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Evaluation and optimization of vitrification for human preimplantation embryos and oocytes

ABSTRACT

of a dissertation for educational and scientific degree
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Cell biology

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The dissertation is written on 129 pages, contains 31 figures, 34 tables and 7 applications. Literature sources used were 274. The numbers of the tables, figures and citations in the abstract do not correspond to those in dissertation. All patients were treated in SAGBAL Dr. Shterev - Sofia, where were received, processed, cryopreserved by vitrification, stored and thawed the analyzed embryos and oocytes.

ABBREVIATIONS USED

- AC artificial collapse
- AH assisted hatching
- ART assisted reproductive technologies
- cFET cancelation of FET - due to poor survival of thawed embryos
- COC cumulus-oocyte complex
- COH controlled ovarian hyperstimulation
- CP clinical pregnancy
- CPR clinical pregnancy rate, percentage of clinical pregnancies
- CPs clinical pregnancies
- ET embryo transfer
- FET frozen- thawed embryo transfer
- FETs frozen- thawed embryo transfers
- hCG human chorionic gonadotropin
- HSA human serum albumin
- ICSI intracytoplasmic sperm injection
- IVF in vitro fertilization
- IVM in vitro maturation
- LAH laser assisted hatching
- NA not analyzed
- OHSS ovarian hyperstimulation syndrome
- PESA percutaneous epididymal sperm aspiration
- PGD preimplantation genetic diagnosis
- PGS preimplantation genetic screening
- PGT-A preimplantation genetic testing for aneuploidies
- PGT-M preimplantation genetic testing for single gene / monogenic disorders
- TESE testicular sperm aspiration

INTRODUCTION

Freezing techniques are part of assisted reproductive technologies (ART), and the areas of their application are increasing. In some cases, they facilitate activities, and in others, assisted reproduction depends on them. There is a significant difference in the possibilities for freezing the two types of gametes - male and female, as well as preimplantation embryos. Freezing and thawing of sperm is a routine method that has proven to be effective and safe, while freezing of oocytes is more difficult task, and has to be improved in order to better results.

Freezing and storage or cryopreservation of oocytes is necessary in various cases, such as difficulty in ejaculation or azoospermia of the male partner on the day of the follicular puncture. It is extremely important in young women without a partner, suffering from a disease that requires radiation or chemotherapy, which in most cases leads to loss of ovarian function, and hence to sterility (fertility preservation for medical reasons). The storage of oocytes gives a chance to realize pregnancy, after successful completion of treatment. Increasingly, in developed countries, where young women have the desire and opportunity for professional realization, oocyte cryopreservation is used in order to delay reproduction termed - elective fertility preservation (EFP) or preservation of fertility for non-medical reasons. Another reason for cryopreservation of oocytes is the use of donor oocytes for heterologous use in recipients who do not produce their own oocytes or is forbidden the usage of their own due to poor quality, genetic diseases or other reasons. Storing donor oocytes facilitates the procedure, shortens the waiting period and avoids the need for synchronization of menstrual cycles between the donor and the recipient of oocytes.

In ART, fertilization of oocytes by conventional in vitro fertilization or intracytoplasmic sperm injection results, in some cases, in more embryos than are transferred to the patient's uterus. Freezing untransferred embryos ensures their storage, providing a chance for a new attempt in case of failure or, if desired, for the next pregnancy, avoiding hormonal stimulation and follicular puncture. In some cases, embryo transfer is contraindicated due to the risk of ovarian hyperstimulation syndrome (OHSS), which is a complication of assisted reproduction, which involves enlargement of the ovaries and the passage of fluid from the intravascular space to various organs due to increased capillary permeability. If a polyp occurs in the uterine cavity during hormonal stimulation, the appearance of uterine bleeding, inability to penetrate the uterus, it is recommended not to apply embryo transfer again. In the cases described, freezing the embryos is the only chance to use them later. Cryopreservation of all good quality embryos

without fresh embryo transfer and their subsequent use is known as freeze all program or freeze all strategy. The possibility of successful cryopreservation in case of need of genetic analysis of the obtained embryos is extremely important. It includes: preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS), which are now called PGT-M - preimplantation genetic diagnosis for monogenic diseases and PGT-A preimplantation genetic diagnosis for aneuploidy. The process involves biopsy of embryonic cells on day five and performing genetic analysis of those cells. The procedure takes several days, which requires freezing the embryos. They are vitrified and used in the patient's subsequent menstrual cycle after receiving the genetic results.

Different clinical and embryological factors can influence the freezing and thawing of oocytes and embryos, as well as the acieved pregnancies. The aim of present dissertation was to analyze such parameters, to determine their effect on the result of cryopreservation, which can be used for its optimization. The data would provide more detailed information, which will be important for successful therapy of patients.

Optimizing cryopreservation methods for oocytes and embryos will lead to greater patient safety, better ART results, and more healthy babies born.

AIM AND TASKS

The aim of this dissertation is to make a detailed analysis, based on our own results, of the cryopreservation method "vitrification" and to optimize the process in human preimplantation embryos and oocytes.

The aim presupposed the solution of the following tasks:

1. To establish survival rate after thawing of vitirfied human preimplantation embryos depending on:

(a) The quality of the embryos before freezing;

b) The patient's age;

(c) Method of fertilization of oocytes: IVF or ICSI;

(d) The day of development of the embryos on which the vitrification was carried out;

e) Type of infertility - primary or secondary;

(f) Storage period of embryos;

g) The application of co-culture of embryos with autologous endometrial cells before vitrification;

h) Application of open and closed vitrification system;

i) Performing an artificial collapse before vitrification.

2. To determine the percentage of clinical pregnancies (CPR) in relation to:

(a) The quality of the embryos before freezing;

b) The patient's age;

(c) Method of fertilization of oocytes: IVF or ICSI;

(d) The day of development of the embryos on which the vitrification was carried out;

e) Type of infertility - primary or secondary;

(f) Storage period of embryos;

g) The application of co-culture of embryos with autologous endometrial cells before vitrification;

h) Application of open and closed vitrification system;

(i) Number of embryos transferred;

j) Survival of the transferred embryos;

(k) Application of assisted hatching (AH) after thawing of embryos before FET;

l) Performing an artificial collapse (AC) before blastocyst vitrification.

3. To analyze the survival rate of embryos and developing clinical pregnancies in "freeze all" program or strategy (cases where no fresh embryo transfer is performed and all developing embryos are frozen and used in a subsequent frozen-thawed embryo transfer).

4. To evaluate and optimize the effectiveness of vitrification in oocyte freezing.

(a) Assessment of their survival rate after thawing;

b) Assessment of fertilization and development of the obtained embryos.

MATERIAL AND METHODS

Clinical material

In the current study 2453 preimplantation human embryos were included. The studied FET cycles were 941 in 844 patients.

Methods

Classical *in vitro* fertilization (IVF)

Oocyte denudation and intracytoplasmic sperm injection (ICSI)

Combination of the two methods of oocyte fertilization (IVF / ICSI)

Biopsy for isolation autologous endometrial cells

Co-cultivation of embryos with autologous endometrial cells before vitrification

Fresh embryo transfer (ET)

Artificial collapse (AC)

Vitrification and thawing of preimplantation embryos

Vitrification and thawing of oocytes

Frozen-thawed embryo transfer (FET)

RESULTS

Measurement of survival of human preimplantation embryos after vitrification

Surviving blastomeres have the characteristics of non-frozen ones - transparent vitelus and regular cell shape. Not survived are dark, with unclear outlines. Salumets and co-authors (1) divided embryos into three groups after thawing: 100% survived, partially damaged (survived over 50% of cells) and degenerates (survivors below 50% of cells). In our study, four groups were formed. After thawing, depending on survival, they were:

Group I (Intact Embryos), 100% post-thaw survival was observed. All cells of the dividing embryo or blastocyst had undergone vitrification and thawing (Fig. 1 a and b).

Group II (Embryos with more than 50% of the cells survived) had good survival, with a small proportion of cells not surviving vitrification and thawing (Fig. 1c).

Group III (embryos with less than 50% of the cells survivied) reported poor survival, with the majority of cells dying during cryopreservation (Fig. 1d).

Group IV (Degeneratde embryos), includes embryos in which no cell survival is observed. It is also defined as zero (0)% or lack of survival (Fig. 1e and f).

Groups I and II were considered good survival (Fig. 1 a, b and c).

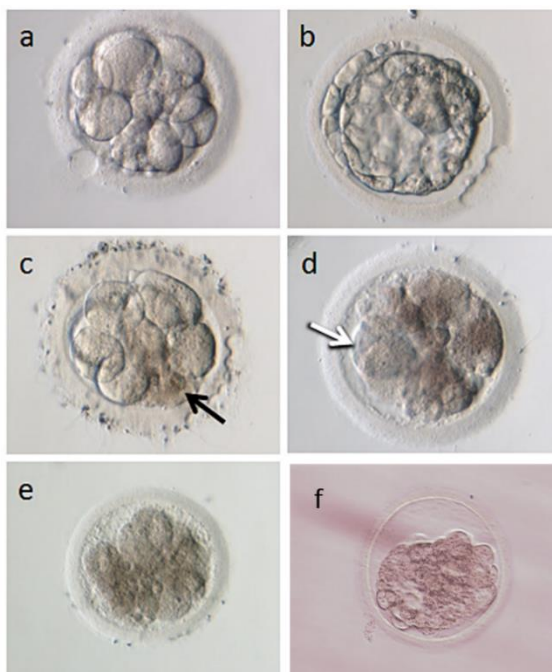


Figure 1. Morphology of thawed embryos. Representatives of group I embryos are shown in panels a and b. a) intact embryo on day 3 and b) intact embryo on day 5 (blastocyst). Panels c and d show embryos of groups II and III: c) embryo with 90% survival (group II) and d) embryo with 14% survival of blastomeres (group III). The last two panels are of group IV embryos: e) embryo on day 3 without survived blastomeres and f) non-survived blastocyst. The black arrow indicates the position of a non-survived blastomere and the white arrow indicates the position of a survived blastomere. 200x magnification, Hoffman modulation contrast

Overall survival was also calculated. It represents all embryos that have survived thawing completely or partially, groups I, II, and III are included. Only completely non-survived (degenerate) embryos (Group IV) were excluded from overall survival. A total of 2453 thawed embryos frozen by vitrification were analyzed. The calculated survival rate of the analyzed embryos is presented in Table 1.

Table 1. Survival of vitrified embryos after thawing

Number of embryos	Overall survival number and %	Intact embryos (Group I) number and %	Survival over 50% of cells (Group II) number and %	Survival below 50% of cells (Group III) number and %	Degenerated embryos (Group IV) number and %
2453	2093	995	616	482	360
	85,3 %	40,6 %	25,1 %	19,6 %	14,7 %

The influence of certain initial parameters on the survival of vitrified embryos after thawing was checked (Table 2).

Table 2. Investigated parameters in the application of vitrification

Investigated parameter	Number of thawed and analyzed embryos
The quality of embryos before freezing	1460
The patient's age	1460
Method of fertilization of oocytes: IVF or ICSI	1148
The day of embryo development on which the vitrification was performed	1460
Modification of the vitrification method on day five	452
Type of infertility - primary or secondary	1460

Storage period of embryos	1460
The application of co-culture of embryos with autologous endometrial cells before vitrification	32
Application of open and closed vitrification system	311
Performing an artificial collapse of blastocysts before vitrification	937

The quality of embryos before freezing

During their initial development, human embryos do not always undergo synchronous division, leading to an even number of blastomeres - 2,4,6,8,10. An odd number of cells is often observed - 3,5,7,9, which reduces the quality of embryos, but there is no reason that these embryos should not be transferred, because they could also lead to pregnancy. An important indicator in assessing the quality of embryos is the number and type of blastomeres up to day three. The quality of the embryos on days 4, 5 and 6 was determined on the basis of the relevant characteristics described below. Another parameter concerning the quality of embryos on each day of their development is the absence or presence of fragmentation, as well as its percentage of the volume of the embryo. It is a small cytoplasmic particle without genetic material, resulting from the division of embryos. It has a negative effect when it is over 15-20% of the embryo's volume. Taking into account all the characteristics of the embryos, they can be with top quality, good quality or poor quality for the relevant day. On the day of freezing before vitrification embryos were divided into three groups depending on their quality: with top, good or poor quality. There were no cryopreservation of zygotes (day one), only dividing embryos from day two to day six.

Day two (Fig. 2):

Embryos with top quality on day two are defined as: embryos with four blastomeres (cells) without fragmentation;

Embryos of two, three or five blastomeres without or with up to 10% fragmentation are with good quality;

Divided into more blastomeres of different size and / or with more than 20% fragmentation are poor quality embryos;

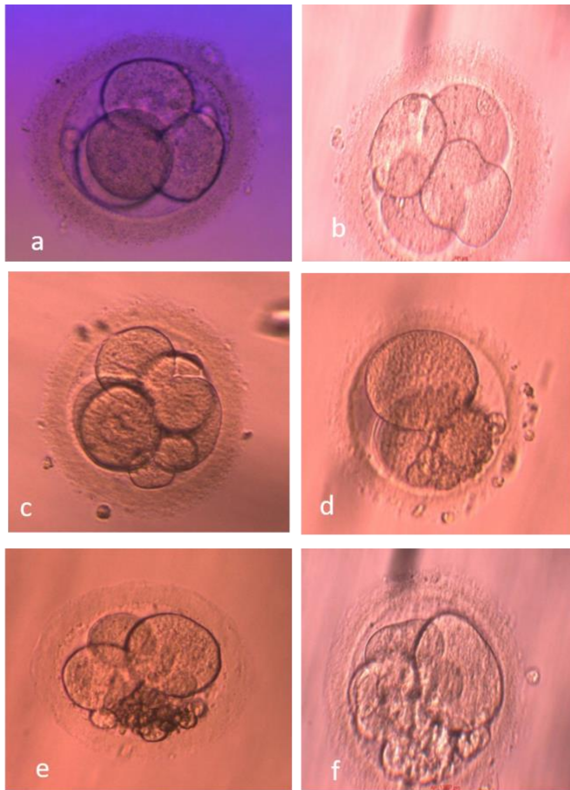


Figure 2. Embryos on day two of their in vitro development. Two-day-old embryos of different quality are presented. Panels a and b are embryos with top quality. Panels c and d are good quality embryos. Poor quality embryos are visible on panels e and f. Magnification 200 x, Hoffman modulation contrast

• **Day three (Fig. 3):**

With top quality on day 3 are embryos divided into 7-10 blastomeres and less than 10% fragmentation;

Good quality embryos on this day are embryos with 6 blastomeres and less than 10% fragmentation or 7-10 blastomeres and 10-20% fragmentation;

Poor quality embryos are defined as those below 4 blastomeres or over 20% fragmentation;

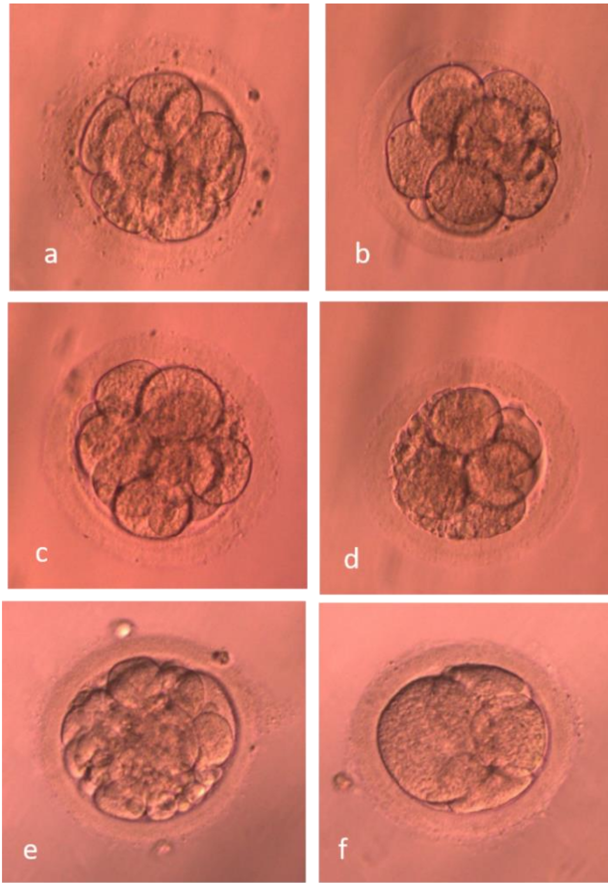


Figure 3. Embryos on day three of their in vitro development. Three-day-old embryos with different quality are presented. Panels a and b are embryos of top quality. Panels c and d are good quality embryos. Panels e and f contain poor quality embryos. Magnification 200 x, Hoffman modulation contrast

• **Day four (Fig.4):**

On day 4, the highest quality embryos are in the compact morula stage with less than 10% fragmentation. This stage is a compact structure, as a result of shortening the distances between the cells. It should include all 16 to 32 blastomeres of the morula stage.

Morula or compact morula with 10% -20% fragmentation are defined as good quality embryos;

Embryos up to eight blastomeres or with more than 20% fragmentation are with poor quality;

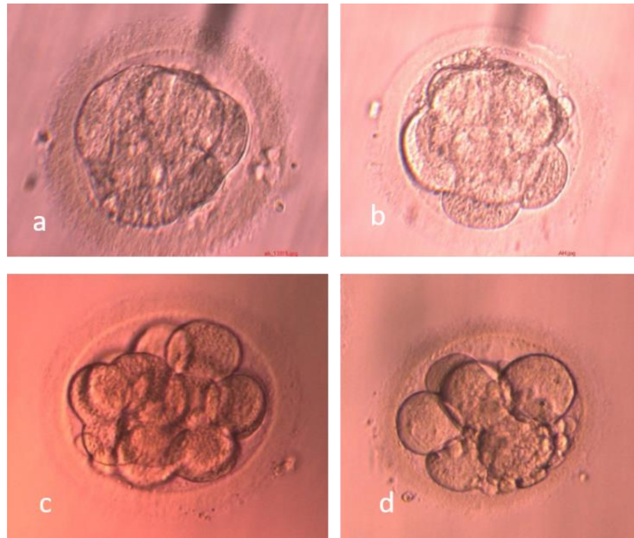


Figure 4. Embryos on day four of their in vitro development. Four-day-old embryos of different quality are presented. Panels a and b are embryos with top. Panel c is a good quality embryo. There is a poor quality embryo on panel d. Magnification 200 x, Hoffman modulation contrast

• **Day five (Fig. 5):**

On day five, developing embryos should be blastocysts with distinction between embryoblast (ICM) from which the embryo develops and the trophoblast cells (trofectoderm) that give the placenta. The Gardner and Schoolcraft scheme (2) was used to evaluate blastocysts. According to it, the following were distinguished:

early blastocyst - when the blastocoel is less than half the volume of the embryo;

blastocyst - the blastocoel is greater than or equal to half of the embryo;

full blastocyst - the blastocoel fills the entire embryo;

expanded blastocyst - the volume of the blastocyst increases and zona pellucida begins to thin;

hatching blastocyst - the blastocyst begins to come out through the zona pellucida;

Embryoblast (ICM) and trofectoderm were evaluated according to the following scheme:

- ICM:

A. Many cells tightly adhered to a ball-like structure;

B. Insufficient number of grouped cells;

C. Composed of few cells.

-Trofoectoderm:

A. Many cells tightly placed next to each other in one layer;

B. Few cells arranged in one layer;

C. Very few cells unable to form a dense layer (3).

In addition to the described blastocysts, compact morula and embryos at the cleavage stage were observed on day five due to their slower development.

The top quality embryos were defined as: hatching blastocyst, expanded blastocyst with ICM and trophoectoderm type A and B, as well as complete blastocyst with <10% fragmentation.

Good quality - expanded blastocyst with ICM and trophoectoderm type C, blastocyst, early blastocyst and complete compaction without or with 10-20% fragmentation.

Poor quality embryos were considered to be those with slower development: with initial or non-started compaction or with> 20% fragmentation.

Some of the full, expanded and hatching blastocysts were pre-prepared for vitrification by performing artificial collapse.

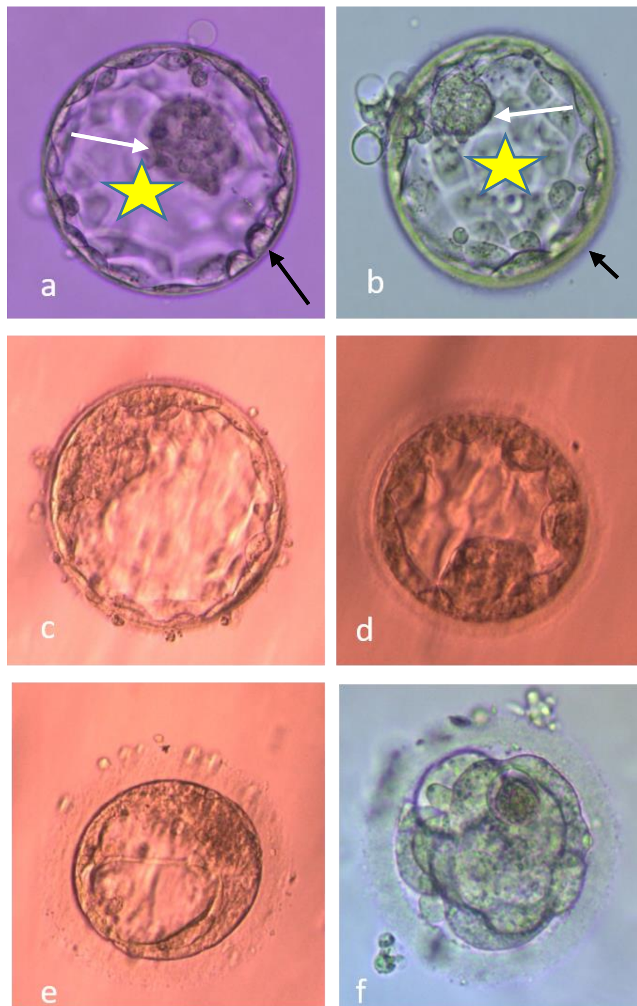


Figure 5. Embryos on day five of their in vitro development. Five-day-old embryos (blastocysts) with different quality are presented. Panels a and b are blastocysts with top quality. Panels c and d are good quality blastocysts. Poor quality embryos are visible on panels e and f. The white arrow indicates the embryoblast (ICM). The black arrow indicates the trofectoderm. The yellow star indicates the blastocele. Magnification 200 x, Hoffman modulation contrast

• **Day six (Fig. 6):**

To day six, embryos were cultured, when on day five had slower growth. Those that continued to develop to the blastocyst stage were frozen on day six.

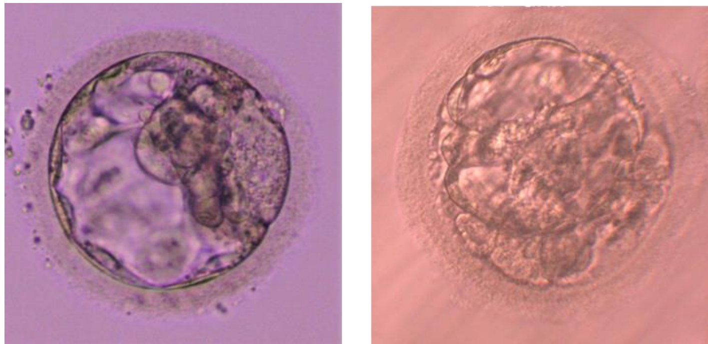


Figure 6. Embryos on day six of their in vitro development. Magnification 200 x, Hoffman modulation contrast

To determine the effect of quality on post-thaw survival, 1460 embryos were examined.

Based on the quality of the embryos from their in vitro development for the respective day on which the cryopreservation was performed and their survival after thawing, three groups were formed, presented in Table 3.

Table 3. Survival of embryos depending on their quality before freezing

Embryo quality before freezing	**Number of embryos**	**Overall survival number**	**Overall survival %**	**Intact embryos number**	**Intact embryos %**
Top	703	586	83,4%	267	38%
Good	587	497	84,7%	223	38%
Poor	170	130	76,5%	39	22,9%

When comparing the overall survival between top and good quality embryos, no statistical significance was found (P = 0.5), while when comparing the poor quality embryos and those from the groups with top (P = 0.04) and good (P = 0.01) quality, the result is significant. Similar correlations, but with even higher statistical significance, were found when comparing the percentages of intact embryos - P = 0.0002 when comparing between

the group of poor and top quality and P = 0.0003 when comparing the embryos with poor and good quality.

The patient's age

1460 embryos were evaluated and divided according to the age of the patients into two groups: patients ≤ 35 years and ≥ 36 years during the assisted reproduction. In the first group (≤ 35 years) the number of embryos was 1199, and the second age group (≥ 36 years) included 261 embryos. Survival is presented in Table 4.

Table 4. Patient age and embryo survival after thawing

Age	Number of thawed embryos	Overall survival number and %	Intact embryos number and %	Survival over 50% of cells number and %
≤ 35 years	1199	1001 (83,5%)	431 (35,9%)	301 (25,1%)
≥ 36 years	261	220 (84,3%)	98 (37,5%)	62 (23,8%)

There was no difference in the percentages of overall survival after thawing (P = 0.8) and in the percentages of intact embryos (P = 0.6) depending on the patient's age.

Method of fertilization of oocytes: IVF or ICSI

The methods used for fertilization of the oocytes were classical in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). The survival of 1148 cryopreserved and thawed embryos was analyzed depending on the method for fertilization (Table 5).

Table 5. Survival of embryos according to the method for fertilization of oocytes

Method	Number of thawed embryos	Overall survival number and %	Intact embryos number and %	Survival over 50% of cells number and %
IVF	585	490 (83,8%)	214 (36,6%)	144 (24,6%)
ICSI	563	461 (81,9%)	195 (34,6%)	139 (24,7%)

Depending on the method for fertilization of the oocytes: IVF or ICSI, no difference in survival was observed after thawing of the embryos (P = 0.4).

The day of embryo development on which the vitrification was performed

We examined 1460 embryos vitrified on different days of their in vitro development: from day two to day six including (Table 6). After fresh ET, the remaining embryos were frozen by vitrification on the same day. The preferred days for fresh ET were the third and fifth, so the number of frozen embryos on the mentioned days was much higher compared to the second, fourth and sixth days.

Table 6. Survival of vitrified embryos after thawing compared to the day of their in vitro cultivation

Day of the vitrification of embryos from in vitro development	Number of thawed embryos	Overall survival number and %	Intact embryos number and %	Survival over 50% of cells number and %	Survival under 50% of cells number and %
Day 2	29	24 (82,8%)	15 (51,7%)	5 (17,2%)	4 (13,8%)
Day 3	771	649 (84,2%)	280 (36,3%)	186 (24,1%)	183 (23,7%)
Day 4	191	157 (82,2%)	62 (32,5%)	54 (28,3%)	41 (21,5%)
Day 5	452	379 (83,8%)	167 (36,9%)	113 (25%)	99 (21,9%)
Day 6	17	12 (70,6%)	5 (29,4%)	5 (29,4%)	2 (11,8%)

There was no statistically significant difference in embryo survival after thawing depending on the day of development on which vitrification was performed.

Modification of the vitrification method on day five

In Table 6, the embryos presented on day five were cryopreserved using two different concentrations of human serum albumin in the vitrification and thawing solutions. Initially, 5% human serum albumin was used. At this concentration, a survival rate of 78.2% of blastocysts was obtained (Table 7). Despite the lack of a statistically significant difference in embryo survival on day 5, it was lower than desired. For this reason, it was decided to apply optimization of the vitrification method by doubling the concentration of human serum

albumin in vitrification and thawing solutions. The aim was to determine whether higher concentrations of human serum albumin had better cryopreservation function and if this would lead to better survival afret thawing. The negative effect of cryopreservation was less pronounced in developing embryos than in other days, so they did not need to use the modified method. To study the effect of increasing concentration of human serum albumin, 452 vitrified and thawed blastocysts were analyzed. Of these, 193 were by the method without modification, and 259 by the already modified method. The results obtained are presented in Table 7, which shows a statistically significant increase in the overall survival of embryos after modification of the vitrification method.

Table 7. Blastocyst survival using two different concentrations of human serum albumin in vitrification and thawing solutions

HSA concentraion	**Number of thawed blastcysts**	**Overall survival number**	**Overall survival %**	**P value**
Before modification (5% HSA)	193	151	78,2%	P=0,005
After modification (10% HSA)	259	228	88%	

Type of infertility - primary or secondary

We aimed to examine the influence of the type of infertility on the survival of embryos. Women without a acieved pregnancy have primary infertility. Secondary infertility is the case of patients who have become pregnant, but trying to achieve a new one have failed. The 1460 embryos studied were divided into two groups. Analyzed embryos from patients with primary infertility were 823 embryos, while those from secondary infertility were 637. There was no difference in the average number of frozen embryos per patient in the two groups, which was 4.5 in the cases of primary and 4.6 embryos in those with secondary infertility. Their survival is presented in Table 8.

Table 8. Survival of embryos according to the type of infertility

Type of infertility	**Number of thawed embryos**	**Overall survival number and %**	**P value**	**Intact embryos number and %**	**P value**
Primary infertility	823	683 (83%)	P=0,4	204 (24,8%)	P<0,0001
Secondary infertility	637	538 (84,5%)		231 (36,3%)	

Although no difference in overall survival was reported depending on the type of infertility (P = 0.4), the percentage of intact embryos after thawing in cases of secondary infertility was significant (P <0.0001).

Storage period of embryos

The storage of the frozen embryos depends on the desire and need of the patients. The survival of 1460 embryos stored from 1 to 28 months was analyzed. Keeping the necessary storage conditions and ensuring that the temperature did not rise, it was checked whether prolonged storage had a negative effect on the embryos. We divided the embryos into two groups: stored in the cryobank for up to 12 months (1366 embryos) and stored for more than 12 months (94 embryos). The calculated survival is presented in Table 9, where there is no difference in the two groups.

Table 9. Survival of embryos at different storage period

Storage period	**Number of thawed embryos**	**Overall survival number and %**	**P value**	**Intact embryos number and %**	**P value**
Less than 12 months	1366	1139 (83,4%)	P=0,3	487 (35,7%)	P=0,08
More than 12 months	94	82 (87,2%)		42 (44,7%)	

Application of co-cultivation of embryos with autologous endometrial cells before vitrification

Analyzed were 32 vitrified embryos co-cultured with autologous endometrial cells (Fig. 7).

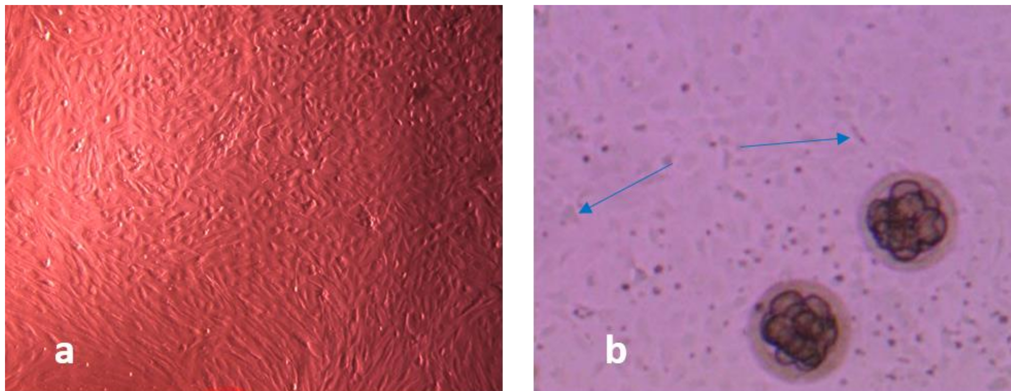


Figure 7. Endometrial cells and embryos co-cultured with them. Presented are: a monolayer of endometrial cells (a) and embryos on day three of their development on a monolayer of autologous endometrial cells (b). The blue arrows indicate the endometrial cells. Magnification 100 x, Hoffman modulation contrast

After thawing, 26 embryos (87.5%) survived. There were 14 (43.8%) intact embryos. These results show similar survival with embryos without co-culture with autologous endometrial cells, although co-culture is used in previous IVF / ICSI attemptes in which the resulting embryos are not with good quality. The embryos were frozen on day three or five of their in vitro development. Nine (28.1%) of them were frozen on day three and 23 (71.9%) on day five. Survival data depending on the day of freezing are presented in Table 10.

Table 10. Co-culture of embryos with autologous endometrial cells and their survival

Day of vitrification	Number of thawed embryos	Overall survival number	Overall survival %	Intact embryos number	Intact embryos %
Day 3	9	8	88,9%	4	44,4%
Day 5	23	20	87,0%	10	43,5%

Application of open and closed vitrification system

In closed system for cryopreservation there is no direct contact between the frozen object and the liquid nitrogen. In this case, the embryos are placed on a single carrier, after which they are stored in an additional container, which is sealed. In this way, the embryos are not in direct contact with the liquid nitrogen. The use of a closed vitrification system ensures greater security of frozen embryos, avoiding the risk of infection of the cells by liquid nitrogen or other cells. The use of an external conteiner, which is sealed, prolongs the freezing time, which could lead to a reduction in the survival of embryos after thawing. Examined were 311 embryos for analyzing the impact of vitrification system on embryo survival. Of these, 158 were vitrified in open and 153 embryos in closed system.

When calculating the survival (Table 11), it was without difference depending on the vitrification system used. In open, the survived embryos were 136 (86.1%), and in closed - 132, which represents 86.3% survival ($p = 1$). No difference was also reported for intact embryos ($p = 1$).

Table 11. Survival of embryos after application of open and closed vitrification system

Type of system	Number of thawed embryos	Overall survival number and %	Intact embryos number and %	Survival over 50% of cells number and %
Open system	158	136 (86,1%)	76 (48,1%)	41 (25,9%)
Closed system	153	132 (86,3%)	80 (52,3%)	39 (25,5%)

Performing artificial collapse (AC) of blastocysts before vitrification

To improve the survival of vitrification of blastocysts, an additional method could be applied - artificial collapse (AC), to remove the fluid from the blastocoel and shrink the blastocyst in order to avoid the formation of crystals (Fig. 8).

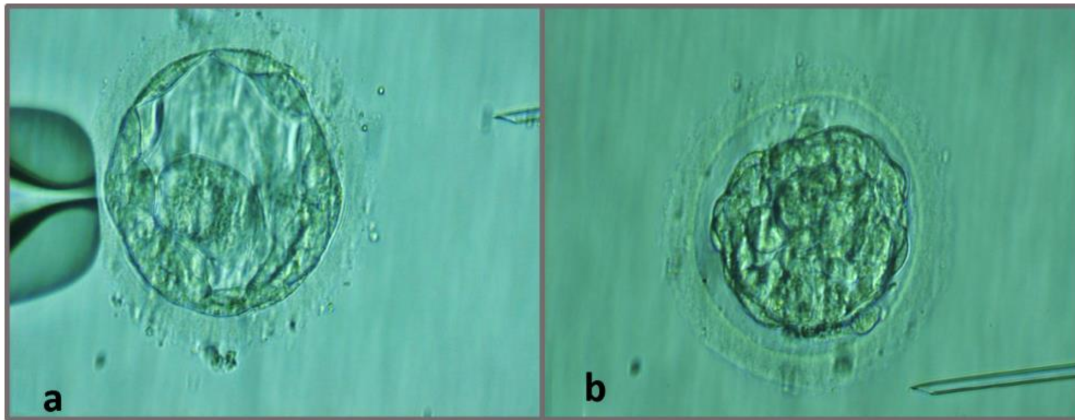


Figure 8. Blastocyst before and after artificial collapse. On panel a) there is a blastocyst with a blastocele formed before the AC. A collapsed blastocyst (b) was observed after using an injection pipette for AC. Magnification 200 x, Hoffman modulation contrast

Were analized 937 blastocysts. Of them, 452 were without use of AC, while 485 were vitrified after performing AC. The results in Table 12 show a statistical increase in overall survival after AC- 85.2% versus 78.1% without prior blastocyst preparation (P = 0.005).

Table 12. Assessment of vitrification of vitrified blastocysts after thawing depending on the application of artificial collapse before vitrification

Method	Number of blastocysts thawed	Overall survival number	Overall survival %	P value
Without AC	452	353	78,10%	P=0,005
With AC	485	413	85,20%	

Acieved clinical pregnancies (CP) after application of cryopreservation method vitrification and subsequent frozen-thawed embryo transfer (FET).

Successful freezing and good embryo survival after thawing are important for subsequent frozen-thawed embryo transfer (FET). It involves placing cryopreserved and thawed embryos in the patient's uterine cavity.

The FET cycle includes ultrasound examination and hormonal monitoring of the development of the endometrium of a patient, performed by an obstetrician-gynecologist

with subsequent thawing of the embryos and FET on the appropriate day of the menstrual cycle. The performance of FET is determined by the survival of the embryos. In cases when all thawed embryos have not survived, or are from group IV (0% survival), despite prior preparation, FET is not performed. This was defined as failure to perform FET or cancelation of FET (cFET). These are canceled FET cycles.

A total of 941 FET cycles were analyzed in the present dissertation, in 844 patients with age from 22 to 46 years. Some patients underwent two or three FETs due to a higher number of frozen embryos. A very important indicator in the cryopreservation of embryos and subsequent FET is the achieved clinical pregnany rate (CPR). Another important marker is the percentage of cases where FET is not performed due to poor embryo survival (cFET). We detected cFET in 105 cycles (11.2%). In the remaining 836 cycles, 299 CPs (35.8%) were achieved. In this way CPR compared to performed FET was defined. Table 13 describes the studied parameters and the number of FETs analyzed.

Table 13. Analyzed parameters when performing FET

Analyzed parameter	Number of FET
Quality of embryos before freezing	372
Cases of FET with frozen embryos with top quality	155
Patient's age	675
Method of fertilization of oocytes	372
Day of embryo development on which the vitrification was performed	610
Vitrification on day five before and after modification of the method	126
Type of infertility - primary or secondary	372
Storage period of embryos	610
Number of frozen embryos	372
Application of co-culture of embryos with autologous endometrial cells before vitrification	22
Use of open and closed vitrification system	87

Use of three different vitrification and thawing media	238
Pregnancies depending on the number of transferred embryos	605
Survival of transferred embryos	610
Application of assisted hatching of embryos after thawing before FET	229
Performing an artificial collapse of blastocysts before vitrification	251

Embryo quality before freezing

A comparison of acieved pregnancies was made depending on the quality of embryos for the corresponding day. 372 FETs were analyzed depending on the quality of the embryos before vitrification (Table 14).

Table 14. CP and cFET in relation to the quality of embryos before freezing

Embryo quality	**Number FET alalized**	**Number FET performed**	**Clinical Pregnancies CP number and %**	**P value**	**cFET number and %**	**P value**
Top	155	141	28 (19,9%)	P=0,8 */**	14 (9,0%)	P=0,06 */**
Good	164	138	29 (21,0%)	P=0,02 */***	26 (15,9%)	P<0,0001 */***
Poor	53	33	1 (3,0%)	P=0,02 **/***	20 (37,7%)	P=0,0008 **/***

* / ** comparison of cFET and CP in FET of embryos with top and good quality

* / *** comparison of cFET and CP in FET of embryos with top and poor quality

** / *** comparison of cFET and CP in FET of embryos of good and poor quality

A similar percentage of pregnancies was observed in FET of top or good quality embryos before cryopreservation (P = 0.8). There was no statistically significant difference (P = 0.06) in cFET when comparing embryos with initial top or good quality. Freezing of poor quality embryos statistically increases the incidence of cFET and decreases in CPR. Cancelation rate of FET reaches 37.7% in vitrified embryos with poor initial quality.

The patient's age

In the analyzis of 675 FETs based on the age of the patients, two groups were formed: Group 1, containing FETs in patients ≤ 35 years of age, and Group 2, patients ≥ 36 years of age during cryopreservation (Table 15).

Table 15. CP and cFET according to the age of the patient during the freezing of the embryos

Age	Number FET alalized	Number FET performed	Clinical Pregnancies CP number and %	P value	cFET number and %	P value
Group 1	518	456	144 (31,6 %)	P=0,04	62 (12 %)	P=0,5
Group 2	157	135	30 (22,2 %)		22 (14 %)	

The obtained results show a statistically higher CPR (P = 0.04) in the group of younger patients, without any difference in canceled FET.

Method of fertilization of the oocytes

For the genetation of embryos, which were later cryopreserved, both methods were used - classical IVF and ICSI, as well as a combination of both methods in the same patient (IVF / ICSI). Table 16 presents the results in terms of cFET and the acieved CP in the analyzed 372 cycles.

Table 16. Method for fertilization of the oocytes - CP and cFET

Fertilization method	Number FET alalized	Number FET performed	Clinical Pregnancies CP number	Clinical Pregnancies CP %	cFET number	cFET %
IVF	139	123	23	18,7%	16	11,5 %
ICSI	163	129	19	14,7 %	34	20,9 %
IVF/ICSI	70	60	16	26,7%	10	14,3%

We reported the highest pregnancy rates when using the combination of the two methods (IVF / ICSI). There was no difference when comparing IVF with ICSI (P = 0.4), IVF with IVF / ICSI (P = 0.2), and between ICSI and IVF / ICSI (P = 0.05).

The day of embryo development on which the vitrification was performed

To assess the effect of the day on the development of the embryos at which they were vitrified, 610 FETs with embryos frozen from day two to day six including were examined.

The obtained results (Table 17) showed no statistically significant difference in acieved CPR when comparing vitrification of embryos on day 2 with 3 (P = 0.9) and day 2 with 4 (P = 0.6). Freezing of embryos on day five resulted in a statistical increase in CPR compared to embryos frozen on day three (P = 0.01) and day four (P = 0.002). No clinical pregnancies occurred on day six.

Table 17. CP and cFET depending on the day of in vitro embryo culture on which vitrification was performed

Day of vitirification	Number FET alalized	Number FET performed	Clinical Pregnancies CP number	Clinical Pregnancies CP %	cFET number	cFET %
Day 2	6	6	1	16,7 %	0	0 %
Day 3	248	214	42	19,6 %	34	13,7 %
Day 4	71	55	5	9,1 %	16	22,5 %
Day 5	274	246	73	29,7 %	28	10,2 %
Day 6	11	7	0	0 %	4	36,4 %

Modification of the vitrification method on day five

Modification of the blastocyst vitrification method by increasing the concentration of human serum albumin resulted in a statistically significant increase in blastocyst survival. For this reason, the results in terms of cFET and CPR were analyzed. The data from the studied 126 FET are presented in Table 18.

Table 18. CP and cFET depending on the modification of method for vitrification of blastocysts

Method	Number FET alalized	Number FET performed	Clinical Pregnancies CP number	Clinical Pregnancies CP %	cFET number	cFET %
Before modification (5% HSA)	54	47	8	19,1%	7	13,0%
After modification (10% HSA)	72	65	24	36,9%	7	9,7%

There was a statistically significant increase (P = 0.04) in the percentage of acieved pregnancies after the modification of method for vitrification of blastocysts.

Type of infertility - primary or secondary

When comparing cFET and the percentage of CP depending on the type of infertility, 372 FETs were considered (Table 19). No statistically significant difference was found, but a lower percentage of cFET and a higher percentage of pregnancies were observed in cases of secondary infertility.

Table 19. Type of infertility - CP and cFET

Type of infertility	Number FET alalized	Number FET performed	Clinical Pregnancies CP number and (%)	P value	cFET number and (%)	P value
Primary	211	174	31 (17,8%)	P=0,8	37 (17,5%)	P=0,4
Secondary	161	138	26 (18,8%)		23 (14,3%)	

Storage period of embryos

Table 20 shows the data from 610 FETs in which the embryos were stored for 1 to 54 months before thawing and use. Two groups were formed: group 1 - used for FET embryos were stored in liquid nitrogen for up to 12 months and group 2 with longer storage - more than 12 months.

Table 20. CP and cFET depending on the storage period of frozen embryos

Storage period	Numbe FET alalized	Number FET performed	Clinical Pregnancies CP number and (%)	P value	cFET number and (%)	P value
Group 1	565	492	108 (22,0%)		73 (12,9%)	
Group 2	45	36	10 (27,8%)	P=0,4	9 (20,0%)	P=0,2

No statistically significant difference was found in the percentages of cFET and CP depending on the storage period.

The application of co-culture of embryos with autologous endometrial cells before vitrification

22 FETs with vitrified embryos co-cultured with autologous endometrial cells were analyzed. Embryos were frozen on day three (8 FETs) or day fifth (14 FETs) of their development. In the case of FET with embryos cultured with autologous endometrial cells we did not have cFET. The acieved CPs were 5 (22.7%). Pregnancies were only in the group with frozen embryos on day five. The pregnancy rate in this group was 35.7%.

The use of open and closed vitrification system

Table 21 presents the results of the analysis performed on 87 FETs depending on the vitrification system used.

Table 21. CP and cFET in relation to the vitrification system

System	Number FET alalized	Number FET performed	Clinical Pregnancies CP number	Clinical Pregnancies CP number %	cFET number	cFET (%)
Open system	44	40	15	37,5%	4	9,1%
Closed system	43	39	15	38,5%	4	9,3%

When comparing the two vitrification systems - open and closed, no difference was found in the percentages of cFET (P = 1) and acieved CP (P = 0.9).

The use of three different vitrification and thawing kits

Initially, the application of cryopreservation was performed with vitrification and thawing media prepared in our embryological laboratory. After the appearance of commercial kits, a comparison was made of the media prepared by us and two commercial kits. Thus, the efficiency of three types of vitrification and thawing media was evaluated and compared. 238 FETs were analyzed, divided into three groups depending on the vitrification and thawing kit used.

1. Group 1 - Vitrification and thawing kit 1 (Prepared in our embryological laboratory);
2. Group 2- Vitrification and thawing kit 2 (Commercial kit 1);
3. Group 3- Vitrification and thawing kit 3 (Commercial kit 2).

The obtained results are presented in Table 22. A statistically significant increase in the acieved CPs in group 3 compared to the other two groups was found.

Table 22. CP and cFET depending on the freezing and thawing kits

KIT	Numbe FET alalized	Number FET performed	Clinical Pregnancies CP number and (%)	P value	cFET number and (%)
Group 1	75	63	12 (19,0%)	м/у 1 и 2 група- p=0.3	12 (16,0%)
Group 2	101	94	24 (25,5%)	м/у 2 и 3 група p=0.01	7 (6,9%)
Group 3	62	59	27 (45,8%)	м/у 1 и 3 група p=0.002	3 (4,8%)

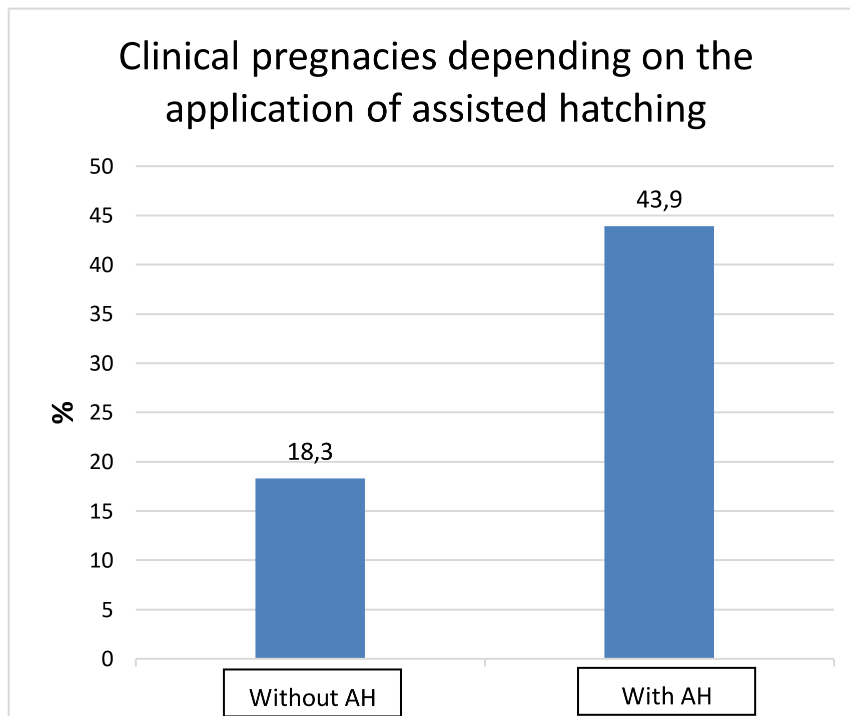
Application of assisted hatching (AH) of embryos after thawing before FET

The effect of application of laser assisted hatching (LAH) to the embryos after thawing and before FET on acieved pregnancies was examined (Fig. 9).

Figure 9. Embryos after laser assisted hatching (LAH). Three-day-old embryo (a) and blastocyst (b). The blue arrow indicates the location of the hole after LAH. Magnification 200 x, Hoffman modulation contrast

FETs analyzed were 229. In 114 FETs, LAH was performed and 50 CP were achieved, while 115 FETs were without LAH, with 21 CP achieved. LAH usage leads to a statistically significant higher pregnancy rate (P <0.0001). Acieved pregnancies are presented in Figure 10.

Figure 10



Embryos were vitrified on day three and day five of their development. It was checked whether there is a difference in terms of acieved CP after LAH depending on the day of vitrification of the embryos. Four groups were formed depending on the day of freezing of the embryos and the administration of LAH (Table 23).

Table 23. CP depending on the day of in vitro development of the embryos on which they were frozen and the application of AHL

Day of vitrification	Number FET	Clinical Pregnancies CP number	Clinical Pregnancies CP (%)
Day 3 without LAH	68	9	13,2%
Day 3 with LAH	49	13	26,5%
Day 5 without LAH	47	12	25,5%
Day 5 with LAH	65	37	56,9%

In vitrified embryos on day three (between Group I and II) no statistically significant difference was found (P = 0.07) after LAH, while in vitrified embryos on day five (Groups III and IV), in those with LAH, CPR was significantly higher (P = 0.001) compared to the group without LAH. There was also a statistically significant increase in CPR (P = 0.001) in FETs with LAH on day five compared to FETs with LAH on day three (Group II and Group IV).

Performing artificial collapse (AC) of blastocysts before vitrification

Analyzed FETs with blastocyst were 251. In 129 of them blastocysts were vitrified without AC, while 122 FETs were frozen after AC. The results are presented in Table 24.

Table 24. CP and cFET according to aplication of artificial collapse before blastocyst vitrification

Method	Numbe FET alalized	Number FET performed	Clinical Pregnancies CP number and (%)	cFET number and (%)
Without artificial collapse (AC)	129	114	30 (26,3%)	15 (11,6%)
With artificial collapse (AC)	122	119	49 (41,2%)	3 (2,5%)

The table shows the achievement of a statistically significantly higher CPR when performing AC before freezing of blastocysts (P = 0.02), as well as a statistically lower percentage (P = 0.005) cFET.

A summary of the effect of various factors on embryo survival and acieved clinical pregnancies (CP) is presented in Table 25.

Table 25. Factors and their influence on embryo survival after thawing and acieved clinical pregnancies

Factor	**Survival**	**Clinical pregnancies**
Quality of embryos before freezing	+	+
Patient's age	-	+
Method of fertilization of oocytes IVF or ICSI	-	-
Day of embryo development on which the vitrification was performed	-	+
Modification of the method for freezing embryos on day five	+	+
The type of infertility - primary or secondary	-	-
Storage period of embryos	-	-
Application of open and closed vitrification system	-	-
Performing an artificial collapse before vitrification	+	+
Application of co-culture of embryos with autologous endometrial cells before vitrification	-	-
Use of different vitrification and thawing media	NA	+
Number of frozen embryos	NA	-
Number of embryos transferred	NA	+
Survival of transferred embryos	NA	+
Application of assisted hatching	NA	+

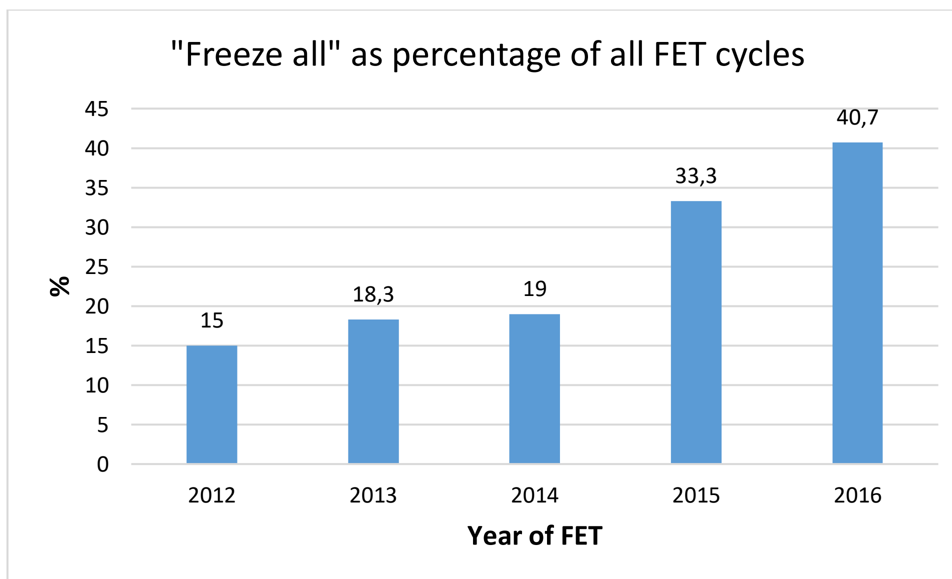
NA- not analyzed

To analyze the survival of embryos and developing pregnancies in "freeze all" program or strategy (cases when no fresh embryo transfer is performed and all developing embryos are frozen and used in a subsequent FET).

This program represents the cases in which after ART procedure no fresh ET is performed, and all obtained embryos with good quality are frozen and used for FET in the following menstrual cycle in the patient, after preliminary hormonal preparation of the endometrium.

For a five-year period (2012-2016) we found that "freeze all" cases were 28.9% of all FETs or 202 FETs of 698. Figure 11 shows the tendency of increasing the "freeze all" over the years. Starting from 15% and reaching 40.7% of all FETs for the corresponding year.

Figure 11



FETs from "freeze all" program, for a four-year period (2013-2016) were studied in detail. They were 189 with 499 embryos thawed. Of all FETs 49 were with embryos frozen on day three and 140 with embryos frozen on day five. The age of the patients was from 24 to 46 years, with a mean age of 33 years. The mean number of embryos transferred was 2.3 (from 1 to 4). There were 9 cases of FETs with 4 embryos, leading to a singleton pregnancy. LAH was performed on all embryos before FET. In 5 cases (2.6%) cFET was registered and acieved CPs were 92 (50%). Two groups were divided according to the age of the woman: Group 1 (women ≤ 35 years) - 132 FETs and Group 2 (women ≥ 36 years) - 57 FETs (Table 26). Each group was

divided into two subgroups depending on the day of development of the embryos on which they were vitrified.

Table 26

	Number FET alalized	**Number FET performed**	**Clinical Pregnancies CP number and (%)**	**cFET number and (%)**
	189	**184**	**92 (50%)**	**5 (2,6%)**
Group 1	**132**	**131**	**70 (53,4%)**	**1 (0,8%)**
Day 5	107	106	63 (59.4%)	1 (0,9%)
Day 3	25	25	7 (28%)	0 (0%)
Group 2	**57**	**53**	**22 (41,5%)**	**4 (7%)**
Day 5	33	31	16 (51,6%)	2 (6,1%)
Day 3	24	22	6 (27,3%)	2 (8,3%)

A statistically significant difference was found in cFET (P = 0.02), and in the group of older women, it was higher (7%) compared to younger - 0.8. No statistical difference was reported in the percentages of CPs in the two groups. In group 1 were achieved 53.4%, and in group 2 they were 41.5% (P = 0.1). There was a statistically significant increase in CPR in FETs with blastocysts compared to day 3 embryos in younger women (Group 1): 59.4% (day 5) and 28% (day 3) (P = 0.005). In Group 2 (older women) the results were: 51.6% (fifth day) and 27.3% (third day), which did not reach a statistically significant difference (P = 0.08), despite a much higher percentage of pregnancies with vitrification of blastocysts (day five).

Assessment of survival, subsequent fertilization and development of human oocytes using vitrification.

64 vitrified and thawed mature (metaphase II, MII) oocytes were analyzed in 14 women. Figure 12 shows the different survival of oocytes after thawing.

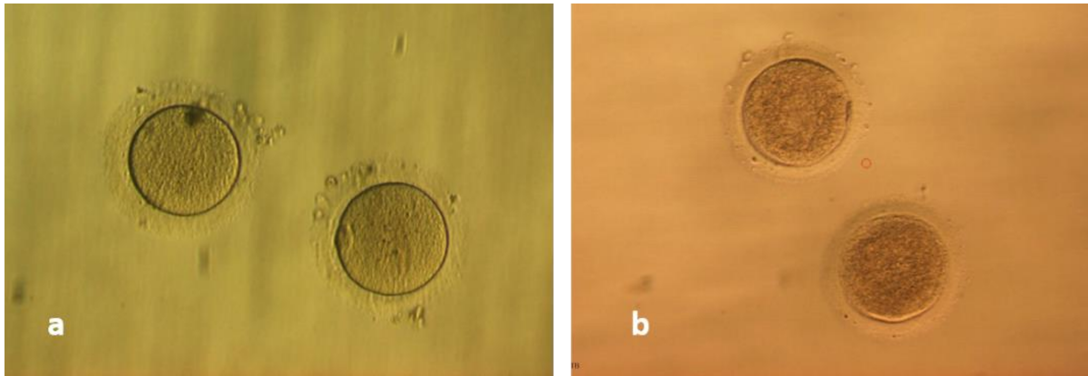


Figure 12. Mature oocytes after thawing. Panel a presents oocytes that have successfully survived their thawing, evaluated on the basis of their morphology. Panel b have not survived the procedure oocytes. Magnification 100 x, Hoffman modulation contrast

After thawing, oocyte survival was 78.1%. After applying ICSI method to the survived oocytes, 72% were correctly fertilized with the presence of two pronuclei 18-20 hours later (Fig. 13).

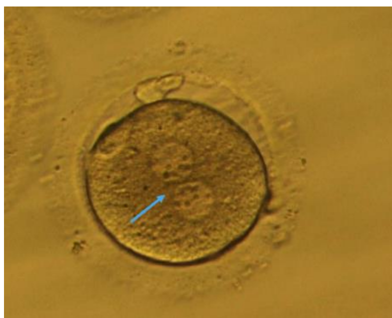


Figure 13. Fertilization. Fertilized with two pronuclei oocyte (2PN). The blue arrow indicates the two pronuclei. Magnification 200 x, Hoffman modulation contrast

From the fertilized oocytes were obtained 77.8% developing embryos, of which 14.3% were with top, 25% with good and 16.7% with poor quality for the corresponding day of development. Vitrified and thawed oocytes were divided into two groups (autologous or own and donor) according to the women from whom they were obtained.

Own oocytes were obtained after hormonal stimulation from patients who would like to presereve them for autologous use after a period of time (in order to maintain fertility) or when it was impossible to obtain sperm on the day of follicle puncture.

The donor oocytes were from young women, again after hormonal stimulation, who donated them for use by recipients. These are women who do not genetare their own oocytes or, for various reasons, do not have to use them. The results are presented in Table 27.

Table 27. Survival and embryos obtained after thawing of own and donor oocytes

Origin of oocytes	Number of warmed oocytes	Number of survived oocytes	Number of fertilized oocytes	Number of developing embryos	Number of embryos with top quality	Number of embryos with good quality	Number of embryos with poor quality
Autologous	31	20	14	10	2	1	7
Donor	33	30	22	18	2	6	10

In terms of survival it was found to be statistically significantly better in donor (90.9%) compared to own oocytes (64.5%) (P = 0.01). For other parameters, such as fertilization after ICSI procedure, development, and quality of embryos, there was no statistically significant difference between the group with donor versus the group with autologous oocytes.

DISCUSSION

In the literature there are studies of various clinical and embryological factors with a possible effect on the outcome of FET. Most of these studies are with embryos frozen by programmed (slow) freezing. A significant part of the studies on the influence of these factors includes embryos frozen at the zygote stage (day one), day two and day three of their in vitro development.

Various studies and meta-analyzes have shown that vitrification is the more successful method for cryopreservation of human cleavage stage embryos and blastocysts compared to slow freezing (4-9).

These two facts determined our interest in examining the influence of important embryological and clinical factors on survival of human preimplantation embryos and acieved CPs in vitrified embryos, frozen on different days (from the second to the sixth day including) of their in vitro development.

Most studies examine the influence of various factors on the outcome of FET, namely acieved pregnancies, so we decided to examine the impact of certain parameters on the survival of vitrified embryos, because we believe that this is the basis for performing FET, which then has directly reflected in the percentage of achieved CP. These data are separated in the first two sections, one examining the survival of embryos and the second acieved CP.

Embryo survival is crucial after thawing. Therefore, we examined which clinical and embryological factors influence it. Our results show a statistically significant effect on the

survival of embryos after thawing of the following factors: quality before freezing, modification of the method of vitrification of blastocysts, and the application of artificial collapse (AC) before vitrification of blastocysts.

Our data showed that the freezing of embryos with top (group 1) and good (group 2) for the respective day quality, leads to a statistically significant higher survival after thawing compared to those with poor quality. Survival was 83.4% and 84.7%, respectively, compared to 76.5% of poor quality embryos (group 3). No statistically significant difference was found between group 1 and group 2 (P = 0.5). Between group 1 and group 3, and group 2 and group 3 was P = 0.03, and P = 0.02, respectively. These results are in accordance with the results of Salumets et al. (1), as well as Veleva et al. Due to the lack of difference in the results between embryos with top and good quality, we started to apply vitrification in the cases of embryos from both groups. In the presence of even one embryo with top quality and others with good quality it is appropriate to perform cryopreservation. We stopped freezing embryos with poor initial quality for the respective day of in vitro development.

Due to the greater sensitivity of blastocysts and the protective capacity of human serum albumin, we decided to test whether increasing its concentration in vitrification and thawing solutions would lead to higher blastocyst survival. When the human serum albumin content doubled from 5% to 10%, we achieved 88% survival, which turned out to be statistically significant (P = 0.005). After obtaining these results, we began routine administration of higher concentrations of human serum albumin in cryopreservation solutions.

The application of artificial collapse (AC) to remove fluid from the blastocoel before vitrification of blastocysts is not a commonly used technique. Some authors (10) did not find an improvement in embryo survival and pregnancies, while others had the opposite opinion (11–15). Our data support the performance of AC in full, expanded and hatching blastocysts, because they show a statistically significant increase in the survival rate when applying AC before freezing (85.20%) compared to 78.10% in cases without AC (P=0,005). Based on the literature and our data, we apply AC before vitrification of blastocysts.

The studied parameters: age of the patient, method of fertilization of oocytes: IVF or ICSI, type of infertility (primary or secondary), day of embryo development on which vitrification was performed, as well as storage period of embryos showed no statistically significant difference in embryo survival.

Co-culture of embryos with autologous endometrial cells is a method used in patients with several failed atemptes or with development of poor quality embryos in a previous ART cycle. The aim of the method is to generate embryos of better quality and above all to increase

the rates of pregnancies and live births. There are publications in the literature on the positive influence on the quality of embryos and acieved pregnancies of co-culture of embryos with autologous endometrial cells (16), according to our data there is not enough research on their influence on vitrified and thawed embryos. Therefore, we analyzed the survival of these embryos. Although co-culture with autologous endometrial cells is used in complicated cases of ART, vitrification and thawing of the resulting embryos showed good results comparable to those of non-co-cultured embryos, namely 87.5% and 85.3% overall survival and 43.8% and 40.6% intact embryos, respectively.

The second section of the present study concerns the influence of the considered factors on the percentage of acieved clinical pregnancies (CP). Only developing CPs, with detectable heartbeat using ultrasound examination were analyzed, described and examined. Biochemical and ectopic pregnancies, as well as very early miscarriages, are not included. In this section, canceled cycles due to poor survival of all embryos (cFET) are also calculated. The lower the percentage of cFET and the higher the percentage of CP, the more secure the cryopreservation program is.

The statistically significant parameters regarding the acieved CP are: the quality of the embryos before freezing, the day of embryo development on which the vitrification was performed, the modification of the blastocyst vitrification method, the patient's age, the application of assisted hatching and artificial collapse, the number of transferred embryos, the survival of the transferred embryos, and the use of certain vitrification and thawing media.

The quality of the embryos before freezing affects both the survival after thawing and the acieved CPs. Embryos with top and good quality before vitrification lead to a significantly higher CPR compared to embryos with poor quality ($P = 0.02$ for both groups). These results again suggest that poor quality embryos should not be cryopreserved. The fact that the acieved CPs are similar in embryos with top and good quality for the corresponding day of in vitro development ($P = 0.8$) makes the group of embryos with good, and not only with top quality, suitable for cryopreservation. These data confirm the results of other authors (17).

Despite the lack of difference in survival, we found a statistically significant difference in CPR depending on the day on which the vitrification was performed. Vitrification of blastocysts resulted in a higher pregnancy rate compared to embryos on days 3 and 4 ($P = 0.02$ and $P = 0.002$, respectively). Based on this and previous results, as well as data that blastocyst transfer does not adversely affect the obstetric result (18), we decided to freeze the embryos on day 5 (blastocysts). Our data showing no pregnancies in FETs with day sixth embryos as well

as those of other authors (19,20) led to termination of culture of embryos until day six and their vitrification.

When modifying the method for freezing and thawing of blastocysts, by increasing the concentration of HSA, statistically significant higher survival also led to a significantly significant higher pregnancy rate (P = 0.04). For this reason, we used these solutions until we started applying vitrification and thawing commercial kit, leading to a higher CPR.

The influence of the patient's age on the ART result has been proven. We aimed to examine the effect of a woman's age on the FET result. When comparing the number of FET performed, they are much higher in the group of women ≤ 35 years. Although there is no difference in embryo survival and cFET (P = 0.5) depending on the age of the patients a statistically significant difference (P=0,04) was observed in CPR (36.1% vs. 22.2%) in favor of younger patients (≤ up to 35 years).

The most commonly used method for assisted hatching (AH) is laser pulsation, or laser assisted hatching (LAH). Although most studies have found an increase in pregnancy rates after AH, it is not routinely administered. Most studies concerned AH in frozen embryos on day two (21) or day three (22,23). We decided to test the effect of AH on vitrified and thawed day 3 cleavage stage embryos and blastocysts. Our results show a significantly higher percentage of pregnant women undergoing LAH with blastocysts after thawing before FET (25.5% vs. 56.9%, with P = 0.001). Based on these and similar results from other authors (24,25), we believe that FET is an indication for routine application of laser assisted hatching.

Our data support the implementation of artificial collapse (AC), showing a statistically significant increase in the survival rate of blastocysts when applying AC before freezing (P = 0.007), as well as an increase in the acieved CP: with AC 41.2% compared to 26, 3% without AC, P = 0.005. These results, as well as those of other authors (11–13,15), led to its routine use in all full, expanded and hatching blastocysts before cryopreservation.

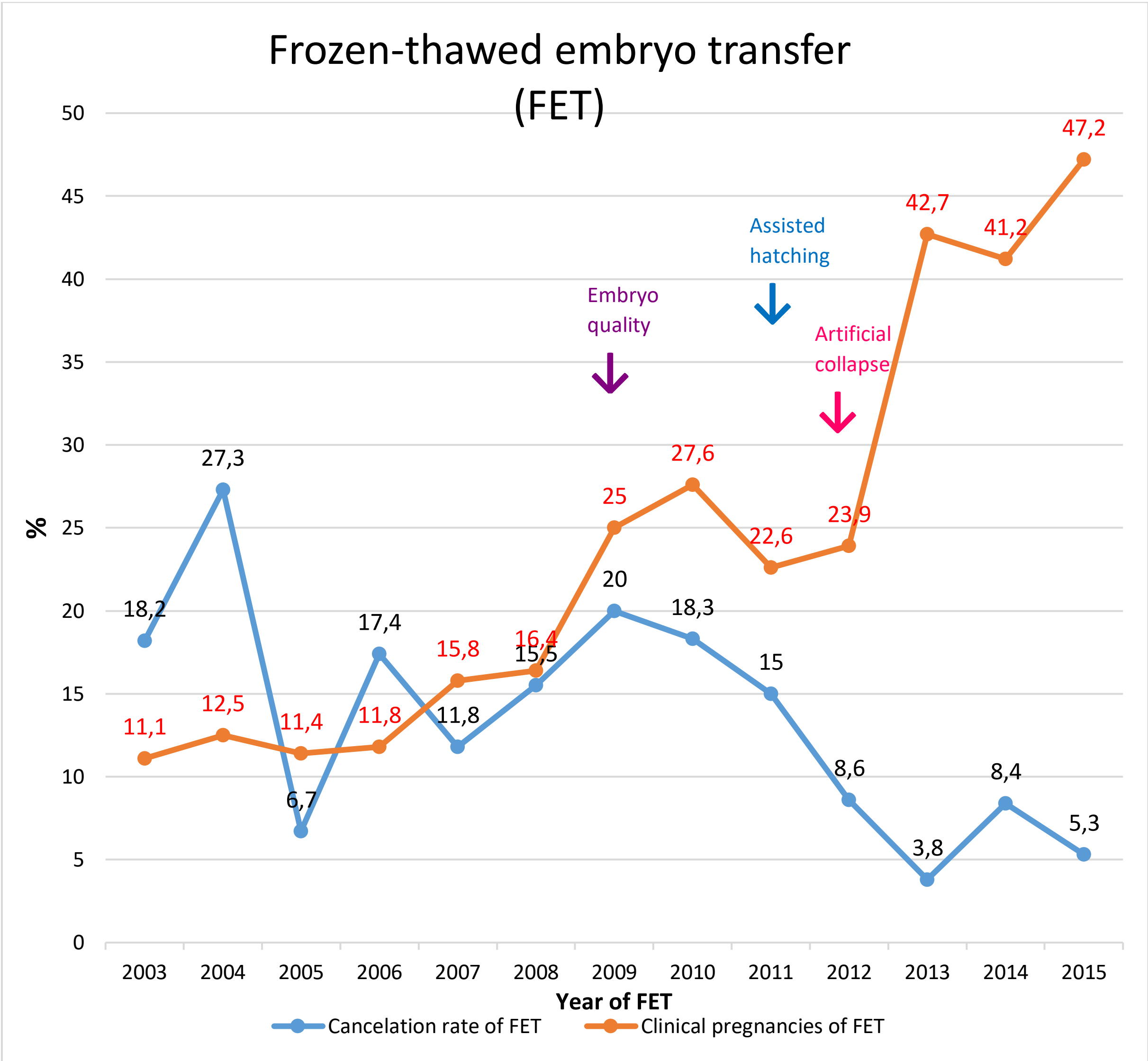
Parameters that do not affect the acieved CPs in FET are: the method of fertilization of oocytes (IVF or ICSI), type of infertility (primary or secondary), the storage period of embryos, the number of cryopreserved embryos and the vitrification system (open and closed).

After analysis FETs with embryos co-cultured with autologous endometrial cells, CPs were found only in cases of FETs with vitrified embryos on day five (blastocysts). Due to the lack of acieved pregnancies from co-cultured embryos on day three, culture is applied until day five and then in the presence of blastocysts of top or good quality they are vitrified.

We analyzed the use of vitrification in preimplantation embryos for a 13-year period from 2003 to 2015. The results of 941 FETs were compared. The data are presented in Figure

14. We found a statistically significant decrease in the percentage of cFET (canceled FET due to lack of survival of thawed embryos) from 18.2% to 5.3% (P = 0.0003) and an increase in acieved CP from 11, 1% to 47.2% (P = 0.03) from 2003 to 2015.

Figure 14. Application of different methods with influence on the FET result



Based on the obtained good results, various parameters were introduced into the routine practice, leading to decrease in cFET and increase in CPs.

A good cryopreservation program and successful application of FET reduces the inconvenience and costs of patients and leads to acieved pregnancies comparable to, or sometimes higher, than fresh ET. The data obtained in these two sections show very good embryo survival and CPR. They support various articles that provide safety data for the

application of cryopreservation of embryos by vitrification, and the results do not differ or are better than those of fresh ET (26-29).

The next part of our results is about the growing popularity of the "freeze all" program or strategy. It is when fresh ET is not performed and all developing embryos of good quality are cryopreserved. In order this program to be used by a particular laboratory, it must have a well-functioning freezing program to ensure good survival of all embryos and no risk of losing a developing embryo. Initially, this strategy was used in women with ovarian hyperstimulation syndrome, presence of a polyp, or atypical uterine bleeding. Now is also applicable for normal responders to stimulation, in whom a large number of oocytes are expected, or in premature luteinisation, which is elevated serum progesterone levels (P> 1.5 ng / ml) during hormonal stimulation (30-32). Most authors get very good pregnancy rates when using the "freeze all" program, and some even reach a higher percentage of pregnancies when compared to fresh ET (30,33).

The cases with "freeze all" for a five-year period (2012-2016) were analyzed. We found a tendency to increase the application of this method. In 2012, 15% of all FETs were freeze all program, while in 2016 they were 40.7%.

Due to the increasing age of patients undergoing ART and the search for successful treatment, with the achievement of pregnancy and childbirth in them, we decided to check whether the age of woman affects the outcome in "freeze all" program. When dividing women into two age groups, no influence of women's age on the CPR was found. We also found that women of advanced reproductive age (≥ 36 years) could participate in this program without compromising their embryos and reducing the likelihood of pregnancy. We checked whether there is a difference in vitrification for "freeze all" of embryos on day three and day five.
There was a statistically significant increase in the CPR in FET of blastocysts compared to day 3 embryos in younger women: 59.4% (day 5) and 28% (day 3) (P = 0.005). In the group of older women the results were: 51.6% (fifth day) and 22.7% (third day), which did not reach a statistically significant difference, despite the much higher CPR in vitrification of blastocysts (P = 0.08).
The last part of this dissertation deals with the vitrification, thawing and fertilization of oocytes, as well as the embryos obtained from them. The vitrified, unfertilized human oocytes studied by us show a satisfactory and comparable to most authors survival rate and fertilization, but an unadequate percentage of developing embryos of good quality. When dividing the groups, it became clear that when using donor oocytes we get a statistically significantly higher survival rate. This result could be explained by the higher average age (36.3 years) of the studied patients

who freeze their own oocytes, compared to the donors of oocytes, which according to the Health Low and Regulation 28, regulating the Assisted Reproduction, must be women. up to 34 years and have at least one live birth. Cryopreservation of oocytes is recommended to be performed till 35 years and with age the required number of frozen oocytes increases to ensure a greater probability of a successful outcome.

The data in this section show the need for more oocytes to be amalyzed. It would be effective to check the studied parameters in different age groups of patients, as well as the the acieved CPs. The difficult accumulation of data in this area is due to the small number of women who cryopreserve there oocytes and delayed use of them.

CONCLUSION

In the present study, various clinical and embryological factors were examined to determine their influence on embryo survival during thawing and on the CPR acieved. Taking them into account during freezing and subsequent FET would lead to a more complete application of cryopreservation, increase the CPR, and hence to increase the percentage of live births, which is the goal of assisted reproduction. With our results, we confirm that the vitrification of embryos and oocytes is an effective and safe method and prove that the usage of additional techniques - LAH, AC, and cryopreservation only of embryos with top or good quality, leads to better outcome.

The implementation of the "freeze all" program provides an opportunity, in certain groups of patients, to delay the embryo transfer, which will increase the chances of pregnancy and childbirth. Of great importance is the successful implementation of this program in women with advanced reproductive age. The number of patients in the mentioned group is constantly increasing due to delay of pregnancy and motherhood. This group of women is a challenge to realize pregnancy and live birth, therefore all methods helping to achieve this result are extremely important.

Future follow-up of children born after vitrification and their development would provide additional information on the safety of the method.

Regarding oocyte freezing, our results are good in terms of survival, fertilization and development of embryos. They could be optimized by analyzing a larger number of oocytes, taking into account the acieved pregnancies and children born. Cryopreservation of female gametes could be applied more widely, and it is recommended to be performed in younger women, due to the already proven effectiveness, but always after fully informing patients about the real possibilities and results.

Inferences

1. The analyzed factors that influence the survival of embryos after thawing and acieved CPs after FET are: the quality of embryos on the day of in vitro development on which vitrification is performed, the increase in the concentration of human serum albumin in freezing and thawing solutions in cryopreservation of blastocysts, as well as performing artificial collapse before vitrification.
2. The survival of embryos after thawing and the acieved CP after FET are not affected by: the method of fertilization of the oocytes, the type of infertility, long-term storage in liquid nitrogen, as well as the application of open or closed vitrification system.
3. The performance of laser assisted hatching (LAH) of the embryos after thawing leads to significant increase in the acieved CP when blastocysts were vitrified.
4. Despite the lack of difference in the survival of embryos depending on the age of the patient, such is reported in acieved CPR. In the cases of cryopreservation of the embryos remaining after fresh ET, the acieved CP after FET are statistically significantly higher in the patients ≤ 35 years, compared to those ≥ 36 years.
5. The survival of embryos after thawing, as well as the number of embryos transferred in FET, affect the acieved pregnancies. Clinical pregnancy is not achieved in FET when embryos with less than 50% of the cells survived were transferred.
6. In the implementation of the program "freeze all" the age of the patients does not have a negative effect on the acieved CP. Vitrification of blastocysts leads to a statistically significant higher CPR compared to embryos on day three, in patients ≤ 35 years.
7. After establishing the factors with a positive effect on the cryopreservation of embryos and their routine application in practice, there is a statistically significant increase in the acieved CP (from 11.1% in 2003 year to 47.2% in 2015 year).
8. Vitrification of donor oocytes compared to own oocytes leads to significantly higher survival rate after thawing.

Contributions to the current knowledge

1. For the first time in Bulgaria a detailed analysis of the vitrification method has been made, determining the factors influencing the survival of embryos after thawing and the acieved clinical pregnancies in frozen-thawed embryo transfer.

2. The analysis of the obtained data led to the optimization of the method with a significant increase in clinical pregnancies after introduction in the clinical practice of:

   - modified method for cryopreservation of blastocysts

   - freezing of embryos only with top or good quality for the corresponding day

   - cancel of frozen-thawed embryo transfer in the presence, after thawing, only of embryos with survived less than 50% of cells

   - routine application of artificial collapse before cryopreservation of blastocysts and laser assisted hatching after thawing of embryos before frozen-thawed embryo transfer.

3. It has been shown that in freeze all strategy, cryopreservation of blastocysts leads to increased clinical pregnancies rate.

Articles related to the topic of the Phd thesis

1. Lyuboslava D. **Valkova**, Petya M. Andreeva, Tanya V. Milachich, Boryana R. Bandreva, Petya V. Penkova, Ivan M. Bochev, Boryana D.Petkova,Tanya N. Timeva Atanas D. Shterev, How many vitrified human autologous or donor oocytes are needed for one live birth, Problems of Cryobiology and Cryomedicine, *in press*, ISSN 23076143

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