Current trends in the laboratory practice of in vitro fertilization treatments

Doctoral theses

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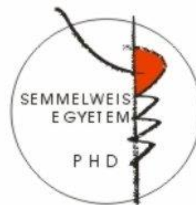

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Introduction

In vitro fertilization (IVF) is the process, during which oocytes are fertilized outside the body, under laboratory conditions, followed by the transfer of the developing embryo into the uterus.

As a result of the collaboration of Robert G. Edwards and Patrick Steptoe, the first IVF baby, Louise Brown was born in 25. July 1978 in the Oldham General Hospital. Since then, over 6.5 million baby have already been born worldwide in the last nearly four decades thanks to this technic. In developed countries, the rate of children fertilized by IVF can reach 5%. The Nobel Prize in Physiology or Medicine 2010 was awarded to Robert G. Edwards "for the development of in vitro fertilization".

Since the first successful treatments, researches are made in every topic of the procedure. In this dissertation, I would like to highlight four important fields in which I researched.

Recently, two different technics are used commonly for the fertilization of the eggs. During conventional in vitro fertilization (IVF), the proper volume of the processed semen sample, that contains mostly progressive motile sperms, is co-incubated with the oocytes. The other method is called intracytoplasmic sperm injection (ICSI) was originally developed to treat severe male factor infertility. For this, cumulus cells surrounding the oocyte are removed, and a single sperm is injected into the cytoplasm using a micromanipulator. This technic gave chance to those patients, who did not have previously because of the low rate of fertilization or failed fertilization with conventional IVF. Even though the higher risk of ICSI, it is chosen as the method of fertilization in two third of the treatments worldwide.

In vitro culture is a key factor in IVF treatment outcome. It is of great importance to provide proper culture conditions, as it effects embryo development and quality. In vitro culture conditions can be manipulated in many ways, for instance with different culture media, different type of incubators or culture method such as individual or group culture. Embryos are placed in separate media droplets. An advantage of this technic is that it makes the identification of the oocytes and developing embryos possible, thus their

development can be followed. Introduction of new culture dishes containing microwells made this identification also feasible. During group culture, paracrine communication between the embryos can be an advantage, as the accumulation of these factors can benefit embryo development. However, embryotoxic and other unfavorable agents can negatively influence embryo quality.

During group culture, it is essential to culture embryos in an adequate media volume, which is big enough to supply the embryos with nutrients and oxygen, and to minimize negative group effect, and at the same time, small enough to keep the benefit of group culture. Embryo density is calculated as the culture volume divided by the number of embryos. In practice, the same density value can be reached in two different ways: number of embryos in a given media volume, or culture volume in case of settled embryo count can also be changed. Proper density can improve embryo development, thus can be applied as an easy-to-use, yet effective tool to optimize culture conditions resulting in better embryo quality.

In many cycle, there are embryos, which are considered viable, but cannot be transferred. Freezing and storing of these supernumerary embryos for a future embryo transfer (cryopreservation) is advantageous. In case of frozen embryo transfer (FET) there is no need to stimulate the ovary again, and higher cumulative pregnancy rate can be achieved, while complicated and expensive laboratory technics are also unnecessary. Recent years, freezing methods have evolved, resulting in more effective processes. During slow freezing, the previously exclusively used method embryos are incubated in cryoprotectant solutions containing permeating and non-permeating agents, followed by computer-controlled slow cooling until the desired temperature. Recent years, conventional technic has replaced in many laboratories by vitrification, an ultra-rapid cooling method. As a result of vitrification, water content of the embryo cells became in a glass-like stage. For this, embryos are exposed to solutions with a high cryoprotectant concentration, and dipped into liquid nitrogen. Vitrification does not require expensive devices and takes much shorter time compared to slow freezing. In the early years, embryos were placed onto small easy-to-cool carriers in order to reach high cooling rates. However, this open

system carries the potential risk of contamination and cross-contamination by microorganisms. Even though the sterilization of liquid nitrogen is possible, closed vitrification system is a safer and more simple opportunity. During the use of a closed system, the straw carrying the embryos is not exposed to liquid nitrogen directly. The drawback of this, is the reduced cooling rate, that can affect embryo survival.

In assisted reproduction institutes, quality control systems are used to ensure safe and high quality services. These systems cover every field of patient care, and include proper infrastructure and working area, skilled and trained staff, appropriate documentation, resource management, as well as protocols for unexpected events. As embryology laboratory plays a key role in treatment success, recognition of discrepancies and identification of the causes is crucial to achieve good and repeatable results. Follow up of values and phenomenons that can refer to treatment outcome or indicate unwanted events is spread. Commonly used indicators are for instance the rate of mature oocytes, fertilization rate, rate of good quality embryos. Giant oocytes are also possible indicators. These giant eggs have an approximately double cytoplasm volume compared to the normal ones, and present sporadically in IVF cycles. Presence of these eggs might indicate the effectiveness of the ovarian stimulation and refer to the quality of sibling oocytes.

Objectives

1. Embryology laboratory methods have gone through serious evolution in the past decades. Researches and developments involve all aspects of in vitro fertilization (IVF) treatments. In our study, four important subfields were examined. The main objectives of the dissertation are:
2. To explore, in what cases can similar or even better results be reached with the use of conventional IVF technic compared to intracytoplasmic sperm injection to decrease the overuse of ICSI.

3. To investigate, whether group culture of embryos in a microwell culture dish influences the fertilization rate following ICSI, and to explore how the culture method influences embryo quality and the number of embryos available for transfer or freezing. Furthermore, to examine implantation and clinical pregnancy rate following embryo transfer.
4. To investigate, whether embryo density influences the development of embryos cultured in a microwell group culture dish, as well as how different density values affect embryo quality.
5. To investigate how closed vitrification system influences survival of the thawed embryos, as well as treatment outcome, compared to conventional slow freezing.
6. To explore what the presence of giant oocytes indicates, and whether they occurrence indicates the quality of sibling eggs and developing embryos, or treatment outcome.

Methods

Data of 1132 IVF cycles were retrospectively analyzed for the comparison of the effectiveness of conventional IVF and intracytoplasmic sperm injection. These cycles were performed between 2009 and 2014. Cycles were divided into 3 groups according to semen quality. As semen samples were processed differently depending on their quality and fertilization method, grouping was made according to the total progressive sperm count, as it is known as a good quality indicator. Groups were as follows SPI: <15 million, SPII: 15-30 million, SPIII: ≥30million progressive motile sperms in the native sample. Cycles were also classified according to patient ages (A1: ≤31, A2: 32-36, A3: 37-40, A4: ≥41) and total egg counts (O1: 1-3, O2: 4-6, O3: 7-9, O4: ≥10).

Prospective randomized trial was performed to compare the two different culture methods. Between September 2012 and April 2015 532 IVF cycles were involved in the study. Culture method was randomly selected following egg collection, if a patient had a minimum of one oocyte. Cycles were divided into

two groups: embryos in the IC Group were cultured conventionally, in single droplets, while embryos in the GC Group were cultured in groups in microwell-containing Petri dishes.

In order to investigate the influence of embryo density on embryo viability, data of IVF cycles where embryos were cultured in groups were analyzed retrospectively. Inclusion criteria was the presence of a minimum of two cleaving embryos on the second day following fertilization. Embryo density was calculated as the total culture volume (25 μl) divided by the number of cleaving embryos. Three groups were formed depending on the number of embryos in one Petri dish: ED1: 2-4 embryos (density of 6.3-12.5 μl), ED2: 5-6 embryos (density of 4.2-5.0 μl), ED3: 7-9 embryos (density of 2.8-3.6 μl).

In order to investigate the effectiveness of closed vitrification system, 96 cycles with transfer of frozen-thawed embryos (FET), preformed during 2013 and 2014 were analyzed. Embryo survival using the two different technics was examined. According to general practice, an embryo is considered survived, if a minimum of 50% of the blastomeres remained intact. For better comparison of the methods, 80% and 100% survivals were also compared.

For the examination of the indicator role of giant oocytes, data of 1512 IVF cycles performed between January 2008 and November 2013 were retrospectively analyzed. Cycles were divided into two groups: GO Group contained all the cycles where giant oocytes occurred, and Normal Group contained cycles without the presence of a giant egg. An oocyte was considered giant, if its diameter was 1.25-1.3-fold bigger than that of normal ones.

In order to a better recognition of the attributes of giant oocytes, measurements were performed with ImageJ v1.48 software of photos showing 11 giant eggs, 40 normal, mature (metahase II) and 20 immature (prophase I) oocytes, as well as 20 zygotes developing from normal size eggs. Photos were taken using Octax Eye USB2.0 (MTG, Bruckberg, Germany) camera, and Octax EyeWare (MTG) imaging software, during the study period. In order to eliminate bias from low case number in the GO Group paired test was also performed, with 30-30 cycles from each group. Pairing of cycles were made by patient age, egg count, stimulation protocol.

Results

Results of comparing conventional IVF and ICSI treatments

Fertilization rate was calculated as the number of normally fertilized oocytes divided by total egg count in case of both technic. using conventional IVF resulted in higher fertilization rates in case of all semen categories (Table I). There was no difference in the fertilization rates in the SPII Group between the two methods in neither age group, however it was higher in O2, lower in O3 Group following conventional IVF. In the SPIII Group significantly higher fertilization could be found in all of the oocyte number and age groups except the A3 Group.

Embryo transfer occurred in 1072 cases (IVF: 315 vs. ICSI 757), while it was not done in 60 cases (IVF: 3 vs. ICSI: 57). Clinical pregnancy rate per embryo transfer differed between the two methods in favor of conventional IVF regarding all of the subgroups (49.4% vs. 37.2%, $p<0.01$). No significance different was found between the two methods in the SPII Group (45.8% vs. 36.1%, $p=0.374$), but it was higher in the SPIII Group following conventional IVF (49.8% vs. 25.9%, $p<0.01$). No difference was likewise found in the implantation rates regarding all subgroups (30.5% vs. 25.4%, $p=0.060$), as in case of moderate sperm quality (SPII: 36.7% vs. 24.1%, $p=0.729$). Implantation rate was significantly higher though in the best sperm quality group (SPIII) following conventional IVF (29.9% vs. 16.8%, $p<0.01$).

Live birth rate per embryo transfer was also higher after IVF regarding all subgroups (40.1% vs. 31.4%, $p<0.01$). The remarkable difference in the moderate sperm quality group (SPII) could not be confirmed statistically (41.7% vs. 27.6%, $p=0.177$). In case of good semen quality (SPIII) live birth rate was also higher following fertilization with conventional IVF technic (39.8% vs. 21.7%, $p<0.01$).

Table I Fertilization rates

Categories		Fertilization rate: IVF	Fertilization rate: ICSI	P-value
All groups (SP I-III)				
No. of oocytes	**O1 (1-3)**	–	57.1%	–
	O2 (4-6)	70.8%	58.9%	<0.01
	O3 (7-9)	66.2%	59.0%	<0.01
	O4 (≥10)	65.6%	56.1%	<0.01
Patient age	**A1 (≤31 years)**	67.5%	57.5%	<0.01
	A2 (32-36 years)	66.1%	56.4%	<0.01
	A3 (37-40 years)	65.4%	58.3%	<0.01
	A4 (≥41 years)	67.0%	57.3%	0.015
SPII Group (15-30 million progressive sperms in the native sample)				
No. of oocytes	**O1 (1-3)**	–	58.7%	–
	O2 (4-6)	79.3%	58.5%	0.036
	O3 (7-9)	49.2%	65.4%	0.034
	O4 (≥10)	65.6%	62.5%	0.480
Patient age	**A1 (≤31 years)**	65.9%	66.0%	1.000
	A2 (32-36 years)	61.9%	62.1%	1.000
	A3 (37-40 years)	63.5%	57.1%	0.337
	A4 (≥41 years)	63.6%	65.0%	1.000
SPIII Group (≥30 million progressive sperms in the native sample)				
No. of oocytes	**O1 (1-3)**	–	56.8%	–
	O2 (4-6)	70.0%	59.7%	<0.01
	O3 (7-9)	67.6%	56.8%	<0.01
	O4 (≥10)	65.6%	58.9%	<0.01
Patient age	**A1 (≤31 years)**	67.6%	55.3%	<0.01
	A2 (32-36 years)	66.3%	59.8%	<0.01
	A3 (37-40 years)	65.5%	61.7%	0.215
	A4 (≥41 years)	67.2%	54.9%	<0.01

SP: Cycles grouped by total progressive sperm count in the native sample
O: Group of cycles classified according to the total oocyte count
A: Group of cycles classified according to patient age

Results of the investigation of group culture

A total of 532 IVF cycles were involved in the study. The number of cycles was 264 in the GC Group and 268 in the IC Group. Fertilization rates were comparable between the groups when conventional IVF was performed (69.0% vs. 66.6%, p=0.361), however, fertilization rate following ICSI treatment was significantly higher in the microwell group culture dish than in the individual culture group (70.6% vs. 64.9%, p<0.01). Morphology score and fragmentation rate were similar between the two groups, however, the number of blastomeres was significantly higher in the microwell group culture than in the individual culture on days 2 (4.1±1.4 vs. 4±1.3, p=0.038) and 3 (4.1±1.4 vs. 4.0±1.3, p=0,038). No difference was found in the proportion of good quality embryos neither on day 2 nor on day 3.

There was no difference between the mean number of transferred embryos (2.1±0.9 vs. 2.1±0.9, p=0.665). Clinical pregnancy rate was significantly higher in the GC Group than in the individual culture group (50.8% vs 40.6%, p=0.022), however, implantation rate did not differ significantly between the groups (30.1% vs. 27.0%, p=0.265). Embryo cryopreservation rate (39.7% vs. 32.1%, p<0.01) and embryo utilization rate (81.3% vs. 74.7%, p<0.01) were significantly higher in the group culture than in the individual culture.

Results of the investigation of embryo density

For the investigation of the effect of different embryo densities on embryo viability, data of 1337 cleavage stage embryos on day 2 (ED1 Group: n=370, ED2 Group: n=486, ED3 Group: n=481), while data of 1229 embryos on day 3 (ED1 Group: n=269, ED2 Group: n=479, ED3 Group: n=481) were analyzed.

No differences between the number of blastomeres were found, however, morphology score was lower (2.2±0.7 vs. 2.3±0.7, p= 0.003), and fragmentation rate was higher (16.1±10.9% vs. 14.2±8.9%, p=0.009) in ED1 than in ED2. Rate of good quality embryos was higher in the moderate density group (ED2) than in any other groups (ED1 and ED2: 18.9% vs. 31.5%, p<0.001 and ED2 and

ED3: 31.5 vs. 24.7%, $p=0.02$), but there was also a slight difference between ED1 and ED3 (18.9% vs. 24.7%, $p=0.043$).

On day 3, cell number was significantly higher in the moderate density group (ED2: 7.3±2.1) than in low (ED1: 6.8±2.2, $p=0.004$) and high groups (ED3: 7.0±2.0, $p=0.014$). Superior average morphology score was also assessed in ED2 (2.3±0.7) compared to ED1 (2.1±0.7, $p=0.021$), but did not differed from that in ED3 (2.2±0.6, $p=0.474$). No significant difference was found in the proportion of fragmentation between ED2 and ED 3 groups (14.7±10.4% vs. 14.2±9.4%, $p=0.768$), but it was remarkably lower in both groups compared to ED1 Group (ED1 and ED2: 16.7±11.5 vs. 14.7±10.4, $p=0.029$, ED1 and ED3: 16.7±11.5 vs. 14.2±9.4, $p<0,01$). Good quality embryo rate shows the same pattern as blastomere number. The highest rate was assessed in ED2 (27.1%), followed by ED3 (21.2%, $p=0.032$) and ED1 (19.7%, $p=0.023$).

Results of the comparison of different freezing methods

A total of 96 FET cycles were performed during the study period (slow freezing: 43, vitrification: 53), while 343 embryos were thawed (slow freezing: 164, vitrification: 149) which were originated from 90 fresh IVF cycles (slow freezing: 52, vitrification: 38).

The 50% survival rate of the embryos was higher following vitrification (55.1% vs.92.1%, $p<0.001$). The same results were found in case of the 80% survival rate (35.1%, vs. 69.1%, $p<0.001$), as well as the 100% survival rate (21.2% vs. 57.2%, $p<0.001$).

Quality of the frozen embryos was not similar in the two groups. Better quality embryos were cooled with vitrification compared to slow freezing, with a higher blastomere number (7.7±2.0 vs. 6.9±1.8, $p<0.001$), higher morphology score (2.3±0.6 vs. 2.2±0.4, $p<0.001$) and lower fragmentation rate (12.2±6.4% vs. 14.1±8.4%, $p=0.039$).

Because of these differences the comparison of embryo survivals was made in case of frozen embryos with blastomeres number of 7-12. Both 50% (80.9% vs. 93.0%, $p=0.004$), 80% (53.9% vs. 69.8%, $p=0.011$) and 100% survival rate (31.3% vs. 56.6%, $p<0.001$) were higher following vitrification.

Significant difference was found in the rate of transferred embryos relative to the thawed ones in favor of vitrification (29.7% vs. 39.4%, $p=0.012$). Even though both were remarkably higher following vitrification, differences in case of neither pregnancy rate (32.7% vs. 47.6%, $p=0.145$) nor implantation rate (16.5% vs. 25.3%, $p=0.125$) did not reach statistical significance. However, live birth rate did not differ (30.8% vs. 31.0%, $p=1.000$).

Results of the investigation of giant oocytes

During the study period, a total of 1521 IVF cycles were performed and 12554 oocytes were collected, from which 37 were giant. The incidence of giant oocytes was 0.3%. The size of giant oocytes was significantly larger in diameter than normal ones' (200.0±12.2μm vs. 161.6±6.1μm, $p<0.01$ with, and 140.8±10.2μm vs. 112.8±2.9μm, $p<0.01$ without zona pellucida). Thickness of zona pellucida was also significantly larger (21.8±2.8μm vs. 17.6±2.8μm vs. $p<0.01$). The average volume of giant oocytes appeared to be 4.19x10-3 μm^3, while the volume of normal oocytes was 2.21x10-3μm^3 ($p<0.01$). The size of germinal vesicles (31.2±2.9μm vs. 32.0±1.4μm, $p=0.06$) and pronuclei (23.6±1.4μm vs. 25.1±1.8μm, $p=0.43$) did not differ.

When applying GnRH agonist "long protocol", giant oocytes occurred in 2.7% of the cycles, whereas when using GnRH antagonist protocol, giant oocytes occurred in 6.6% of the cycles ($p=0.31$). Occurrence of giant oocytes was also similar when HMG or FSH was used for ovarian stimulation (1.6% vs. 1.9% $p=0.22$). Mean patient age was lower in the GO Group than in the Normal Group (33.5±3.9 vs. 35.3±4.9, $p=0.02$). E2 levels (1954±903pg/l vs. 1488±909pg/l, $p<0.01$) and the number of retrieved oocytes (12.7± vs 8.1±5.1, $p<0.01$) were significantly higher compared to the Normal Group. Clinical pregnancy rates were similar in the two groups (37,8% vs. 37,4%, $p=1.00$).

On Day 2 only the presence of fragmentation differed between the two groups in favor of Normal Group (16.1±11.4% vs. 14.4±10.2%), and this difference rose remarkably on Day 3 (17.9±13.0% vs. 15.6±11.5%, $p<0.01$) Number of blastomeres were significantly lower in the GO Group (6.2±2.0 vs. 6.6±2.1, $p<0.01$) on Day 3.

In case of paired cycles, no differences were found between the quality parameters neither on Day 2, nor on Day 3. Mean cell number of the transferred embryos in the GO Group was lower (7.0±1.8 vs.7.8±1.6, p=0.03).

Conclusions

1. The use of conventional IVF in case of non-male factor infertility results in similar or even better outcomes compared to ICSI. In case of good quality semen sample fertilization rate, quality of the transferred embryos, as well as clinical pregnancy rate is superior, and also not poorer in case of moderate semen quality following conventional IVF compared to ICSI. Elevated patient age itself does not indicate the need of ICSI, which we recommend to use only in case of severe male factor infertility or previous IVF failure.
2. Culturing embryos in groups in a microwell culture dish improves fertilization rate following ICSI. Group culture also promotes embryo development, and results in a higher number of embryos available for cryopreservation.
3. Embryo density affects embryo development when culturing in a microwell culture dish. Culturing 5-6 embryos together in a media volume of 25 μl (4.2-5.0 μl/embryo) benefits embryo quality.
4. Cleavage stage embryos have a higher chance to survive cryopreservation after vitrifiing them using closed system vitrification with Rapid-I carrier compared to slow freezing, moreover vitrification results in improved embryo quality after thawing. Clinical pregnancy rate and implantation rate showed a favorable trend in case of vitrification using Rapid-I system, although difference in live birth rate could not be detected. However, in order to transfer the same number of embryos less embryo needs to be thawed, and thud cumulative pregnancy rate, as well as cumulative live birth rate could be improved.

5. Giant oocytes occur most often in cycles of patients with high number of oocytes collected. Presence of giant oocytes reflect neither to the quality of sibling oocytes, nor the quality of the developing embryos and nor treatment outcome.

List of own publications

Publications related to the doctoral dissertation

- Lehner A, Kaszas Z, Murber A, Rigo J Jr, Urbancsek J, Fancsovits P. (2017) Embryo density may affect embryo quality during in vitro culture in a microwell group culture dish. Arch Gynecol Obstet, *DOI: 10.1007/s00404-017-4403-z*
  IF: 2,090
- Lehner A, Kaszas Z, Murber A, Rigo J Jr, Urbancsek J, Fancsovits P. (2015) Giant oocytes in human in vitro fertilization treatments. Arch Gynecol Obstet, 292(3): 697-703.
  IF: 1,680
- Lehner Á, Kaszás Z, Murber Á, Rigó J Jr, Urbancsek J, Fancsovits P. (2015) Az embriók vitrifikációja és hagyományos mélyfagyasztásuk eredményességének összehasonlítása in vitro fertilizációs kezelések során. Magy Nőorv L, 78(4): 210-217
- Lehner Á, Kaszás Z, Murber Á, Rigó J Jr, Urbancsek J, Fancsovits P. (2015) Az intracitoplazmatikus spermiuminjekció (ICSI) és hagyományos in vitro fertilizációs (IVF) kezelések eredményeinek összehasonlítása az ondóminta minőségének függvényében. Magy Nőorv L, 78(6): 310-318.

Other publications that provide scientific work

- Fancsovits P, Urbancsek J, Fónyad L, Sebestyén A, Csorba G, Lehner Á, Kaszás Z, Rigó J Jr, Bokor A. (2016) Kezdeti tapasztalataink a petefészeszövet-fagyasztás beveztésével. Orvosi Hetilap 157(49): 1947-1956.
  IF: 0,349
- Fancsovits P, Lehner A, Murber A, Kaszas Z, Rigo J Jr, Urbancsek J. (2014) Effect of hyaluronan-enriched embryo transfer medium on IVF outcome: a prospective randomized clinical trial. Arch Gynecol Obstet, 291(5): 1173-1179.
  IF: 1,364

- Fancsovits P, Lehner Á, Murber Á, Rigó J Jr, Urbancsek J. (2013) Az ondóminták minőségének megítélése a WHO-referenciaértékek változásának tükrében. Magy Androl, 18(2): 29-34.

Scientific publications:

Publications: 7 (IF: 5.483)

In international journal: 3

In Hungarian journal: 4

Quotable abstracts: 15