^6$ < |
| *Motility (%)* | 1-9<br>10-19<br>20-29<br>30-40<br>40-49<br>50 % < |
| *Normal morphology (%)* | 30 % ><br>30 % ≤ |

**Table 2** Subgroups based on the type of azoospermia in the Group 4

| |
|---|
| Obstructive |
| Non-obstructive |

**Table 3** Subgroups based on sperm count in the Group 4

| *Subgroups* | *Sperm count* |
|---|---|
| 0 | No spermatozoa could be found |
| 1 | Only a few spermatozoa could be found in the whole sample |
| 2 | One or more spermatozoa per more than 10 viewfield (200x magnification) |
| 3 | One or more spermatozoa per 10 viewfield (200x magnification) |

**Table 4** Subgroups based on motility in the Group 5

| *Subgroups* | *Motility* |
|---|---|
| 1 | Motile spermatozoa after thawing |
| 2 | Motile spermatozoa induced by pentoxifyllin |
| 3 | Inmotile spermatozoa selected by hypoosmotic swelling test (HOST) |

***Oocyte retrieval***

**Ovarian hyperstimulation**

Controlled ovarian hyperstimulation was carried out using long/ultrashort protocol with GnRH agonist or Clomiphen citrate in combination with recombinant FSH. Follicle development was evaluated directly with transvaginal ultrasound imaging of follicular growth and by measurement of serum estrogen and progesterone levels. Final oocyte maturation was induced with an intramuscular dose of hCG (5–10,000 units) when optimal follicular development was obtained.

**Follicle aspiration**

Retrieval of oocytes was performed by transvaginal follicular aspiration using ultrasound guidance with intravenous sedation. Oocytes were washed in GMOPS medium (Vitrolife, Scandinavia), then placed into GIVF medium (Vitrolife, Scandinavia) and incubated (37 ºC, 6 % $CO_2$) for 2-3 hours before denudation.

***Sperm retrieval***

**Sperm examination**

Semen specimen was obtained by masturbation after 3-5 days of sexual abstinence. Semen analysis was carried out under phase contast microscope. Sperm concentration and motility was determined using Makler counting chamber. When it was possible morphology was also examined following fixation and staining with eosin of the sample.

**Sperm preparation**

*Density gradient centrifugation*

Two layer (0,5-1,5 ml of 90 % and 45 % SpermGrad; Vitrolife, Scandinavia) gradient was applied. 0,5-2 ml of liquified semen was gently placed on the top of the layers and the sample was centrifuged at 300 **g** for 20 minutes. Pellet was removed with Pasteur pipette and placed into 3 ml GSperm medium (Vitrolife, Scandinavia). Then the sample was centrifuged at 300 **g** for 10 minutes. Supernatant was removed and depending on the sediment, 0,5-1,0 ml GIVF medium was placed carefully on the pellet. The centrifuge tube was placed in incubator (37

ºC, 6 % $CO_2$) and motile spermatozoa were allowed to swim up for 1 hour. Supernatant was used for fertilization.

*Washing*

In the case of poor quality semen or testicular tissue, samples were only washed twice in GSperm medium at 300 **g** for 10 minutes. Pellet was then diluted in GIVF medium.

*Preparation of testicular tissue*

Fresh testicular tissue was dissected by surgical blades and examined under inverted microscope. If an appropriate number of spermatozoa was found, gradient centrifugation was performed using 0,5-1,0 ml layers. In cases of extremely low sperm count, washing was only repeated. Final dilution was carried out in GIVF medium depending on the initial mass of the testicular tissue.

*Preparation of frozen samples*

Freezing was performed in SpermFreezing medium (MediCult, Denmark) in the vapoure phase of liquid nitrogen (rapid freezing). Frozen semen and testicular tissue was prepared in similar way. For thawing frozen samples in cryogenic ampoulles were held at room temperature for 1 minute, then immersed into water bath (37 ºC) for 5 minutes. Thawed sample then was prepared by gradient centrifugation or washing depending on the quality.

*Pentoxifyllin treatment*

If motile spermatozoa could not be found after incubation of the prepared sample for 1 hour (37 ºC, 6 % $CO_2$), motility was induced with pentoxifyllin (3mM final concentration). Sample was examined again one hour later under inverted microscope.

*Hypoosmotic swelling test*

In the case of immotile spermatozoa, hypoosmotic swelling test (HOST) was performed in Hypo-10 medium (Vitrolife, Scandinavia). Spermatozoa with swollen tail were selected for ICSI.

***Fertilization, embryo culture, embryo transfer, cryopreservation***

**Intracytoplasmic sperm injection (ICSI), round spermatid injection (ROSI), elongated sperm injection (ELSI)**

Oocytes were prepared by removing the cumulus mass and corona radiata with hyaluronidase. All ICSI, ROSI or ELSI was performed on MII. oocytes wich had been reached the methaphase of second meiotic division. ICSI was carried out in GMOPS medium under paraffin oil (Ovoil; Vitrolife, Scandinavia). The oocyte was stabilized with a holding micropipette (Humagen, USA) and injected with an injection micropipette of 4-5 μm of inner diameter (Humagen, USA or Swemed, Scandinavia) under an inverted microscope. In the case of round or elongated spermatids a thicker micropipette was used with a 9 μm of inner diameter. Motile spermatozoa were initially immobilized with the injection micropipette. The individual single sperm or sperm precursor cell was aspirated and directly injected into the oocyte. The polar body was held at the 12 or 6 o'clock position, and the injection micropipette containing the single sperm was pushed through the zona pellucida and oolemma into the cytoplasm of the oocyte at the 3 o'clock position. Further handling of injected oocytes was similar to that for oocytes in standard IVF (see Embryo culture).

**Petri dish intracytoplasmic sperm injecsion (PICSI)**

In the case of PICSI spermatozoa were selected by hyaluronan binding. The PICSI dish is a polystyrene culture dish with three microdots of hyaluronan attached to the interior bottom. Hyaluronan dots were hydrated with 10-10 μl GMOPS at room temperature. 10 μl prepared sperm suspension was added to the GMOPS microdroplet and mixed. The PICSI dish was carefully flood with paraffin oil (Ovoil) and incubated for 10-30 minutes at room temperature. To collect a bound sperm the tip of the ICSI micropipette was positioned next to the sperm and it was gently drawing in. Injection was carried out int he same way as in conventional ICSI.

**Embryo culture and embryo grading**

Injected oocytes were placed into preincubated GIVF medium and incubated (37 ºC, 6 % $CO_2$). Fertilization was checked 16-18 hours later. Fertilized oocytes were separated from infertilized ones and were transferred into 50 μl micdrodroplets of G1 medium (Vitrolife, Scandinavia) under paraffin oil (Ovoil). Fertilized oocytes were cultured in groups of 4-5 for 3-5 days. Embryonic development was routinely checked once a day. Embryo grading was

carried out as follows: *Grade 1* (even or uneven blastomeres, fragmentation ≥50 %), *Grade 2* (even or uneven blastomeres, fragmentation ≥ 20 % < 50 %), *Grade 3* (even or uneven blastomeres, fragmentation < 20 %), *Grade 4* (even blastomeres, no fragmentation). In the case of blastocyst transfer 6-8 cell embryos were placed into 50 µl micdrodroplets of G2 medium (Vitrolife, Scandinavia) under paraffin oil (Ovoil) on day 3. Blastocyst scoring was based on the stage (*Grade 1*: early blastocyst; *Grade 2*: blastocoel more than half of the blastocyst; *Grade 3*: blastocoel fills the blastocyst; *Grade 4*: expanded blastocyst), the quality of the inner cell mass (*A*: numerous and tightly packed cells; *B*: several and loosely packed cells; *C*: few cells) and the quality of the trophoectoderm cells (*A*: many cells organized in epithelium; *B*: several cells organized in loose epithelium; *C*: few cells).

**Laser assisted hatching (LAH)**

Assisted hatching was performed with a non-contact laser equipment (Hamilton-Thorne, USA). Indications for assisted hatching were the maternal age (35<), two previous failured IVF cycles or irregularity of the zona pellucida.

**Embryo transfer**

Embryotransfer was carried out in G1 or G2 medium depending on the day of ET. Up to 4 embryos were aspirated in 15-20 µl culture medium between two small air bubbles into Wallace catheter (UK). The position of the air bubbles was determined by transvaginal ultrasound imaging.

**Embryo freezing**

Supernumerous embryos were frozen with slow freezing protocol using Embryo Freezing Kit (Medicult, Denmark) or BlastFreezing Kit (Medicult, Denmark). Day2/day3 embryos were dehydrated in 1,3-propanediol and sucrose (1,5M PROH and 1,5M PROH+ 0,5 M sucrose stepwisely) and day5/day6 embryos in glicerol and sucrose (7,5 % glicerol and 15% glicerol+0,1 M sucrose stepwisely). Freezing procedure was carried out in programmable freezing equipment (Planer, UK).

**Statistical analysis**

Statistical analysis was performed using $\chi^2$-test, Student's t-test and Fisher's exact test, where appropiate. A P value < 0.05 was considered to be statistically significant. Analysis was carried out using Statview Software, Version 5.0 (SAS Institute Inc., USA).

## Results

### ICSI with fresh ejaculated spermatozoa in mixed (male and female) factor infertility

Data of 128 IVF-ET cycles have been analysed where fertiliazition of oocytes was performed with ICSI of fresh ejaculated spermatozoa. The indication of IVF treatment was mixed (male and female) factor infertility. Mean age of the women was 34,8 (± 4,9 SD) years, mean number of cycles 2,0 (± 1,1 SD) and mean basal FSH and LH levels 10,3 (± 4,4 SD) mIU/l and 7,2 (± 3,9 SD) mIU/l respectively. A total of 844 oocytes (mean: 6,6 ± 4,1 SD) were collected and 668 MII. oocytes were injected. Fertilization rate related to MII. oocytes was 88,2 (± 22,9 SD) % and the proportion of good quality embryos was 69,2 (± 28,5 SD) %. In the course of 125 embryo transfer 302 embryos (mean: 2,42 ± 0,75 SD) were transferred and 93 supernumerous embryos derived from 16 cycles were cryopreserved. IVF traetments have resulted 31 singletons, 7 twins and 1 triplet. Clinical pregnancy rate related to ET was 31,2 %. The effect of the examined parameters on the pregnancy rate is shown in **Table 5** while **Table 6** demonstrates the influence of sperm parameters regarding fertilization, in vitro embryonic development and pregnancy rate. Finally, Table 7 presents the effect of maternal age on the quality and maturation of oocytes and embryonic development. Regression analyses of the proportion of matured (MII.) oocytes and the proportion of good quality embryos also proved the strong correlation of these two parameters (P< 0,0001).

**Table 5** Effect of the examined parameters on the pregnancy rate
(*continued on the next page*)

| *Parameter* | *Pregnancy rate* |
|---|---|
| Sperm concentration | N.S. |
| Motility | N.S. |
| Normal morphology | N.S. |
| Paternal age (mean: 37,6 ± 5,7 year) | N.S. |
| Maternal age (mean: 34,8 ± 4,9 year) | P=0,0206 |
| Basal FSH level | N.S. |
| Stimulation protocol | N.S. |
| Number of collected oocytes | N.S. |
| Number of matured (MII.) oocytes | N.S. |
| Number of normally fertilized oocytes | P=0,0294 |

| | |
|---|---|
| Number of good quality embryos | P=0,0419 |
| Number of transferred embryos | P< 0,0001 |
| Number of transferred good quality embryos | N.S. |
| Day of ET (2.,3.,4.,5.) | P=0,0113 |
| Endometrium thickness | N.S. |
| Application of laser assisted hatching | N.S. |

**Table 6** Effect of sperm parameters on fertilization, embryonic development and clinical pregnancy rate

| *Parameter* | *n* | *Fertilization rate/MII. oocytes (%)* | *Cleavage rate (%)* | *Good quality embryos (%)* | *Clinical pregnancy rate/ET (%)* |
|---|---|---|---|---|---|
| *Sperm concentration* | | | | | |
| 1-100 spermatozoa | 12 | 80,8 [a,b] | 99,1 | 75,4 | 58,3 |
| 100-100.000 sp. | 3 | 90,3 | 100 | 66,7 | 66,7 |
| 0,1-1x$10^6$ /ml | 23 | 85,9 [c] | 95,5 | 69,6 | 58,3 |
| 1-4 x$10^6$/ml | 4 | 84,4 | 100 | 54,4 | 25,0 |
| 5-9 x$10^6$/ml | 13 | 86,1 | 100 | 70,5 | 8,3 |
| 10-14 x$10^6$/ml | 22 | 99,5 [a] | 96,2 | 61,2 | 36,4 |
| 15-19 x$10^6$/ml | 10 | 83,4 | 96,3 | 76,3 | 30,0 |
| 20 x$10^6$/ml < | 40 | 98,2 [b,c] | 100 | 72,9 | 27,5 |
| | | [a] P= 0,0499 | | | |
| | | [b] P= 0,0160 | | | |
| | | [c] P= 0,0341 | | | |
| *Motility (%)* | | | | | |
| 1-9 | 1 | 80,0 | 100 | 40,0 | 100 |
| 10-19 | 10 | 88,9 | 100 | 74,8 | 11,1 |
| 20-29 | 15 | 81,6 | 97,0 | 75,6 | 13,3 |
| 30-39 | 27 | 100 | 97,5 | 67,1 | 32,1 |
| 40-49 | 14 | 100 | 100 | 76,2 | 42,9 |
| 50 % < | 22 | 93,0 | 97,6 | 61,9 | 22,7 |
| *Normal morphology* | | | | | |
| 30 % > | 98 | 91,9 | 100 | 68,9 | 31,6 |
| 30 % ≤ | 17 | 88,2 | 95,8 | 78,5 | 36,4 |

**Table 7** Effect of maternal age on the number and quality of oocytes, fertilization and embryonic development

| | | |
|---|---|---|
| Maternal age | Number of oocytes | P< 0,0001 |
| | Number of MII. oocytes | P< 0,0001 |
| | Proportion of MII. oocytes (%) | P< 0,0001 |
| | Fertilization rate related to MII. oocytes (%) | P< 0,0001 |
| | Proportion of good quality embryos (%) | P< 0,0001 |

**ICSI with fresh ejaculated spermatozoa in male factor infertility**

Data of 205 IVF-ET cycles have been analysed where fertiliazition of oocytes was performed with ICSI of fresh ejaculated spermatozoa. The indication of IVF treatment was male factor infertility. Mean age of the women was 31,9 (± 4,1 SD) years, mean number of cycles 2,0 (± 1,2 SD) and basal FSH and LH levels 7,1 (± 1,7 SD) mIU/l and 6,2 (± 3,0 SD) mIU/l respectively. A total of 2524 oocytes (mean: 10,1 ± 9,7 SD) were collected and 1785 MII. oocytes were injected. Fertilization rate related to MII. oocytes was 87,2 (± 29,6 SD) % and the proportion of good quality embryos was 68,1 (± 25,8 SD) %. In the course of 249 embryo transfer 605 embryos (mean 2,43) were transferred and 276 supernumerous embryos derived from 52 cycles were cryopreserved. IVF treatments have resulted 77 singletons, 24 twins and 2 triplets. Clinical pregnancy rate related to ET was 41,4 %. The effect of examined factors on the pregnancy rate is shown in **Table 8** while **Table 9** demonstrates the influence of sperm parameters regarding fertilization and embryonic development.

**Table 8** Effect of the examined parameters on the pregnancy rate (*continued on the next page*)

| *Parameter* | *Pregnancy rate* |
|---|---|
| Sperm concentration | N.S. |
| Motility | N.S. |
| Normal morphology | N.S. |
| Paternal age (mean: 36,2 ± 5,2 years ) | N.S. |
| Maternal age (mean: 31,9 ± 4,1 years) | P=0,0319 |

| | |
|---|---|
| Basal FSH level | N.S. |
| Stimulation protocol | N.S. |
| Number of oocytes | N.S. |
| Number of MII. oocytes | P=0,0510 |
| Number of normally fertilized oocytes | P=0,0066 |
| Number of good quality embryos | P=0,0004 |
| Number of transferred embryos | P< 0,0001 |
| Number of tansferred good quality embryos | P< 0,0001 |
| Day of ET (2.,3.,4.,5.) | N.S. |
| Endometrium thickness | N.S. |
| Application of laser assisted hatching | N.S. |

**Table 9** Effect of sperm parameters on fertilization, embryonic development and clinical pregnancy rate (*continued on the next page*)

| *Parameter* | *n* | *Fertilization rate/MII. oocytes (%)* | *Cleavage rate (%)* | *Good quality embryos (%)* | *Clinical pregnancy rate/ET (%)* |
|---|---|---|---|---|---|
| *Sperm concentration* | | | | | |
| 1-100 spermatozoa | 43 | 80,0 [a,b] | 100 | 61,7 [a] | 36,4 |
| 100-100.000 sp. | 20 | 80,5 | 98,3 | 62,6 | 55,0 |
| 0,1-1x$10^6$ /ml | 45 | 83,2 | 99,6 [a] | 64,4 | 52,2 |
| 1-4 x$10^6$/ml | 15 | 88,6 | 95,5 | 73,8 | 20,0 |
| 5-9 x$10^6$/ml | 29 | 92,0 | 100 [b] | 73,4 | 51,7 |
| 10-14 x$10^6$/ml | 38 | 97,4 [a] | 99,4 [c] | 66,7 | 44,7 |
| 15-19 x$10^6$/ml | 16 | 82,5 | 90,9 [a,b,c] | 70,2 | 37,5 |
| 20 x$10^6$/ml < | 42 | 91,3 [b] | 95,1 | 76,0 [a] | 33,3 |
| | | [a] P= 0,0493<br>[b] P= 0,0435 | [a] P= 0,0115<br>[b] P= 0,0499<br>[c] P= 0,0343 | [a] P= 0,0081 | |

| | | | | | |
|---|---|---|---|---|---|
| *Motility (%)* | | | | | |
| 1-9 | 3 | 52,4 | 83,3 | 80,6 | 0,0 |
| 10-19 | 24 | 96,6 | 94,3 | 72,1 | 41,7 |
| 20-29 | 25 | 97,3 | 95,6 | 69,0 | 40,0 |
| 30-39 | 29 | 86,7 | 99,4 | 75,7 | 41,4 |
| 40-49 | 14 | 100 | 100 | 73,3 | 42,9 |
| 50 % < | 45 | 88,4 | 97,4 | 70,3 | 37,8 |
| *Normal morphology* | | | | | |
| 30 % > | 88 | 88,3 | 98,0 | 69,7 | 41,9 |
| 30 % ≤ | 52 | 85,4 | 99,4 | 65,4 | 43,6 |

**ICSI with cryopreserved ejaculated spermatozoa in male factor infertility**

Data of 86 IVF-ET cycles have been analysed where fertiliazition of oocytes was performed with ICSI of cryopreserved ejaculated spermatozoa. The indication of IVF treatment was male factor infertility. Mean age of the women was 32,5 (± 5,2 SD) years, mean number of cycles 2,1 (± 1,7 SD) and basal FSH and LH levels 7,8 (± 3,7 SD) mIU/l and 5,7 (± 3,1 SD) mIU/l respectively. A total of 676 oocytes (mean: 7,9 ± 4,8 SD) were collected and 546 MII. oocytes were injected. Fertilization rate related to MII. oocytes was 77,8 (± 38,6 SD) % and the proportion of good quality embryos was 67,8 (± 31,8 SD) %. In the course of 82 embryo transfer 194 embryos (mean 2,36 ± 0,87 SD) were transferred and 50 supernumerous embryos derived from 10 cycles were cryopreserved. IVF treatments have resulted 12 singletons, 3 twins and 1 triplet. Clinical pregnancy rate related to ET was 19,5 %. The effect of examined factors on the outcome is shown in **Table 10.**

**Table 10** Effect of the examined parameters on the pregnancy rate (*continued on the next page*)

| *Parameter* | *Pregnancy rate* |
|---|---|
| Maternal age (mean: 32,5 years) | N.S. |
| Paternal age (mean: 35,9 years) | N.S. |
| Basal FSH level | N.S. |
| Stimulation protocol | N.S. |
| Number of oocytes | N.S. |
| Number of MII. oocytes | N.S. |

| | |
|---|---|
| Number of normally fertilized oocytes | N.S. |
| Number of good quality embryos | N.S. |
| Number of transferred embryos | P< 0,0001 |
| Number of transferred good quality embryos | P< 0,0001 |
| Day of ET (2.,3.,4.,5) | N.S. |
| Endometrium thickness | N.S. |
| Application of laser assisted hatching | N.S. |

**ICSI with fresh testicular spermatozoa in male factor infertility (TESE+ICSI)**

Testicular biopsies were retreived by open surgical method (TESE) in the course 273 IVF-ET cycles of 189 couples. Diagnosis of azoospermia was certified by andrologist following fully comprehensive examination. 105 obstructive and 168 non-obstructive cases of azoospermia was the indication for IVF treatment. Testicular tissue was transported in SpermPrep medium (Medicult, Denmark) to the IVF center. In 218 cases (146 patients) testicular biopsy was successful provided spermatozoa for fertilization. Mean age of the women was 30,0 (± 3,8 SD) years, mean number of cycles 2,3 (± 1,4 SD) and basal FSH and LH levels 8,1 (± 2,1 SD) mIU/l and 6,9 (± 2,3 SD) mIU/l respectively. A total of 1668 oocytes were retrieved during 218 IVF-ET cycles and 1283 MII. oocytes were injected with fresh testicular spermatozoa. Fertilization rate related to MII. oocytes was 55,4 (± 27,1 SD) % and the proportion of good quality embryos was 54,4 (± 31,2 SD) %. 567 embryos (mean: 2,9 ± 1,2 SD) were transferred during 198 ET and 237 supernumerous embryos were cryopreserved in 39 cycles. IVF treatments have resulted 31 singletons, 14 twins, 3 triplets and 2 quadruplets. Clinical pregnancy rate related to ET was 27,8 %. **Table 11, Table 12** and **Table 13** contain data regarding to the quality of testicular biopsies of obstructive and non-obstructive azoospermic cases. Data related to the IVF-ET treatment are shown in **Table 14** and **Table 15**.

**Table 11** Results of TESE according to the type of azoospermia

| | *Total* | *Obstructive* | *Non-obstructive* | *P-value* |
|---|---|---|---|---|
| Testicular biopsies(n) | 273 | 105 | 168 | - |
| Successful retrieval (n/%) | 218/79.8 | 101/96.2 | 117/69.6 | P< 0,05 |

**Table 12** Reason of azoospermia

| *Obstrutive azoospermia (n=71)* | | *Non-obstruktiveazoospermia (n=75)* | |
|---|---|---|---|
| Obstruction of ductus deferens | 20 | Partial maturation arrest | 31 |
| Congenital absence of vas deferens | 13 | SCOS | 26 |
| Aspermia | 12 | Hypospermatogenesis | 18 |
| Unsuccessful recanalisation | 7 | | |
| Unsuccessful VEA | 6 | | |
| Retrograde ejaculation | 5 | | |
| Agenesia of epididymis | 5 | | |
| Unsuccessful allopl. spermatokele impl. | 3 | | |

**Table 13** Subgroups according to the sperm count in obstructive and non-obstructive azoospermia (see **Table 3**)

| *Groups* | *Obstructive* | *Non-obstructive* | *P-value* |
|---|---|---|---|
| 0 (n/%) | 4/3,8 | 51/30,3 | P<0,05 |
| 1 (n/%) | 13/12,4 | 47/28 | P<0,05 |
| 2 (n/%) | 19/18,1 | 32/19 | N.S. |
| 3 (n/%) | 69/65,7 | 38/22,6 | P<0,05 |

**Table 14** Impact of obstructive and non-obstructive azoospermia on IVF-ET treatment

| *Parameter* | *Obstructive azoospermia* | *Non-obstructive azoospermia* | *P-value* |
|---|---|---|---|
| Biopsies (n) | 105 | 168 | - |
| Successful cases (n/%) | 101/96,2 | 117/69,6 | P<0,05 |
| Mean paternal age (ys) | 33,5 | 37,9 | N.S. |
| Mean maternal age (ys) | 29,9 | 30,0 | N.S. |
| Injected MII. oocytes | 587 | 706 | - |
| Fertilization rate (%) | 61,1 | 51,7 | N.S. |
| Cleavage block (%) | 11,2 | 16,6 | N.S. |
| Good quality embryos (%) | 57,8 | 51,4 | N.S. |
| Clinical pregnancy rate/ET (%) | 29 | 26,7 | N.S. |

**Table 15** Outcome of IVF-ET cycles

| | |
|---|---|
| Clinical pregnancies | 55 |
| Singleton | 31 |
| Twin | 14 |
| Triplet | 3 |
| Quadruplet | 2 |
| Missed ab. | 5 |
| Extrauterine gravidity | 1 |
| Delivery | 49 |
| Children born | 65 |

**Table 16** Correlation between the sperm count, the number of transfered embryos, the age of the females, the application of assisted hatching and the outcome

| **Parameters** | **Clinical pregnancy rate (%)** |
|---|---|
| **Groups according to sperm count** | |
| Class I (n=52) | 23.1 |
| Class II (n=46) | 28.3 |
| Class III (n=100) | 29.0 |
| **Number of transfered embryos** | |
| 1 (n=20) | 10.0 [a] |
| 2 (n=42) | 14.3 [a] |
| 3 (n=79) | 33.0 (one triplet) [a] |
| 3< (n=57) | 36.8 (two triplets and one quadruplets) [a] |
| **Number of transfered good embryos** | |
| 0 (n=135) | 19.2 (two triplets and one quadruplet) [b] |
| 1 (n=30) | 40.0 (one triplet) [b] |
| 2 (n=25) | 40.0 [b] |
| 3 (n=8) | 75.0 [b] |
| **Female age** | |
| ≤ 35 years (n=178) | 29.2 [c] |
| > 35 years (n=20) | 15.0 [c] |
| **Application of assisted hatching** | |
| No (n=121) | 22.3 [d] |
| Yes (n=77) | 36.3 [d] |

[a,b,c,d] Values with the same superscript were significantly different ($p < 0.0001$, $p < 0.0001$, $p < 0.0500$ and $p = 0.0041$ respectively)

**ICSI with cryopreserved testicular spermatozoa in male factor infertility (cryoTESE+ICSI)**

A total of 509 oocytes (mean: 6,8 ± 4,3 SD) were retrieved in 75 IVF-ET cycles in wich oocytes were fertilized with frozen-thawed testicular spermatozoa. Mean age of the women was 30,8 (± 3,8 SD) years and of the men was 35,4 (± 6,1 SD) years, mean number of cycles 2,4 (± 1,3 SD) and basal FSH and LH levels 7,6 (± 3,2 SD) mIU/l and 6,2 (± 2,3 SD) mIU/l respectively. In 32 cases motile, in 34 cases motile after induction with pentoxifyllin treatment and in 9 cases inmotile (selected with HOST) spermatozoa were used for ICSI. Fertilization rate regarding to MII. oocytes was 64,5 (± 24,6 SD) % and proportion of good quality embryos was 54,7 (± 29,1 SD) %. A total of 163 embryos (mean: 2,6 ± 0,57 SD) were transferred in 64 ET and 47 supernumerous embryos were cryopreserved in 10 cycles. 11 singletons and 3 twins were diagnosed by ultrasonography. Clinical pregnancy rate related to ET was 21,9 %. The influence of different parameters on the IVF treatment are shown in **Table 17** and **Table 18**.

**Table 17** Outcome of cryoTESE+ICSI cycles according to motility

| *Motility* | *n* | *Fertilization rate (%)* | *Good quality embryos (%)* | *Clinical pregnancy rate (%)* |
|---|---|---|---|---|
| 1 | 32 | 65,3 | 53,20 | 25,50 |
| 2 | 34 | 66,9 | 61,80 | 17,50 |
| 3 | 9 | 52,3 N.S. | 33,33 N.S. | 0,00 N.S. |

1 Motile spermatozoa after thawing
2 Motile spermatozoa after pentoxifyllin treatment
3 Inmotile spermatozoa selected with hypoosmotic swelling test (HOST)

**Table 18** Clinical pregnancy rate (CPR) related to different factors

| *Parameter* | *CPR/ET (%)* |
|---|---|
| Day of ET | N.S. |
| Maternal age | N.S. |
| Paternal age | N.S. |
| Application of laser assisted hatching | N.S. |
| Number of transferred embryos | N.S. |
| Number of transferred good quality embryos | P=0,0063 |

**Injection of round and elongated spermatids (ROSI, ELSI)**

Oocytes were fertilized with round spermatids (26 cycles) and elongated spermatids (3 cycles) in the course of 29 IVF-ET cycles of 26 couples because matured sperms could not be found in the processed samples. In 14 cycles some of the oocytes were fertilized with donor sperm s it was requested by the couples. Sperm precursor cells were retrieved from fresh semen in 3 cases, from fresh testicular biopsies in 21 cases and from frozen testicular tissue in 5 cases. Mean age of the women was 31,2 (± 4,2 SD) years and of the men was 37,1 (± 7,2 SD) years, mean number of cycles 2,1 (± 1,2 SD) and basal FSH and LH levels 7,3 (± 2,9 SD) mIU/l and 6,3 (± 2,1 SD) mIU/l respectively. A total of 294 oocytes were aspirated in 29 IVF-ET cycles. 124 MII. oocytes were injected with round spermatids, 12 MII. oocytes with elongated spermatids and 76 MII. oocytes were fertilized with donor spermatozoa. Fertilization rate regarding to MII. oocytes was 37,0 (± 19,6 SD) % and proportion of good quality embryos was 36,0 (± 21,1 SD) %. Results are summerized in **Table 19**. 61 embryos (mean: 2,1) were transferred in 29 ET. Clinical pregnancy rate related to ET was 10,3 %. IVF treatments have resulted 4 singletons and 1 twin. It should be noted that in 3 out of the 5 successful cases, embryos fertilized with donor semen were transferred along with the embryos derived from round spermatid injection.

**Table 19** Outcome of ROSI and ELSI cycles

| | *Round spermatid (n=26)* | *Elongated spermatid (n=3)* | *Donor sperm (n=14)* |
|---|---|---|---|
| MII. oocyte | 124 | 12 | 76 |
| 2PN fertilization(n/%) | 29/23,38 | 4/33,33 | 54/71,05 |
| 1PN fertilization (n/%) | 18/14,51 | 2/16,66 | 3/3,94 |
| Trploid fertilization (n/%) | 1/0,8 | 0/0 | 2/2,63 |
| Cleavage/2PN fertilization (n/%) | 25/86,2 | 4/100 | 50/92,59 |
| Good quality embryos (n/%) | 9/36 | 0/0 | 28/56 |
| Clinical pregnancy (n/%) | 3(5)/10,34* *1 missed ab.* | 0/0 | 2/14,28* |

** Mixed ET (embryos derived from homologues and heterologues fertilization)*

**ICSI with ejaculated spermatozoa selected by hyaluronan binding (PICSI)**

Data of 88 IVF-ET cycles have been analysed where fertiliazition of oocytes was performed with ICSI of fresh ejaculated spermatozoa selected by hyaluronan binding. The indication of IVF treatment was mixed (male and female) factor infertility. Mean age of the women was 35,6 (± 3,9 SD) years, mean number of cycles 3,7 (± 2,1 SD) and basal FSH and LH levels 7,9 mIU/l (± 3,3 SD) and 6,3 (± 3,2 SD) mIU/l respectively. A total of 781 oocytes (mean: 8,9 ± 4,0 SD) were collected and 582 MII. oocytes were injected. Fertilization rate related to MII. oocytes was 88,0 (± 29,9 SD) % and the proportion of good quality embryos was 79,8 (± 28,2 SD) %. In the course of 83 embryo transfer 216 embryos (mean 2,6 ± 0,9 SD) were transferred and 67 supernumerous embryos derived from 18 cycles were cryopreserved. IVF treatments have resulted 22 singletons, 2 twins and 3 triplets. Clinical pregnancy rate related to ET was 32,5 %. The results are shown in **Table 20, Table 21** and **Table 22.**

**Table 20** Effect of the examined parameters on the pregnancy rate

| *Paremeter* | *Clinical pregnancy rate* |
|---|---|
| Sperm concentration | N.S. |
| Motility | N.S. |
| Normal morphology | N.S. |
| Maternal age (mean: 35,6 years) | N.S. |
| Paternal age (mean: 39,2 év) | N.S. |
| Basal FSH level | N.S. |
| Stimulation protocol | N.S. |
| Number of retrieved oocytes | N.S. |
| Number of MII. oocytes | P=0,03714 |
| Number of normally fertilized oocytes | N.S. |
| Number of good quality embryos | N.S. |
| Number of transferred embryos | N.S. |
| Number of transferred good quality embryos | N.S. |
| Day of ET (2.,3.,4.,5.) | N.S. |
| Endometrium thickness | N.S. |
| Application of laser assited hatching | N.S. |

**Table 21** Outcome of PICSI cycles

| | |
|---|---|
| PICSI cycles (n) | 88 |
| Total number of oocytes retrieved | 781 |
| Injected MII: oocytes | 512 |
| Normal fertilization (%) | 88,0 |
| Number of cleaved embryos | 491 |
| Good quality embryos (%) | 79,8 |
| Clinical pregnancy | 27 |
| Clinical pregnancy rate/ET (%) | 32,5 |

**Table 22** Effect of sperm parameters on fertilization, embryonic development and clinical pregnancy rate

| *Parameter* | *n* | *Fertilization rate/MII. oocytes (%)* | *Cleavage rate (%)* | *Good quality embryos (%)* | *Clinical pregnancy rate/ET (%)* |
|---|---|---|---|---|---|
| *Sperm concentration* | | | | | |
| 1-4 x$10^6$/ml | 3 | 100 | 100 | 95,2 | 33,3 |
| 5-9 x$10^6$/ml | 6 | 79,5 | 100 | 92,4 | 16,7 |
| 10-14 x$10^6$/ml | 12 | 88,9 | 96,3 | 78,3 | 25,0 |
| 15-19 x$10^6$/ml | 6 | 100 | 66,7 | 49,3 | 25,0 |
| 20 x$10^6$/ml < | 61 | 86,5 | 96,1 | 81,0 | 36,2 |
| *Motility (%)* | | | | | |
| 1-9 | 1 | 25,0 | 100 | 100 | 0 |
| 10-19 | 3 | 94,1 | 97,6 | 74,0 | 0 |
| 20-29 | 7 | 100 | 100 | 98,0 | 14,3 |
| 30-39 | 10 | 90,3 | 88,0 | 71,2 | 11,1 |
| 40-49 | 11 | 100 | 87,3 | 67,0 | 44,4 |
| 50 % < | 56 | 82,4 | 96,2 | 81,5 | 38,9 |
| *Normal morphology* | | | | | |
| 30 % > | 83 | 89,2 | 94,4 | 78,5 | 34,6 |
| 30 % ≤ | 5 | 68,3 | 96,0 | 100 | 0 |

**Discussion**

Data accumulated between 2000-2008 has provided solid funds to determine the efficiency of intracytoplasmic sperm injection and related techniques in the treatment of male factor infertility. Main parameters (fertilization rate, proportion of good quality embryos, clinical pregnancy rate) in the examined groups are summerized in **Table 23**.

**Table 23** Main parameters in the examined groups

| *Group* | *n* | *Ferilization rate/MII. oocytes (%)* | *Good quality embryos (%)* | *Clinical pregnancy rate/ET (%)* |
|---|---|---|---|---|
| 1. ICSI (mixed factor infertility, ejaculated spermatozoa) | 128 | 88,2 | 69,5 | 31,2 |
| 2. ICSI (male factor infertility, ejaculated spermatozoa) | 250 | 87,2 | 68,1 | 41,4 |
| 3. ICSI (frozen-thawed, ejaculated spermatozoa) | 86 | 77,8 | 67,5 | 19,5 |
| 4.a TESE+ICSI (obstructive azoospermia) | 101 | 61,1 | 57,8 | 29,0 |
| 4.b TESE+ICSI (non-obstructive azoospermia) | 117 | 51,7 | 51,4 | 26,7 |
| 5. cryoTESE+ICSI | 75 | 64,5 | 54,7 | 21,9 |
| 6. ROSI | 29 | 37,0 | 36,0 | 10,3 |
| 7. PICSI | 88 | 88,0 | 79,8 | 32,5 |
| | | P< 0,05 | P< 0,05 | P< 0,05 |

In accordance with our expectation and international experiences higher fertilization rate could be achieved with ejaculated spermatozoa than with testicular spermatozoa or round spermatids. The use of fresh samples proved to be superior in achieving fertilization compared to frozen-thawed samples except when cryopreserved testicular spermatozoa (64,5 %) were used instead of fresh (61,1 %). Higher proportion of good quality embryos was found if ejaculated spermatozoa were used. Fresh and frozen-thawed samples provided similar results. This parameter was the highest in the PICSI group. Clinical pregnancy rate was higher if ejaculated fresh spermatozoa were injected. In the next paragraphs the groups summarized in the **Table 23.** are discussed individually.

1. In the first group (ICSI, mixed factor infertility) the effect of advanced maternal age on the pregnancy rate could be demonstrated. It seems that paternal age does not have similar impact in ICSI cycles. However effect of advanced paternal age was clearly demonstrated in a recent study when data of 17000 intrauterine insemination (IUI) were analysed (11). Authors agreed that paternal effects come from the structural change of sperm DNA and the higher frequency of necrospermia. The effect of maternal factors (age, basal FSH/LH etc.) on the clinical pregnancy rate (CPR) was truly conspicuous comparing the first two groups (mixed and male factor infertility). More than 10 % higher CPR could be achieved if only male factor was the indication of the IVF treatment (41,4 % vs. 31,2 %), although the fertilization rate (87,2 % vs. 88,2 %) and the proportion of good quality embryos (68,1 % vs. 69,5 %) were similar. In the latter group (male factor infertility) the maximum age of women was 37 years with normal basal FSH/LH levels and at least five MII. oocytes at the time of fertilization. Even so the influence of maternal age on the pregnancy rate in the second group could be detected too (**Table 8**). A number of studies have clearly demonstrated that advancing female age is a negative prognostic factor (75, 88). Advanced maternal age has negatively influenced the number and quality of oocytes retrieved (**Table 7**). When ICSI is applied a few viable spermatozoa are enough to fertilize the oocytes. After all the negative effect of extremely low sperm concentration on fertilization rate (1-100 sperms in the whole sample) on fertilization rate could be demonstrated (**Table 6**).

2. The effect of paternal factors on the fertilization, early embryonic development and pregnancy rate without the effect of maternal factors, was monitored in the second group (ICSI, male factor infertility) and is demonstrated in **Table 9**. Fertilization rate and proportion of good quality embryos was significantly lower in the case of extremely low sperm count (1-100 spermatozoa/sample) in comparision with normal sperm concentration ($20x10^6$ spermatozoa/ml). Conversely similar effect could not be observed with motility and morphology. Embryonic genome is inactive until the 6-8 cell stage (98), thus early cleavage depends on the cytoplasmic and nuclear maturity of oocyte if the fertilization was successful. Contribution of spermatozoon to this period is limited to the oocyte activating factor (OAF) and centrosome. Scientific researches of recent years revealed that sperm's mRNA can play an important role during the early cleavage satge (115). Impairment of the DNA of sperm is not correlated with the morphologic disorders of the zygote and early cleavage stage (early paternal effect). However early paternal effect was shown in a study when oocytes of the same egg donor were fertilized with sperms from different males (102). Paternal effect could

be even more demonstrated in blastocyst development (71). Objective evaluation of the male factors is more important these days since increasing incidence of subfertility has been reported in young men too (3, 5, 8, 86).

3. Fertilization rate was 10 % lower in the third group (ICSI, frozen-thawed ejaculated spermatozoa) compared to the first and second groups. It could be explained with the quality of the frozen semen samples. In many cycles the quality of the semen was poor before freezing. For instance freezing was sometimes performed after the initiation of the patient's chemotherapy (7). Recent freezing techniques can guarantee 70 % survival rate of frozen-thawed spermatozoa in optimal cases (73). Our experience certify that the efficiency of cryopreservation is lower in case of poor quality samples (3, 4, 5, 7). Pregnancy rate (19,5 %) in this group is far from the first group's (ICSI, fresh ejaculated spermatozoa, mixed factor infertility) outcome (32,1 %). The most probable explanation for this difference is the poor quality of frozen samples since maternal age was lower (mean: 32,5 years vs. 64,8 years) and the number of oocytes retrieved was higher (mean: 7,9 vs. 6,6) in this group. The quality of spermatozoa did not show effect in the early stage of embryonic development as the proportion of good quality embryos was similar in the third and first groups (67,8% vs. 69,5 %). It should be noticed that in the case of frozen ejaculated spermatozoa high proportion of the frozen samples were transported from other institutes. We have frequently found that inadequate freezing protocol was used and sometimes the sign of previous melting of the samples was observed. Offering an adequate cryopreservation program for reproductive-age men and women with the diagnosis of cancer should be mandatory in our days (3, 45). It was impossible to investigate the impact of sperm parameters on fertilization, embryonic development and clinical pregnancy rate since sperm count and motility could not be exactly measured in many samples (only a few motile spermatozoa in the whole sample). On the other hand important data regarding the sperm parameters before freezing of these samples were often missing from the data sheet of the samples wich were transported from other institutes.

4.a-b. In obstructive azoospermia a mechanical pathology is present. In this case spermatogenesis is more or less normal, spermatozoa are usually motile (40), and the recovery rate approaches 100 %. Patients suffering from non-obstructive azoospermia have pathological spermatogenesis. Three different pathologies can be described: Sertoli cell-only syndrome, maturation arrest and hypospermatogenesis (104). Most of the Sertoli cell-only

syndrome patients have small testes and elevated FSH levels. In total Sertoli cell-only syndrome there is a complete absence of germ cells. Even repeated testicular biopsies may lead to false diagnosis, since total or partial germ cell aplasia is questionable, as it has been clearly demonstrated (105). Maturation arrest is characterized by an arrested development of spermatogenesis. These patients generally have normal testicular size and FSH levels. In my study testicular spermatozoa could be retrieved almost in every cases of obstructive azoospermia (96.2 %), but only 69.6 % of the non-obstructive cases were succesful. Since a small testicular tissue represents only 1/1000 of the total testicular volume, the question pops up wether a prior biopsy has any predictive value. Silber (93) indicated that there is indeed a minimum quantitative threshold of spermatogenesis which must be exceeded for any spermatozoa to reach the ejaculate. When the mean number of matured spermatids/seminiferous tubule is less than 4-6, the quantity of spermatozoa is insufficient to reach the ejaculate after transit from the testicle. The cause of the non-obstructive azoospermia had no effect on this threshold concept. The normal range of spermatogenesis in fertile men or in obstructive azoospermia appears to be 17-35 spermatids/seminiferous tubule (91). As it was expected we found extreme low sperm count more frequently in the case of non-obstructive azoospermia (**Table 13**). However our results (**Table 16**) demonstrate that low sperm count does not effect significantly the pregnancy rate. On the other hand there is a correlation between the sperm count and the post-thaw motility of frozen testicular spermatozoa (55). Fertilization rate in the TESE+ICSI group was lower than at ICSI with ejaculated or epididymal spermatozoa as it was previously demonstrated by other authors (59, 109). 121 oocytes (9.3 %) had one pronucleus which was higher proportion than in the case of ICSI with ejaculated spermatozoa (4.0-5.0 %). Some oocytes developed three pronuclei. Fertilization failure after ICSI is caused by disruption of the normal sequence of events that lead to oocyte activation, fertilization and syngamy or ejection of the sperm from the oocyte. Previous studies suggested that in one-pronuclear oocytes the oocyte was activated, ejected a second polar body and formed a female pronucleus (30, 31). Maturation of mammalian spermatozoa depends on their interaction with epididymal proteins (1). To achieve higher fertilization rate and reduce fertilization failure, in vitro maturation of testicular spermatozoa can be an alternative way (106). Distribution of good quality embryos (Grade3 and Grade4) was similar to our conventional ICSI program, and the type of the azoospermia had no effect on the quality of the embryos (**Table 14**).

5. Freezing of testicular tissue allows asynchronous testicular biopsy and follicular aspiration. It is also crucial to limit the testicular injury, since testicular sperm extraction can also produce devascularization and inflammation of the testis (63). Good quality samples can be frozen in parts and used in subsequent cycles, but compared to ejaculated spermatozoa, we rarely found motile spermatozoa in the samples immediately after thawing. Epididymal proteins play an important role in the maturation of spermatozoa (1). During the maturation process, substantial alteration occurs in the membrane constitution of the spermatozoon, that affects its cryobiological properties. In 32 cases, motile spermatozoa were found after two hours of incubation. In the other 34 cases, motility could be induced by pentoxifyllin. On the whole, motile spermatozoa were available for ICSI in 88 percent of the samples. Pentoxifyllin inhibits phospho-diesterase, and slows down the degradation of cAMP (112). This will result in significantly more hyperactivated spermatozoa after 1-2 h of incubation (70). In 9 cases, only immotile spermatozoa were found even after treatment with pentoxifyllin. In these cases spermatozoa with functionally intact membrane were selected by hypoosmotic swelling test (HOST). Surprisingly, there was no significant difference between the fertilization rates obtained with immotile (52.3 %) or motile (66.1 %) spermatozoa. However, there were fewer good quality embryos (not significant) when immotile spermatozoa were used for fertilization (**Table 17**). In my study pregnancy was only achieved following fertilization with motile spermatozoa, although pregnancies and deliveries with immotile spermatozoa had been reported previously (17). Our data demonstrates a reduced fertility potential of immotile spermatozoa. The lack of statistical significance could be explained by the limited number of cycles with immotile spermatozoa. The current findings are similar to our previous experience with immotile, hypoosmotically reactive spermatozoa (54).

6. Arrested spermatogenesis can be complete or incomplete. It should be determined at wich level the development is arrested. It is widely accepted that maturation arrest occurs in meiosis (57), wich indicate that the use of round spermatids should be limited to the rare occasion where only round spermatids are found (100). Our experiences has proved that the efficiency of round spermatid injection (ROSI) is substantially lower compared to matured sperms (66), thus we should make an effort to find the more matured formats for fertilization. Sampling of different areas of the testicles can provide mature sperms or at least elongated spermatids even in the case of previously failed biopsy. Fishel (28, 29) has found higher fertilization rate with elongated spermatids from testicular tissue, although it was the opposite with semen. In the present study elongated spermatid injection (ELSI) was performed only in

three cases, wich means that in the presence of elongated spermatids usually associated with matured spermatozoa in the same sample. Higher frequency of 1PN status 16-18 hours after injection and cleavage blockade also refer to the suboptimal activation. On the other hand individual variation of spermatogenetic failure of different patients also explain these problems since I have found adequate fertilization and cleavage rate in some cycles. Yanagida (114) has observed altered $Ca^{2+}$-oscillation more often with spermatids and suggested artificial activation. Technical problems of round spermatid injection sholud be also noted. It is considerably difficult to distunguish round spermatids from other round cells using standard optical system. Presence of acrosome in the form of luminous spot or cytoplasmic mound would help in recognition. However similar selection markers does not occur at the Golgi phase. There are other possibilioties to improve the efficiency of ROSI beside better selection (101). In vitro maturation is another way to achieve better fertilization (102) and the improvement of artificial activation of oocytes is also promising (50). Three components of spermatozoa are essential for successful fertilization: the centrosome, the oocyte activating factor (oscillin) and the genetic material. At the end of the second meiotic division the newly evolved spermatid has two centriolums. The flagellum develops from the distal centriolum and the centrosome from the proximal centriolum (94). Based on the successful fertilization with round spermtids it can be stated that round spermatids have all the three factors (28). The lower fertiolization rate following round spermatid injection can be explained with the suboptimal function of the oocyte activating factor. On the other hand DNA structure of of round spermatid differs from the matured sperm's. 90 % of histamines are replaced with protamines during the maturation, then an adverse process occures after fertilization (78). Modification of the genetic material takes place before the second meiotic division resulted in the altered expression of the modified genes later (2). The birth of healthy offsprings confirms that the chromosomes of round spermatid can mate with the chromosomes of the oocyte and can function normally during the development of the fetus. It is questionable that chroomosome aberration can happen more often to round spermatids. Oppedisano (76) has found significantly more aneuploid spermatids in infertile mice. However it was the same in matured sperms of oligozoo- and azoosperm men (34). Looking at different studies it seems that the chromosome disorders are originated rather from the reason of infertility than the round spermatid stage of the gametes. Conversely microdeletions of the long arm of the Y chromosome are thought to be inherited in male offsprings (24).

7. In the present study indication for Petri Dish Intracytoplasmic Sperm Injection (PICSI) was at least one failed IVF-ET cycle when low fertilization rate and/or poor embryo quality was observed. 77,4 % of fertilization rate and 32,5 % of clinical pregnancy rate could be achieved in this group with mean number of cycles of 3,7. The proportion of good quality embryos (79,8 %) was superior in the PICSI group, 10 % higher than in the first group (ICSI, mixed factor infertility: 69,5 %). As a consequence of the better embryo quality it was possible to freeze supernumerous embryos in 21,7 % of the PICSI cycles in contrast to the first group (ICSI, mixed factor infertility: 12,8 %). Cryopreservation of these embryos is a useful tool to increase the cumulative pregnancy rate of IVF cycles (53). Sperm parameters have not effected fertilization and early embryonic development in this group (**Table 22**). Application of PICSI in its present form is limited by low sperm concentration and/or poor motility (69). In the case of few progressive motile spermatozoa the sediment should be dropped directly to the hyaluronic acid layer in a small droplet. However this method has no benefit if spermatozoa does not have appropriate motility. The method of sperm selection is a subject of intensive research. Traditionally the selection is based on the morphologic and motility properties of the spermatozoon. Normal morhology does not mean normal genetic material (19). Numerical chromosome disorders exist in morphologically normal spermatozoon too, however the frequency is lower than in amorf gametes. PVP is used in most IVF laboratory to make sperm trapping easier. Conversely, natural motility of spermatozoa could not be evaluated prior injection as PVP is a highly viscous solution. In turn, hyaluronic acid binding capacity of spermatozoa is related to a number of important biochemical markers (CK activity, HspA2 level, AR status etc.). HspA2 is chaperone protein wich is produced in the testes. The first wave of its productuion is occured during meiosis since HspA2 is the part of the synaptonemal complex. The second wave takes place in the late phase of spermiogenesis synchnronized with the alteration of sperm mebrane and appearance of hyaluronic acid (HA) receptors. Thus HspA2 level is an important indicator of genetic and functional maturity of spermatozoa. It has been certified that the frequency of diploidy and disomy is 4-6-fold less in HA binding spermatozoa (37, 39). Intracytoplasmic morphologically selected sperm injection (IMSI) is a new method based on motile sperm organellar examination (MSOME) performed with an inverted light microscope equipped with high power Nomarski optics enhanced by digital imaging to achieve a magnification of 6600x. In a number of studies it was demonstrated that fine morphological integrity of human sperm nuclei was positively associated with fertilization and pregnancy rate (10, 12). Although IMSI makes possible to decrease the probability of injection of a DNA-damaged

spermatozoon even in the case of extremely low sperm count, it does not seem to be a routinely applicable method. It needs expensive optical equipment and highly trained manpower. On the other hand it is time consuming technique (2,5-3 hours per cycle) and the procedure has additional cost. Furthermore prospective randomized studies are not yet available.

Clinical pregnancy rate has been effected almost in every groups by the number and quality of the transfered embryos (**Tables 5, 8, 10, 16, 18**). The incidence of multiple pregnancies has increased when three or four embryos were transfered. It should be noted that most of these patients had at least three previous failed attempts before multiple pregnency occured. Data of this study and our previous paper (44) also suggest that the transfer of two good quality embryos is enough to achieve high clinical pregnancy rate in most cases without the risk of multiple gestation in younger patients. In turn, transferring more than two embryos is beneficial in older women (72). Data in **Table 7** clearly demonstrates that advanced maternal age has negative effect on the whole process of oocyte maturation, fertilization and embryonic development.

In the examined groups no significant association between stimulation protocol/endometrial thickness and pregnancy outcome was shown. In our previous studies significantly higher pregnancy rates were found when GNRH agonist ultrashort protocol was used in contrast to CC + gonadotrophin (42, 43). Moreover improved pregnancy rates were found when the endometrium thickness reached at least 10 mm (42). In the present study the lack of statistically significant difference can be axplained by the relatively low number of cycles in certain groups.

LAH (laser assisted hatching) was applied when at least one of the following indications existed: at least two previous failed IVF-ET cycles, elevated FSH level or maternal age (≥ 35 years). Benefits of assisted hatching in certain groups of patients have been widely reported (14, 20, 21, 52). Our initial results in 1995 encouraged us to apply assisted hatching for all patients, but we found no benefits to good prognosis patients. We used to open the zona by partial zona dissection (51) at the beginning. We have been using a non-contact laser system (ZLTS-II., Hamilton-Thorne Research; USA) since 2001. It is is equipped with a non-contact, infrared laser (1480 nm) supplemented with a useful measurment software. It has been designed with power settings matched to the safety factor of the human cells (33). Using the ZLTS-II., standardization of hole size is easy to achieve. The

inefficiency of human IVF is largely due to implantation failure. It was supposed that assisted hatching (AH) would improve implantation. The landmark study regarding assisted hatching in the human was reported by Cohen and his fellow workers (20). Opening the zona pellucida may overcome the mechanical barrier to embryonic hatching presented by an abnormally hard and thick zona. In addition it may decrease the energy required by an embryo to complete the hatching process. The ATP content in embryos is a predictor of embryo viability (107). Liu (49) have shown that embryos subjected to assisted hatching implant on the average one day earlier than embryos wich are not micromanipulated. This earlier implantation may improve the synchrony between the endometrium and the embryo necessary for implantation success. In contrast, in this study application of assisted hatching has improved the clinical pregnancy rate only in the TESE+ICSI group (**Table 16**). Furthermore the timing of ET has effected the pregnancy outcome only in the first group (**Table 5**). It can be explained with the low number of fifth day transfer in the other examined groups. Blastocyst transfer has certain advantages, although it is not applicable for all patients (58).

ICSI is a delicate procedure requiring considerable skills of the person performing it. Theoretically, the injection procedure could damage cytoplasmic structures in the oocyte, resulting in sublethal cellular injury and/or numerical chromosomal abnormalities that could lead to impaired embryonic development. Positioning of the oocyte for injection of spermatozoon seems to be an important factor. The oocyte can be held during the injection with the polar body directed to 12 o'clock or 6 o'clock position. The later positioning has been found to produce better quality embryos than other placings (74). The clinical success of ICSI itself could thus depend on the rotation of ooplasm by a correctly-positioned sperm centrosome. In another study, development to the blastocyst stage was found to be compromised in a group of surplus embryos originating from oocytes in which >6 pl of cytoplasm was aspirated into the injection pipette during the ICSI procedure. However neither the volume of cytoplasm aspirated during the injection procedure, nor the position of the polar body (6 o'clock or 12 o'clock) influenced the mean incidence of disomic cells per blastocyst as revealed by fluorescence in-situ hybridization using probes specific for chromosomes X, Y and 18 (26). Application of ICSI has been crabbed since certain steps of normal fertilization are lacking. The fact that ICSI still works has shed new light upon fertilization. Contrary to the previous conception, Dozortsev (25) has certified that a heat sensible sperm citosolic protein (oscillin) is responsible for oocyte activation. Competence to undergo nuclear and cytoplasmic maturation are aquired independently during oocyte maturation (18). Some of the

oocytes from follicles of varying size may be cytoplasmically immature despite having reached nuclear maturity (MII). Not all the injected oocytes exhibit multiple $Ca^{2+}$ oscillations and the periodicity of $Ca^{2+}$ transients varies between oocytes (96). Amplitude and frequency of the rises are important for oocyte activation (110). This suggests that not all oocytes respond to sperm penetration or injection in the same manner. Defficiency or absence of the activator factor from some spermatozoa can also prevent normal fertilization (25, 99). Micromanupulation of oocytes has its own risk. Oocytes can be injured mechanically during denudation or injection, moreover meiotic spindle is also vulnerable (27). There are many publications regarding to the genetic risk of ICSI (32). MII. oocytes have their diploid complement of chromosomes delicately arranged on the metaphase plate near the polar body. Mechanical disruption of the metaphase plate can occur by injury from the injection pipette or by the presence of a motile sperm in the oocyte cytoplasm. A newly developed microscopic method (Spindleview system) has made meiotic spindle visible, so the position of the injection could be choosen more precisely (111). Beyond these technical issues ICSI candidates have to face other genetic risks. Since it is known that approximately 3 % of the karyotypes are abnormal, in the case of severe oligozoospermia (sperm concentration$< 5x10^6$ /ml) or non-obstructive azoospermia consideration of karyotypic abnormalities is appropriate (46, 68). The cause of obstruction should be also diagnosed, because cystic fibrosis can be related to infertility caused by bilateral congenital absence of the vas deferens. Detection of microdeletions of the long arm of the Y-chromosome is advisible since it can be inherited in the male offsprings (116).

Cryopreservation can generate ultrastructural damage of plasma membrane, acrosome, mid-piece or tail. Swelling of plasma membrane or acrosome, lack of acrosome or recessed mitochondrial sheath indicates the impairment (9). Since mitochondria provide energy of spermatozoa, lesions of these cell organelles induce declined viability after thawing. In a previous study when mitochondrial activity of fresh and frozen-thawed sperms were measured by MTT test, we found that other factors like ultrastructural damage of the cells are more reponsible for the loss of motility after freezing and thawing (62). Spermatozoa of severe oligozoospermic and azoospermic patients were found to be more sensitive to the detrimental effects of freezing and thawing (63). During the last decades application of computer controlled freezers was the main progression in the field of semen cryopreservation, however it has not solved the problem of the low survival rate of the extra sensible samples. Glicerol remained the primary cryoprotective agent (79). This relative idleness compared to the other

fields of assisted reproduction techniques derives from the acceptable survival rate of spermatozoa with traditional freezing techniques. In the case of serious oligozoospermia or azoospermia traditional freezing techniques often do not preserve enough motile spermatozoa for ICSI. Recent freezing techniques are inappropriate to avoid excessive osmotic and mechanical stress of frozen cells (4, 6). Development of an adequate vitrification protocol might solve this problem in the future (38, 60). Treatment of infections of the genital tract or eliminating of reactive free radicals from the semen could also improve the efficiency of cryopreservation (64, 65). There is another promising new technique wich has been developed in Hungary by Pribenszky and his collegues. They have found that a sublethal stress treatment - using high hydrostatic pressure - before freezing would increase the survival rate of different types of cells (35, 80, 81, 82).

Finally, the applied culture techniques in the IVF laboratory has a dramatic influence on the overall efficiency of the IVF cycles too. Most of the IVF centers use high quality ready to use culture media, even so quality control is crucial (67). Aside from the culture system technical backround (e.g. incubators, micromanipulators, heating stages, CODA tower etc.) is also relevant. One of the most important factor is the selection of the best embryos, since clinical pregnancy rate has been effected almost in every groups by the number and quality of the transfered embryos. Embryo scoring is based on their clevage rate and morphology. Omics technologies, including transcriptomics, proteomics, metabolomics (41) and Time Lapse video techniques (83) are promising alternatives of existing scoring systems.

**Summary**

Data accumulated between 2000-2008 has provided solid evidence to determine the efficiency of intracytoplasmic sperm injection and related techniques. Based on the data of 874 IVF-ET cycles I have concluded that ICSI and related micromanipulation techniques (ROSI, ELSI) can be effectively used in the treatment of male factor infertility. The next paragraphs summerize the findings in a thematic manner.

***Concerning the etiology***

- *Does the type of male factor infertility have an effect on the efficiency of intracytoplasmic sperm injection and related micromanipulation techniques?*

**Micromanipulation was less effective in the treatment of azoospermic men compared to other type of male factor infertility (oligozoo-, asthenozoo-, teratozoospermia).** Higher fertilization rate  higher proportion of good quality embryos  and higher clinical pregnancy rate was obsereved in oligozoo-, asthenozoo-, teratozoospermia compared to obstructive and non-obstructive azoospermia.

- *What is the effect of the female factors in case of mixed (male and female) origin infertility?*

**Female factors, especially the age of women (p=0,02) have significant effects on the clinical pregnancy rate, wich have manifested in the number and quality of oocytes.** ICSI has resulted 10 % higher pregnancy rate without female factors.

- *How effective is intracytoplasmic sperm injection (ICSI) in obstructive and non-obstructive azoospermia?*

**Fertilization rate, the proportion of good quality embryos and clinical pregnancy rate  have not differed significantly between obstructive and non-obstructive azoosperma.** However, the lower fertilization rate and the fewer good quality embryos indicate spermatozoa's poorer quality in the case of non-obstructive azoospermia. Moreover only 69,6 % of the testicular sperm retrievals were successful in these cases.

***Concerning the type and the quality of gametes used for fertilization***

- *Do sperm concentration, motility and morphology effect the efficiency of ICSI cycles?*

**Fertilization rate (mixed factor infertility and male factor infertility, ejaculated spermatozoa) and the proportion of good quality embryos (male factor infertility, ejaculated spermatozoa) were significantly lower in the case of extremely low sperm concentration in comparision to normal sperm concentration.** I have not found similar impact of motility and morphology.

- *How effective is intracytoplasmic sperm injection (ICSI) if fresh or cryopreserved spermatozoa are used for fertilization?*

**The use of fresh ejaculated or testicular spermatozoa proved to be superior in achieving pregnancy compared to frozen-thawed spermatozoa.** It should be noted that many of the frozen semen samples derived from other institutes, where inappropriate freezing technique and/or storage condition was applied. Post-thaw motility is crucial to achieve pregnancy if frozen samples are used. Outcome was similar whether motility returned spontaneously or it needed to be induced with pentoxifyllin after thawing.

- *How effective is intracytoplasmic sperm injection (ICSI) if testicular spermatozoa were used for fertilization?*

**I have found significantly lower fertilization rate, proportion of good quality embryos and pregnancy rate if testicular spermatozoa were used for fertilization instead of ejaculated ones.** These results indicate that testicular spermatozoa have less potential to activate oocyte and initiate normal embryonic development.

- *How effective is the applied micromanipulation technique if round (ROSI) or elongated spermatids (ELSI) are used for fertilization?*

**The use of round or elongated spermatids resulted in the worst outcome of the examined groups in every aspect.** The lowest fertilization rate, proportion of good quality embryos and pregnancy rate could be achieved in this group. This technique in its recent form should only be applied in those cases when the couple definitely refuses the use of donor sperm.

***Concerning the type of gamete micromanipulation***

- *How effective is intracytoplasmic sperm injection (ICSI)?*

**Almost every type of male factor infertility can be successfully treated with the application of intracytoplasmic sperm injection or related micromanipulation techniques.** The efficiency of the these techniques particularly depends on maternal and paternal factors.

- *How effective is round spermatid (ROSI) and elongated spermatid (ELSI) injection?*

**These techniques are the least effective ones in the examined groups.** In the case of ROSI, artificial activation should be taken into consideration due to the low fertilization rate.

- *Perspectives of sperm selection based on hyaluronan binding capacity (PICSI)?*

**Highest proportion of good quality embryos was achieved in the PICSI group, however fertilization rate and pregnancy rate were similar compared to conventional ICSI.** It should be noted that PICSI technique was applied in difficult cases after previous fertilization failure and/or poor embryonic development. Unfortunately, PICSI technique could be not applied in cases of low sperm concentration and/or motility.

- *Wich technical aspects should be taken into consideration in order to improve the efficiency of gamete micromanipulation?*

**The following technical aspects should be taken into consideration:**

1. **Method of sperm selection,**
2. **Sperm freezing techniques,**
3. **Artificial oocyte activation in certain cases.**

Efficiency of different techniques applied in IVF have great importance since 2-3 % of the newborns in Hungary are conceived following assisted reproduction. In recent years up to five IVF attempts are covered by the National Insurance Company without the cost of the medicines, which is partially reimbursed too. Moreover these children will be taxpayer citizens in 20-25 years. From this viewpoint assisted reproductive techniques have both social and economic significance. The aim of the study was to evaluate and compare the efficiency of ICSI and related techniques, including a new sperm selection method (PICSI). Based on

these data, present study has demonstrated the potential and the barrier of the existed techniques.

Technical aspects of ICSI remained unchanged until the past few years. PICSI is a new technique to improve the efficacy of sperm selection. As far as I know PICSI technique as well as ICSI, TESE+ICSI, ROSI techniques were first applied in our institute in Hungary. Further research should be focused on the extension of the PICSI technique, since it does not work in low sperm concentration and/or motility. On the other hand a prospective randomized study is also needed to reveal its real potential. Recent freezing techniques turned out to be not adequate for all samples, especially in the case of low sperm concentrion and testicular spermatozoa. Vitrification is widely applied in the freezing of embryos and oocytes with high efficacy. Unfortunately, a completely aseptic vitrification method does not exist yet. Apart from this, research work should be focused on the innovate of a unique method for spermatozoa. In certain cases artificial oocyte activation may help to avoid fertilization failure. Several methods exist, however most of them are used mainly in animal research. It should be investigated which method can be safely adapted for human oocytes in routine IVF cycles. Culture technique and the method of embryo selection are also important factors, since the quality of the transferred embryos has had significant impact on the clinical pregnancy rate in all groups.

In summary, I have concluded that ICSI and related techniques are effective in the treatment of most types of male factor infertility. In order to improve the efficiency of these techniques, research should be focused on the development of new sperm and embryo selection methods, freezing techniques and artificial oocyte activation.

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