Early miscarriage after single and double blastocyst transfer – An analysis of 1020 blastocyst transfers

Zgodnji spontan splav po prenosu ene ali dveh blastocist – Analiza rezultatov 1020 prenosov blastocist

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Izvleček

Izhodišča: V dosedanjih študijah o spontani prekinitvi nosečnosti po oploditvi z biomedicinsko pomočjo (OBMP) so večinoma preučevali le cikle IVF (oploditev *in vitro*) s prenosom zarodkov drugega ali tretjega dne po punkciji foliklov. Z našo retrospektivno raziskavo smo želeli ugotoviti, kateri dejavniki napovedujejo zgodnji spontani splav po prenosu ene ali dveh blastocist.

Metode: Z multivariatno logistično regresijo smo analizirali 1020 nosečnosti, ki smo jih dosegli po prenosu ene ali dveh blastocist iz zaporednih IVF/ICSI postopkov. Kot možne napovednike SM smo ocenjevali starost zdravljenih žensk, način oploditve, število jajčnih celic, skupno število blastocist, število zamrznjenih blastocist ter število ter kakovost prenesenih blastocist.

Rezultati: Delež biokemijskih nosečnosti pri ženskah, ki so zanosile po prenosu ene ali dveh blastocist, je znašal 6,6 % (67/1020), in sicer 9,05 % (23/254) po prenosu ene blastociste ter 5,8 % (44/763) po prenosu dveh blastocist. Delež zgodnjih kliničnih spontanih splavov je bil pogostejši po prenosu ene blastociste (15,3 % (39/254)) kot po prenosu dveh blastocist (12,7 % (97/763)), v celem vzorcu pa je znašal 13,3 % (136/1020). Do zgodnje izgube nosečnosti (biokemijska nosečnosti in/ali zgodnji klinični spontani splav) je prišlo v 24,4 % (62/254) pri zanositvah po prenosu ene blastociste in v 18,4 % (141/763) pri zanositvah po prenosu dveh blastocist (delež zgodnje izgube nosečnosti v celotnem vzorcu je znašal 19,9 % (203/1020)). Ob upoštevanju različnih spremenljivk smo dokazali, da so bile spremenljivke starost ženske (OR = 1,098; 95-odstotni IZ 1,057–1,140, P < 0,001), število prenesenih blastocist (OR = 0,592; 95-odstotni IZ 0,412–0,851, P = 0,005), kakovost prenesenih blastocist (OR = 0,666; 95-odstotni IZ 0,468–0,949; P = 0,024) in število blastocist za zamrzovanje (OR = 0,912; 95-odstotni IZ 0,832–0,999; P = 0,048) statistično značilno povezani s SM.

Zaključek: Prenos blastociste optimalne kakovosti oziroma prenos dveh blastocist sta dejavnika, ki sta povezana z manjšim deležem spontanih splavov pri nosečnostih s pomočjo OBMP.

Abstract

Background: Occurrence of early miscarriage in pregnancies accomplished by *in vitro* fertilisation procedures was studied, almost exclusively, in women in whom cleavage stage embryos were transferred. The primary objective of our retrospective study was to identify the factors predicting early miscarriage following the transfer of one or two blastocysts.

Materials and Methods: Using the multivariate logistic regression model, we analyzed the parameters of 1020 sequential IVF/ICSI cycles with the transfer of one or two blastocysts, in which positive levels of βhCG were observed. Age of the patient, fertilization method, number of retrieved oocytes, number of developed, frozen and transferred blastocysts and their quality were analyzed as possible predictors for early spontaneous miscarriage.

Results: The overall frequency of biochemical pregnancies in the observed group of patients was 6.6 % (67/1020). Biochemical pregnancies occurred somewhat more frequently following the transfer of one, rather than two blastocysts (9.05 % (23/254) vs. 5.8 % (44/763)). The rate of early clinical miscarriage was 13.3 % (136/1020), with the rate of miscarriage higher following the transfer of one as opposed to two blastocysts (15.3 % (39/254) vs. 12.7(97/763)). If biochemical pregnancies and early clinical miscarriages were observed together, the rate of early pregnancy losses in the overall sample studied was 19.9 % (203/1020), following the transfer of one blasto-

cyst 24.4 % (62/254) and following the transfer of two 18.4 % (141/763). Using the multivariate logistic regression, we demonstrated that statistically significant predictors for early spontaneous miscarriages were the patient's age (OR = 1.098; 95 % CI 1.057–1.140, P < 0.001), the number of blastocysts transferred (OR = 0.592; 95 % CI 0.412–0.851, P = 0.005), the quality of blastocysts transferred (OR=0.666; 95 % CI 0.468–0.949; P = 0.024), as well as the number of blastocysts frozen (OR = 0.912; 95 % CI 0.832–0.999; P = 0.048).

Conclusion: The transfer of optimal quality blastocyst(s) and transfer of two blastocysts were associated with a reduced rate of spontaneous miscarriages in pregnancies achieved by ART.

Introduction

Early miscarriage (early spontaneous abortion) is a very common event in early pregnancy.[1] This high frequency of early miscarriage is characteristic of our species.[1-5] According to the results of several studies which evaluated frequency of miscarriage in the first trimester of pregnancy after spontaneous conception, approximatelly 30 % of total fertilized ova were lost prior to the next expected menstruation (i. e. prior to the first measurement of the level of βHCG 14 days after ovulation–the so-called premenstrual losses), another 30 % were spontaneously miscarried prior to the ultrasound confirmation of successful implantation (biochemical pregnancies), and only 10–15 % of all losses were spontaneous miscarriages recognized after the first ultrosonographic confirmation of viable intrauterine pregnancy.[1-7,51-54] Ackowledged unmodifiable risk factors for miscarriage in general population are maternal age, previous history of spontaneous miscarriages, certain anatomical, endocrine and autoimmune disorders, trombophilia and infections. Modifiable risk factors are pronounced maternal obesity, cigarette smoking, use of alcohol, recreational drugs and high coffein intake.[3,7-10] The most common cause of miscarriage after spontaneous conception is a chromosomal abnormality of the conceptus, which has been detected in approximatelly 50 % of cytogenetically examined miscarried fetuses.[11-12,24] It has been observed that this high aneuploidy rate is mainly the consequence of high incidence of meiotic errors (mostly chromosome nondisjunction) in oocytes of older mothers.[13] The precise mechanism of meiotic nondisjuction is not completely elucidated, but it has been noted that there is significantly less chiasmata in oocyte chromosomes of older women than in oocyte chromosomes of younger ones.[14,44]

Miscarriage in pregnancies achieved by assisted reproductive technology (ART) procedures is slightly more frequent than in general population and occurs in 16–30 % of patients with confirmed pregnancy.[15-20] If one considers only losses of embryos prior to the first measurement of bHCG 14 days after embryo transfer, the difference between pregnancies achieved by ART and those after spontaneous conception is even more pronounced–the rate of premenstrual losses is 50 % after embryo transfer and only 30 % after spontaneous conception.[21] One explanation for this increase of early pregnancy loss rate may be the intense follow up of ART pregnancies. Another reason of this phenomenon could be that risk factors for infertility may considerably overlap with those for miscarriage.[15] Risk factors for early miscarriage after ART are age of the mother,[15,25-26] age of the father,[27] number of previous spontaneous miscarriages,[7,15,25-26,28] pronounced obesity, cigarette smoking,[29-31] alcohol consumption,[8,32-33] use of recreational drugs[29] and high doses of caffeine.[34] Another group of risk factors for early miscarriage in ART pregnancies originates from ART itself. The characteristics of stimulated cycles, which could be risk factors for early miscarriage after ART, are method of fertilisation (IVF/ICSI),[15,40,49] protocol of stimulation[48] and the level of controlled ovarian hyperstimulation (estradiol level at oocyte retrieval, ocurrence of ovarian hyperstimu-

Table 1: Age of female patients.

Variable	SBT group	DBT group	Overall
Patient's age (mean ± SD; range)	31.2 ± 4.3 (21–41)	32.3 ± 4.3 (20–44)	32.0 ± 4.3 (22–44)

** SBT–single blastocyst transfer; DBT–double blastocyst transfer*

Table 2: Etiology of infertility in the examined cohort.

Etiology of infertility in the examined cohort	Frequency (%)
Tubal factor	36.6 % (371)
Idiopathic female infertility	20.0 % (203)
Anovulation	11.3 % (115)
Uterine factors	5.9 % (60)
Infertility due to male factor	21.3 % (216)
Other main diagnosis of infertility	4.8 % (49)
Overall	100.0 % (1020)

lation syndrome, number of developed/punctured follicules, number of retrieved oocytes). Important parameters of the developing embryos, which could be risk factors for early miscarriage after ART, are total number of developed embryos, developmental stage of transferred embryos, number of transferred embryos and their quality, number of embryos suitable for freezing and freezing/thawing of embryos.[40,45-50] As in pregnancies after spontaneous conception, the most common cause of early miscarriage in ART pregnancies is chromosomal abnormality of the embryo,[22-24] which has been detected by citogenetic methods in 55 % of miscarried fetuses.[24] The distribution of abnormalities among affected chromosomes in the studied miscarried fetuses is virtually the same in both spontaneous and assisted conception group of patients.[24]

Risk factors for early miscarriage in ART pregnancies were evaluated almost exclusively in cycles in which cleavage stage embryos had been transferred (embryo transfer on the second or third day following follicle aspiration). The primary objective of our study was to identify factors predicting early miscarriage after transfer of one or two blastocysts.

Material and methods

Patients

Retrospective clinical study encompassed 1020 stimulated IVF or ICSI cycles, performed at the Department of Reproductive Medicine and Gynecological Endocrinology at the University Medical Centre of Maribor, Slovenia. The data was gathered from January 2001 to December 2005.

Patients included were under 43 years of age and, prior to entering an IVF/ICSI treatment, underwent all tests prescribed by the protocol for clinical examination of infertile couples.

Only the patients meeting the following criteria were included in the study:

1. embryo transfer was performed on day 4 or 5 after follicle aspiration (blastocyst stage embryos);
2. only one or two fresh blastocysts were transferred (single blastocyst transfer–SBT or double blastocyst transfer–DBT);
3. serum βhCG levels exceeded 15 IU/l on the fourteenth day following the blastocyst transfer; and
4. information on the pregnancy outcome was known and documented.

From the group of pregnant patients meeting these criteria, those in whom the pregnancy ended as an extrauterine pregnancy, spontaneous abortion after week 13 or delivery were excluded from the study.

Stimulation protocols

Patients were most frequently stimulated according to the protocol involving gonadotrophin-releasing hormone agonists (GnRH-a) (86 %) (almost exclusively using the long protocol). In the remaining patients, the protocol with gonadotrophin-releasing hormone antagonists (GnRH-ant) was applied.[35] GnRH agonists used were triptorelin (Diphereline®, Ipsen Pharma Biotech, France) (48 %), gosereline (Zoladex®, Zeneca Pharmaceuticals, England) (23 %) or buserelin (Suprefact®, Sanofi Aventis, France) (15 %). Cetorelix (3 mg) (Cetrotide®, Merck Serono, Switzerland) was used as GnRH antagonist. Follicle growth was predominantly stimulated by recombinant FSH (Gonal F®, Merck Serono, Switzerland), while human menopausal gonadotrophin (HMG) (Menopur®, Ferring Pharmaceuticals, Switzerland) was used infrequently. On the day when at least two follicles reached an average diameter of 18 mm, final maturation of the oocyte was stimulated by the urinary human HCG (Profasi®, Merck Serono, Switzerland, using

Table 3: Etiology of infertility in patients' partners.

Etiology of infertility in partners of the patients	Frequency (%)
Oligozoospermia	29.9 % (304)
Azoospermia	10.2 % (104)
Normoastenozoospermia	7.3 % (74)
Oligoastenozoospermia	5.7 % (58)
Oligoastenoteratozoospermia	3.6 % (37)
Oligoteratozoospermia	1.1 % (11)
Normoastenoteratozoospermia	0.8 % (8)
Normoteratozoospermia	0.6 % (6)
Female infertility	37.3 % (379)
Other main diagnosis of infertility	3.8 % (39)
Overall	100.0 % (1020)

a dose of 10,000 IU) or human recombinant HCG (Ovitrelle®, Merck Serono, Switzerland, 250 mg dose). A detailed description of the laboratory procedures can be found elsewhere.[36] Approximately 36 hours (36 ± 1) following the administration of HCG, oocytes were recovered by ultrasound-guided trans-vaginal follicle aspiration. Fertilization was performed through IVF or ICSI. Medicult® media (MediCult, Denmark) were used for oocyte culturing. Pursuant to the protocol of our centre, only one or maximally two blastocysts were transferred on the fifth, exceptionally on the fourth day (1.8 %) following follicle aspiration. Labotec® catheter (Labotec, Germany) was used for blastocyst transfer. According to the legislation in force at the time of the study, the couple was allowed to decide on the number of embryos to be transferred. Embryos were transferred only after both partners signed the official consent form for the transfer of embryos. A day after the follicle aspiration, all patients started receiving didrogesterone (30 mg/day) (Dabroston®, Belupo, Croatia) or micronized progesterone (600 mg/day) (Utrogestan®, Laboratories Besins International, France) for luteal support.

Blastocyst quality evaluation system

The quality of transferred blastocysts was evaluated by a blastocyst classification system based on morphological criteria, developed by our Centre.[37] This classification is a modification of the blastocyst evaluation system introduced by Gardner and Schoolcraft.[38] The classification used in our laboratory takes into consideration four parameters: blastocoel expansion, inner cellular mass (ICM) form, morphology and cohesion of the trophoectoderm (TE) as well as the degree of embryo fragmentation.

B1 class blastocysts were those in which blastocoel was completely expanded, ICM was round or oval, TE was seen as cohesive epithelium and there were no excluded blastomeres or cytoplasmatic fragments. B2 class blastocysts were expanded with optimal ICM, but suboptimal TE. In B3 class were grouped morphologically optimal unexpanded blastocysts and compact morulae (embryos formed from more than 10 blastomeres tightly connected into a compact formation). Expanded blastocysts with normal TE, but suboptimal ICM (ICM smaller in size than the blastomere of a correctly divided 16-cell embryo) were arranged in the B4 class. All expanded blastocysts with suboptimal ICM and with fragments or necrotic foci in the TE were included in B5 class. Morulae and early blastocysts, which had up to 20 % blastomeres or fragments excluded in the periviteline space were classified as B6 class. B7 class comprised necrotic blastocysts (more than 50 % of embryonic material was necrotic, and abnormal ICM was present; blastocysts with normal ICM but necrotic TE were included in B2 class). Small blastocysts and morulae (more than 20 % of excluded blastomeres) and also very fragmented embryos in which some sign of blastocoel or ellipsoid TE cells were noted were included in B8 class. Non-compact morulae and fewer than 12-cell embryos on day 5 were considered as arrested embryos and had never been used for transfer.

Pregnancy confirmation

Fourteen days after the transfer of embryos, serum βhCG levels were determined in all patients. Pregnancy was considered confirmed if the level exceeded 15 UI/l. Total βhCG levels were determined by Architect i2000™ (Abbott diagnostics, USA) analyzer,

Table 4: Pregnancy outcome in the observed group of patients.

Variable	SBT group	DBT group	Overall
Delivery rate (livebirths) (%)			
singletons	75.6 % (192/254)	45.3 % (347/766)	52.8 % (539/1020)
twins	0	34.9 % (267/766)	26.2 % (267/1020)
triplets	0	1.0 % (8/766)	0.8 % (8/1020)
Spontaneous miscarriages (%)			
biochemical pregnancy	9.05 % (23/254)	5.8 % (44/763)	6.6 % (67/1020)
early clinical spont miscarriage	15.3 % (39/254)	12.7 % (97/763)	13.3 % (136/1020)
Overall spont. miscarriages	24.4 % (62/254)	18.4 % (141/763)	19.9 % (203/1020)

** SBT–single blastocyst transfer; DBT–double blastocyst transfer*

using immunochemical hemiluminescent methods (CMIA: acridine; analytical sensitivity < 1.2 IU/l).

Definitions of miscarriage and exclusion criteria

Early miscarriage (early spontaneous abortion in earlier nomenclature) is generally defined as an unprovoked termination of pregnancy prior to the end of first trimester of pregnancy (13^{+6} weeks of gestation). A distinction should be drawn among premenstrual pregnancy loss (loss of conceptus prior to the first measurement of βhCG level 14 days after ovulation or embryo transfer), biochemical pregnancy (loss of conceptus after the first measurement of βhCG level but before the ultrasound (US) confirmation of implantation) and early clinical miscarriage (pregnancy loss after US confirmation of viable pregnancy but before the beginning of the second trimester). Biochemical pregnancies and early clinical miscarriages are commonly identified together as early pregnancy losses (EPL).

The definition of spontaneous miscarriage, which was used in our study, included the following clinical outcomes of pregnancy following its confirmation by positive serum βhCG levels:

1. a decrease in serum βhCG levels compared to the values found at the first measurement prior to the first US control (biochemical pregnancy);
2. presence of a gestational sac without an embryo at the first ultrasound (US) control 14 days after the positive results of the serum βhCG levels (anembrionic pregnancy);
3. presence of a gestational sac with an embryo with no cardiac action, after the previous US control has confirmed heart action (missed abortion);
4. spontaneous expulsion of the conception products from the uterus, following an US confirmation of the presence of a gestational sac with or without an embryo (complete or incomplete clinical miscarriage).

In the case of an anembryonic pregnancy and missed abortion, it was noted that the miscarriage occurred on the day that the definitive clinical diagnosis was made by trans-vaginal ultrasound examination, regardless of when the expulsion of the conception products (spontaneous or induced) actually occurred.

The following outcomes of pregnancy were excluded from the definition of miscarriage:

1. clinically confirmed extra uterine pregnancy;
2. miscarriage after 13^{+6} week of gestation (late miscarriage); and
3. spontaneous loss (spontaneous reduction) of one embryo with the survival of the other, in case of twin pregnancy, determined at the first ultrasound control.

Late miscarriages (those occurring after 13^{+6} week of gestation) were excluded

Table 5: Characteristics of the observed cycles–method of fertilization.

Method of fertilization *(%)*	SBT group	DBT group	Overall
IVF	23.2 % (59/254)	25.3 % (194/766)	24.8 % (253/1020)
ICSI	76.8 % (195/254)	74.7 % (572/766)	75.2 % (767/1020)

** SBT–single blastocyst transfer; DBT–double blastocyst transfer*

from the analysis due to the specific nature of their etiology, which is significantly different from those of early miscarriages. In addition, we were unable to include in our study pregnancy losses which occurred after the transfer of blastocyst into the uterus, but prior to the determination of serum βhCG 14 days after the blastocyst transfer (the so-called premenstrual miscarriages).

Pregnancies in which the blastocyst spontaneously divided into two embryos (monozygotic twins) after the transfer of one or two blastocysts were excluded from the analysis.

Our centre's database did not record the number of previous miscarriages, so that repeated miscarriages could not be included into the multivariate statistical analysis model.

Data quality

Data used in this analysis were received from the centre's database on couples whose infertility was treated by medically assisted fertilization techniques. If there was any data missing in the database for any variable, the patient's documentation (paper records) was checked. If it was still impossible to find the missing data, the patient was excluded from further analysis.

The miscarriages were either confirmed clinically at our centre or were reported by patients in the form of a questionnaire that we routinely sent to each patient whose pregnancy was confirmed by the first test of serum βhCG.

Statistical analysis

We examined the following parameters as potential factors predicting the occurrence of an early spontaneous miscarriage following medically assisted fertilization: patient's age, method of fertilization (IVF or ICSI), number of oocytes retrieved by follicle aspiration, total number of blastocysts, number of blastocysts frozen, number of blastocysts transferred and quality of transferred blastocysts.

Quantitative variables were presented in the form of mean value ± standard deviation (SD) and category variables were presented as ratios.

Bivariate analysis of the correlation between risk factors and miscarriages was performed using the logistic regression model. In addition, an analysis using multivariate logistic regression model was also conducted. All risk factors were first entered into the log-likelihood fitting model unconditionally. Backward stepwise logistic regression was used subsequently at the removal probability of P=0.1. The results were interpreted as the odds ratios (OR) for the observed event, with a confidence interval (CI) of 95 %.

The study had a 95 % power to detect a difference of 10 % (27 % vs. 17 %) in the rate of miscarriages in the analyzed sample of 709 cycles with the transfer of optimal quality blastocysts and 311 cycles with the transfer of suboptimal quality blastocysts.

P value of under 0.05 was considered to be statistically significant. Statistical analysis was performed using STATISTICA® software, version 8.0 (StatSoft Inc., OK, USA).

Results

A. Demographic and clinical characteristics of the patients

In the period covered by our research, pregnancy was confirmed in 1020 patients who underwent IVF/ICSI cycles with the transfer of one or two blastocysts.

Average age of the patients in the studied group was 32 years (32.0 ± 4.3; the youngest patient was 22 and the oldest 44 years old), with no significant difference in age between

Table 6: Characteristics of the observed cycles–number of retrieved oocytes, number of developed and frozen blastocysts.

Variable	SBT group	DBT group	Overall
Number of retrieved oocytes (mean ± SD; range)	11.5 ± 6.7 (1–37)	9.9 ± 4.2 (2- 27)	10.3 ± 5.0 (1–37)
Number of developed blastocysts (mean ± SD; range)	4.5 ± 2.6 (1–19)	4.7 ± 3.8 (1–19)	4.5 ± 2.1 (1–11)
Number of frozen blastocysts (mean ± SD; range)	3.1 ± 3.4 (0–16)	1.7 ± 2.0 (0–9)	2.1 ± 2.5 (0–16)

** SBT–single blastocyst transfer; DBT–double blastocyst transfer*

patients in whom one blastocyst was transferred vs. those in whom two blastocysts were transferred (Table 1).

Main diagnoses in patients and their partners are presented in Tables 2 and 3.

In 254 (24.9 %) patients a single blastocyst was transferred, while in 766 (75.1 %) two blastocysts were transferred. The blastocysts were transferred almost exclusively on the fifth, and only exceptionally (in 1.8 % of cases) on the fourth day after the retrieval of oocytes.

In the studied group of women in whom single blastocyst transfer was performed, in 75.6 % of the cases (192/254) the pregnancy resulted in live birth and in 24.4 % of the cases (62/254) in an early spontaneous miscarriage (as a biochemical pregnancy in 9.05 % (23/254) and as an early clinical spontaneous miscarriage in 15.3 % (39/254)). Spontaneous division of the blastocyst into two embryos (monozygotic twins) was not observed in any patient following the transfer of one blastocyst (Table 4).

In patients in whom two blastocysts were transferred, the pregnancy resulted in live birth in 81.5 % (622/763) patients and in a miscarriage in 18.4 % (141/763) (as a biochemical pregnancy in 5.8 % (44/763) and as an early clinical spontaneous miscarriage in 12.7 % (97/763)) (data on three patients were incomplete). Spontaneous division of the blastocyst into two embryos (monozygotic twins) was observed in 8 cases following the transfer of two blastocysts (the rate of triplet pregnancies in this subgroup was 1.0 % (8/763)) (Table 4).

In most patients (approximately 75 % of the women) ICSI was used as the fertilization technique, while IVF was used in the remaining cases. There was no significant difference in the frequency of a certain method of fertilization between SBT and DBT subgroups (Table 5).

Values of the remaining parameters examined (except blastocyst quality, which is described separately, for the sake of clarity) are presented in Table 6.

B. Bivariate analysis

Prior to multivariate analysis, bivariate logistic regression was used for a preliminary assessment of the predictive value of each of the risk factors for early spontaneous miscarriage following medically assisted conception.

Quality of blastocysts and early spontaneous miscarriage

Within the bivariate analysis, special attention was given to the examination of the correlation between the quality of transferred blastocysts and early spontaneous pregnancy loss, due to the great theoretical importance of this parameter for the occurrence of miscarriage. Information on blastocyst quality were analyzed in the whole group (both SBT and DBT subgroups were included). Therefore, the data on the quality of blastocysts expressed using the previously presented system of blastocyst quality classification (categories B1–B8) had to be transformed. This transformation was performed in two steps. First, the blastocysts from B1 category were designated as optimal quality blastocysts, while those in categories B2–B8 were classified as being of suboptimal quality. In the second step, DBT subgroup in which blastocysts of defferent quality were

Table 7: Quality of transferred blastocysts after final conversion of quality grade.

Quality of blastocyst	SBT group	DBT group	Overall
Optimal	65.7 % (167/254)	70.8 % (542/766)	69.5 % (709/1020)
Suboptimal	34.3 % (87/254)	29.2 % (224/766)	30.5 % (311/1020)

** SBT–single blastocyst transfer; DBT–double blastocyst transfer*

transferred, was merged with the subgroup in which two optimal quality blastocysts were transferred. This transformation, i. e. data regrouping was performed in line with the assumption that in those cases in which multiple embryos of different quality were transferred and only one of them was implanted, the higher quality embryo (so-called leading embryo) had the highest probability of implantation. Data on blastocyst quality following an additional transformation of the data is shown in Table 7.

Application of a logistic regression in bivariate model showed that there was a high statistically significant correlation between the quality of transferred blastocysts, defined in the aforementioned manner (blastocyst quality designated as optimal or suboptimal in the whole group) and the rate of early spontaneous miscarriages (OR = 0.560; 95 % CI 0.407–0.770, P < 0.001) (Table 8).

Other studied parameters and miscarriage

Association of each of the examined parameters (patient's age, fertilization method, number of oocytes retrieved, total number of blastocysts, number of frozen blastocysts, number of transferred blastocysts and the quality of transferred blastocysts) was examined using the logistic regression. Except for the number of oocytes, all other parameters showed a statistically significant correlation with the occurrence of early spontaneous miscarriage. The results of the bivariate logistic regression for the studied parameters are shown in Table 8.

Multivariate analysis

All parameters that were shown to have a statistically significant association with the occurrence of early spontaneous miscarriage by the analysis in bivariate model, were subsequently included in the multivariate logistic regression model. The only exception was the parameter *total number of blastocysts*, which was excluded due to its statistically significant correlation with the parameter *number of blastocysts frozen* (r = 0,966). We decided to include the parameter *number of blastocysts frozen* into the multivariate model, since this variable was more strongly associated with the frequency of early spontaneous miscarriage.

In the first step, all selected variables were included in the multivariate analysis model. The results were then confirmed using the backward elimination model, as a stepwise method for the selection of variables.

The analysis showed that statistically significant factors predictive of the early miscarriage in the studied group were the patient's age (OR = 1.098; 95 % CI 1.057–1.140, P < 0.001), the number of blastocysts transferred (OR = 0.592; 95 % CI 0.412–0.851, P = 0.005), the quality of blastocysts transferred (OR = 0.666; 95 % CI 0.468–0.949; P = 0.024), as well as the number of blastocysts frozen (OR = 0.912; 95 % CI 0.832–0.999; P = 0.048) (Table 9).

Discussion

Frequency of early miscarriage

Techniques of assisted reproductive technology (ART) are multiphase procedures. The outcome of ART also depends on factors that are highly specific to these techniques. Partial or complete modification of these factors is possible in a large number of cases, which is why their identification and study represents an opportunity for the improvement of ART success.

Ability to predict the outcome of ART procedures based on the value of numerous parameters related to the stimulation of fol-

liculogenesis and transfer of embryos was the subject of much study. Previous research included almost exclusively those patients in whom the cleavage stage embryos were transferred (i.e. embryos on the third day after follicle aspiration). The main objective of our study was to assess the risks of early miscarriage as well as to determine the predictive value of different risk factors for the occurrence of early miscarriages in women who conceived after the ART procedures involving the transfer of blastocyst(s).

When comparing data from different studies, we must be very mindful of the precise definition of pregnancy and miscarriage. We should differentiate among premenstrual pregnancy loss, biochemical pregnancy, early and late clinical miscarriage (definitions were given in previous section).

Premenstrual pregnancy loss

Unfortunately, we were unable to assess the frequency of premenstrual pregnancies (i.e. pregnancies lost prior to the first measurement of βhCG level). In the literature published so far, information on this form of early pregnancy loss is very scarce. Taking into consideration that the patients must frequently submit their blood or urine for analysis around the period immediately after the ovulation in spontaneous conception, i.e. following the transfer of embryos in ART procedures, it is easy to understand why these studies are so few. In one of these rare researches in this field, it was shown that the rate of premenstrual pregnancy losses was significantly higher following the transfer of embryos than following spontaneous conception (50 % vs. 30 %).[21]

Biochemical pregnancies

In our study, the total frequency of biochemical pregnancies was 6.6 % (67/1020) (Table 4). Biochemical pregnancies were somewhat more frequent following the transfer of one than following the transfer of two blastocysts (9.05 % (23/254) vs. 5.8 % (44/763)). In most studies published so far, these early pregnancy losses were not analyzed separately.

In most studies published so far, biochemical pregnancies were either excluded from the final analysis,[15] or more commonly, they were included in the same group as early clinical miscarriages.[26,40,46,48-49] There were, however, studies that addressed the problem of biochemical pregnancy separately from other forms of early miscarriage. For example, Tummers *et al.*[47] observed the frequency of biochemical pregnancies in patients following *in vitro* fertilization techniques of 5.8 %, while De Neubourg *et al.*[49] observed this outcome in 8.1 % of the studied pregnant patients following the transfer of the highest quality embryos. The frequency of biochemical pregnancy observed in our study was comparable to the results of these researchers.

Early clinical miscarriage

The rate of early clinical miscarriages in the studied group of women was 13.3 % (136/1020). The frequency of early clinical miscarriages was higher following the transfer of one than following the transfer of two blastocysts (15.3 % (39/254) vs. 12.7 % (97/763)) (Table 4).

Our results were comparable to the observation of Wang *et al.* who examined the occurrence of early clinical miscarriages in a group of women that had conceived following assisted fertilization, as well as in two cohorts of pregnant women who had conceived spontaneously (the so-called Ford's and Treloar's cohort).[15] Biochemical pregnancies and miscarriages prior to the 6th week of gestation were excluded from the analysis. It was shown that there was a small, but statistically significant additional risk for the occurrence of an early miscarriage in women who had undergone ART procedures. Specifically, in the first trimester of gestation, the observed frequency of early miscarriage was 16.5 % following assisted conception vs. the frequency of 14.0 % (Ford's cohort) or 11.3 % (Treloar's cohort) following spontaneous conception.[15]

Early pregnancy loss

In our research, early pregnancy loss (EPL–defined as biochemical pregnancy and clinical miscarriage taken together), was observed in 19.9 % of the patients (203/1020). This outcome of pregnancy was more common following the transfer of one (24.4 % (62/254)) than following the transfer of two

blastocysts (18.4 % (141/763)). EPL rate remained constant throughout the study period.

The results of published studies, which included biochemical pregnancies and early miscarriages together showed that early pregnancy loss after ART occurred with a frequency of 15–30 %.[15,26,40,47,49-50] There were, however, significant differences between these studies in the definition of early miscarriages, as well as in the number of observed patients.

Winter *et al*[40] recorded the frequency of early miscarriage of 16.0 % in a group of 1196 pregnancies following *in vitro* fertilization (the definition of an early miscarriage included biochemical pregnancies and early clinical miscarriages up to weeks 6–7 of gestation; extra-uterine pregnancies were excluded). Hourvitz *et al.*[26] analyzed 1471 patients who conceived following the transfer of cleavage stage embryos and found a frequency of early miscarriage of 31.7 % (definition of an early miscarriage included biochemical pregnancies, extra uterine pregnancies and clinical miscarriages up to 12^{+6} weeks of gestation). In patients under 35 years of age, early miscarriage frequency was 28.5 %, while in those 35 years of age and older it amounted to as high as 38.6 %. Tummers *et al.*[47] analyzed 1597 single and twin pregnancies following *in vitro* fertilization. They reported that the frequency of early miscarriage in the whole group was 17.7 %, with the EPLs significantly more frequent in women with single pregnancies than in those with twin pregnancies (21.8 % vs. 5.1 %) (definition of an early miscarriage included biochemical pregnancies and clinical miscarriages up to 11^{+6} weeks of gestation; extra uterine pregnancies were excluded). Schieve *et al.*[55] studied the occurrence of miscarriages in the total of 48043 patients to whom fresh embryos, conceived by the fertilization of their own oocytes, were transferred; they found the frequency of miscarriage of 14.5 % (definition of an early miscarriage included the early and the late clinical miscarriage; biochemical and extra-uterine pregnancies were excluded). In the subgroup of patients from 20–29 years of age, the miscarriage rate was 10 %, while in the 40–47 year age subgroup, this rate amounted to 39.3 %.[55] La Sala *et al.*[50] analyzed a group of 407 pregnant women following *in vitro* fertilization and reported the frequency of early miscarriage of 23.1 % (their definition of early miscarriage included the losses detected up to the ultrasound control in the second trimester, while biochemical and extra-uterine pregnancies were excluded).[50] De Neubourg *et al.*[49] studied a group of 370 patients who conceived after an elective transfer of a single top quality embryo and reported the rate of early miscarriages of 29.7 % (definition of early miscarriage encompassed biochemical pregnancies, early clinical miscarriage and extra-uterine pregnancies).

Whether we take into account solely the frequency of early clinical miscarriages, or the frequency of EPL (early clinical miscarriages and biochemical pregnancies together), the rate of miscarriages in the sample we observed was smaller than the one reported in literature and showed no practical discrepancy from that observed following spontaneous conception.

Age of the mother

While controlling the mutual influences of potential risk factors for the occurrence of early miscarriage using the multivariate analysis model, in our study we showed that the mother's age was the most significant positive predictor of sporadic early miscarriage (OR = 1.098; 95 % CI 1.057–1.140, $P < 0.001$). The results observed were in complete accordance with the findings of other researchers.[15,40,25-26]

Cytogenetic studies provided explanation of this strong association between patients' age and miscarriage rate. It was observed that older women had higher incidence of meiotic errors than younger ones and that these errors were the cause of a higher incidence of chromosomal abnormalities in miscarried fetuses and livebirth children of older women.[20-21,25] It was also shown that chromosomal abnormalities of the fetus were the most common cause of early miscarriage.[22-24] Taking into account all these data, the pathophysiologic mechanism, which explains the influence of

mother's age on the frequency of early miscarriage is quite straightforward.

Number of blastocysts transferred

In our study, we also showed that the number of transferred blastocysts was a significant negative predictor for the occurrence of early miscarriage (OR = 0.592; 95 % CI 0.412–0.851, P = 0.005). The rate of early miscarriage following SBT was 24.4 % vs. 18.4 % following DBT.

Research results on the association between the number of embryos transferred and the frequency of early miscarriage published to date are also scarce. Our results confirm the findings of the study by Balen *et al.*[45] These researchers showed that the rate of early miscarriage was statistically more significant in the group of pregnant women in whom one or two embryos were transferred than in those in whom three or four embryos were transferred. It should be noted that this study was performed at a time when the transfer of multiple embryos was a common practice. The rate of early miscarriage following the transfer of one embryo was 37.7 %, following the transfer of two embryos 34.6 %, following the transfer of three embryos 22.5 %, and following the transfer of four embryos 25.2 %.[45] Similar results were observed by La Sala *et al.*,[50] in their analysis of a group of 962 pregnant women who had 1 to 4 embryos transferred. Results of this research indicated that the frequency of miscarriage declines with an increase in the number of embryos transferred. Following the transfer of one embryo, the frequency of miscarriage was 25.8 %, following the transfer of two embryos 11.5 %, following the transfer of three embryos 8.1 % and following the transfer of four embryos 11.8 %. Unfortunately, these investigators did not statistically analysed the significance of the association between the number of embryos transferred and the frequency of early miscarriage.

Contrary to these results, in the aforementioned study by Hourvitz *et al.*,[26] multivariate logistic regression model showed no association between the number of embryos and the frequency of early miscarriage.

Theoretically, according to our results, transfer of two blastocysts could be used as a tool for reducing early miscarriage rate. But we should be very cautious about this assumption. Transfer of two blastocysts of optimal quality in younger women carries a significant risk for twin or even triplet pregnancy.[58] The ideal goal of an ART procedure should be to get healthy child after uncomplicated pregnancy, which ends up as vaginal delivery at term. Keeping in mind this goal, reduction of early miscarriage rate on the account of growth of risky multifetal pregnancy rate is not justifiable. There are, however, special groups of patients (for example, older women, patients with embryos of poor quality or ones with a history of repeated early miscarriages) who could benefit of this approach.

Blastocyst quality

In the case of single blastocyst transfer, analysis of the association between its quality and the frequency of early miscarriage was a relatively simple task. In patients who have two blastocysts transferred, the situation is somewhat more complicated. First, morphological grades of blastocyst quality are categorical variables, so in the case of the transfer of two or more blastocysts of different quality, mean values of their morphological quality grades cannot be taken into consideration. Second, in the case of transfer of two blastocysts of different qualities, in which only one of these eventually implants, it is impossible to determine exactly which one of them has implanted.

The transfer of two (or more) blastocysts is (still) a common clinical practice. If we were to exclude, for the sake of simplicity of the analysis, the information on patients in whom two blastocysts were transferred, we would reduce the number of cycles observed significantly, while at the same time, investigation of the influence of other significant variables (such as the number of transferred embryos) would be impossible.

Researchers who have come across with this issue tried to overcome it in different ways. Lambers *et al.*[48] used a cumulative embryo score, previously introduced

Table 8: Evaluation of the association between the observed variables and spontaneous miscarriage by bivariate logistic regression.

Variable	B	Standard Error (SE)	Level of significance (P)	Odds ratio (OR)	95 % Confidence interval (CI) for OR	
					Lower	Upper
Patient's age	0.088	0.019	< 0.001	1.092	1.053	1.133
Method of fertilization (IVF / ICSI)	0.439	0.197	0.026	1.551	1.055	2.280
No. of retrieved oocytes	-0.006	0.016	0.693	0.994	0.964	1.025
No. of developed blastocysts	-0.071	0.032	0.027	0.932	0.875	0.992
No. of frozen blastocysts	-0.083	0.035	0.017	0.920	0.860	0.985
No. of transferred blastocysts (SBT or DBT)	-0.359	0.173	0.038	0.699	0.297	0.981
Quality of transferred blastocysts	-0.580	0.163	< 0.001	0.560	0.407	0.770

** IVF–in vitro fertilization; ICSI–intracytoplasmic sperm injection; SBT–single blastocyst transfer; DBT–double blastocyst transfer*

by Steer *et al.*[56] Cumulative embryo score was defined as an additive parameter (i.e. following the transfer of two embryos with scores of 1 and 3, the total score of embryos transferred was 4). Winter *et al.*[40] assessed embryo quality with relation to the number of embryos transferred and the possibility of elective transfer. According to this system, embryos were scored 1 in the case of an elective transfer of one or two embryos (highest score); elective transfer of 3 embryos yielded a score of 2; if two or three embryos had been transferred non-electively, the score was 3, and if only a single embryo was transferred non-electively, it was scored 4 (worst score). La Sala *et al.*[46] excluded from their study patients in whom embryos with different quality scores had been transferred.

In their study, Hourvitz *et al.*[26] assumed that in cases in which more than one embryo was transferred, the highest quality embryo (the so-called leading embryo) had the highest likelihood for implantation. This assumption was based on the well documented association between embryo quality and implantation rate suggesting that the implanting embryo was the best quality embryo transferred with a high level of probability.[49,57] We considered this approach to be more logical and better documented than the others. We also did not accept the approach used by La Sala *et al.*,[46] i. e. to investigate only cycles involving exclusively the transfer of embryos of the same quality, since these situations were rare in clinical practice.

In our study, we demonstrated that the quality of blastocysts was a statistically significant negative predictor for the occurrence of early miscarriage (OR = 0.666; 95 % CI 0.468–0.949; P = 0.024).

Our results confirmed the findings of the aforementioned study by Hourvitz *et al.*[26] These authors used the number of cells in the leading embryo and the degree of its fragmentation as indicators of embryo quality, but found that only the number of cells had a significant association with the rate of early miscarriages. Similarly, Winter *et al.*[40] reported that the transfer of lowest quality embryos lead to a three-fold increase in the occurrence of early miscarriages, compared to the group in which only the highest quality embryos were transferred. On the other hand, Lambers *et al.*[48] failed to show a statistically significant association between the early miscarriage frequency and the quality of embryos, expressed as a cumulative embryo score. This result was unexpected for the researchers themselves, who assumed that the cause was in the small number of patients in the multiple pregnancy group, combined with the even lower early miscarriage rate in this group. Also, there was a possibility that cumulative embryo score wasn't the most appropriate method to express embryo quality. Association between miscarriage rate and quality of the transferred embryos was not found by De Neubourg *et al.*[49] in their study on the transfer of exclusively one highest quality embryo, as the model of early pregnancy outcome.

Table 9: Evaluation of the relationship between the observed variables and spontaneous miscarriage by multivariable logistic regression.

Variable	B value	Standard Error (SE)	Level of significance (P)	Odds ratio (OR)	95 % Confidence interval (CI) for OR	
					Lower	Upper
Patient's age	0.093	0.019	< 0.001	1.098	1.057	1.140
Method of fertilization (IVF / ICSI)	0.325	0.202	0.108	1.384	0.931	2.057
No. of retrieved oocytes	0.028	0.020	0.163	1.028	0.989	1.069
No. of frozen blastocysts	-0.092	0.047	0.048	0.912	0.832	0.999
No. of transferred blastocysts (SBT or DBT)	-0.524	0.185	0.005	0.592	0.412	0.851
Quality of transferred blastocysts	-0.406	0.180	0.024	0.666	0.468	0.949

* IVF–*in vitro* fertilization; ICSI–intracytoplasmic sperm injection; SBT–single blastocyst transfer; DBT–double blastocyst transfer

These authors concluded that the quality of embryos, based on morphological criteria, was a good predictor for the implantation rate, but not for the early miscarriage frequency.[24]

We think that embryo quality determined by morphological criteria is a useful tool for predicting pregnancy outcome. Which one of different approaches to express embryo quality in the case of transfer of more than one embryo/blastocyst is the most useful and successful remains an open question and it should be the focus of future studies.

Number of blastocysts frozen

We also showed that the number of frozen blastocysts represented a significant negative predictor for early miscarriage (OR = 0.912; 95 % CI 0.832–0.999; P = 0.048). Similar results were found by Lambers *et al.*,[48] who reported that positive predictors for the continuation of pregnancy were the number of frozen embryos and the younger age of the mother. We agree with the assumption of these authors that the big number of embryos/blastocysts suitable for freezing represents an indirect sign of developmental potential of the obtained embryos, but also of a reproductive potential of a given patient.

Fertilization method (IVF or ICSI)

In our study, we were unable to demonstrate a statistically significant association between the fertilization method (IVF or ICSI) and the rate of early miscarriage (OR = 1.384; 95 % CI 0.931–2.057, P = 0.108). These results confirmed the findings of practically all other studies dealing with this issue.[15,26,46,49-50]

Conclusion

In the analyzed group of pregnant women, the total rate of biochemical pregnancies was 6.6 % (67/1020). Biochemical pregnancies were somewhat more frequent following the transfer of one than following the transfer of two blastocysts (9.05 % (23/254) vs. 5.8 % (44/763)). Early clinical miscarriage rate was 13.3 % (136/1020). The frequency of early clinical miscarriage was higher following the transfer of one than following the transfer of two blastocysts (15.3 % (39/254) vs. 12.7 % (97/763)). If both biochemical pregnancies and early clinical miscarriages are included in the definition of early miscarriages, the rate of EPL in the whole examined group was 19.9 % (203/1020), following the transfer of one blastocyst 24.4 % (62/254) and following the transfer of two blastocysts 18.4 % (141/763).

By applying the multivariate analysis model, we showed that statistically significant factors that predict the occurrence of early miscarriages were the patient's age (OR = 1.098; 95 % CI 1.057–1.140, P < 0.001), the number of blastocysts transferred (OR = 0.592; 95 % CI 0.412–0.851, P = 0.005), the quality of the transferred

blastocysts (OR = 0.666; 95 % CI 0.468–0.949; P = 0.024), as well as the number of frozen blastocysts (OR = 0.912; 95 % CI 0.832–0.999; P = 0.048). In addition, we demonstrated that the number of oocytes retrieved by follicle aspiration (OR = 1.028; 95 % CI 0.989–1.069, P = 0.163) and fertilization method (IVF or ICSI) (OR = 1.384; 95 % CI 0.931–2.057, P = 0.108) were not statistically significantly associated with the rate of early miscarriages in the observed group of patients.

Transfer of a single, optimal quality blastocyst, as well as the transfer of two blastocysts, were associated with a lower frequency of early miscarriages in pregnancies achieved by *in vitro* fertilization.

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