Determination of criteria for the assessment of embryo quality with good implantation predictability on a model of unstimulated *in vitro* fertilization cycles – Prediction of implantation in unstimulated cycles

Določitev meril za ocenjevanje kakovosti zarodka z možnostjo napovedovanja ugnezditve na modelu nespodbujenih ciklih zunajtelesne oploditve – napovedovanje ugnezditve pri nespodbujenih ciklih

Mojca Čižek Sajko, Veljko Vlaisavljević

Department of Reproductive Medicine and Gynecologic Endocrinology, University Clinical Centre Maribor, Slovenia

Korespondenca/ Correspondence:
Veljko Vlaisavljević, Department of Reproductive Medicine and Gynecologic Endocrinology, University Clinical Centre Maribor
Ljubljanska 5,
SI-2000 Maribor,
Slovenia
Tel: +386 2 321 100
E-mail: vlai@ukc-mb.si

Ključne besede:
en zarodek, napoved ugnezditve, naravni cikel, ocena zarodka

Key words:
embryo score, natural cycle, prediction of implantation, single embryo

Citirajte kot/Cite as:
Zdrav Vestn 2011; 80: I-39–I-45

Prispelo: 2. nov. 2009,
Sprejeto: 18. nov. 2010

Abstract

Background: In unstimulated *in vitro* fertilization cycles we analysed which criteria for the assessment of day-3-embryo quality were the most important predictive factors of successful implantation.

Methods: A retrospective analysis of 115 unstimulated *in vitro* fertilization (IVF) cycles, terminated by embryo transfer (ET) on day 3 post follicular aspiration, was done. The embryos were evaluated according to three criteria: (i) blastomere fragmentation, (ii) blastomere number on the day of ET and (iii) blastomere cleavage dynamics. The impact of individual criteria, and of combinations of several criteria for embryo quality assessment, on implantation was established.

Results: The impact of embryo quality on implantation could only be confirmed in the case when the embryo was evaluated on the basis of blastomere fragmentation and blastomere cleavage dynamics simultaneously ($\chi^2 = 5.23$; $P < 0.05$). In such cases the implantation rate amounted to 27.0 % (20/74) in good quality embryos and 9.8 % (4/41) in poor quality embryos. The predictive value of implantation for the so defined embryo quality was 3.43 (95 % CI = 1.08–10.84).

Conclusion: Embryo quality, defined by blastomere fragmentation and blastomere cleavage dynamics simultaneously, proved to be a good predictive factor of implantation. In good quality embryos, the odds for implantation were 3.43 times higher than in embryos of poor quality.

Izvleček

Izhodišča: Ugotavljali smo, katera merila za določanje kakovosti zarodka tretjega dne so bili pri nespodbujenih ciklih zunajtelesne oploditve najboljši napovedni dejavniki za uspešno ugnezditev.

Metode: Retrospektivno smo analizirali 115 nespodbujenih ciklov zunajtelesne oploditve, ki so se zaključili s prenosom zarodka tretji dan po aspiraciji folikla. Zarodke smo ocenjevali na osnovi treh meril: (i) fragmentacija blastomer; (ii) število blastomer na dan prenosa zarodka in (iii) dinamika delitve blastomer. Ovrednotili smo vpliv posameznih meril in kombinacije več meril za določanje kakovosti zarodkov na ugnezditev.

Rezultati: Vpliv kakovosti zarodka na ugnezditev smo lahko potrdili le v primeru, ko smo jo opredelili na podlagi fragmentacije blastomer in dinamike delitve blastomer hkrati ($\chi^2 = 5{,}23$; $P < 0{,}05$). Pri tako opredeljeni kakovosti zarodka je bila stopnja ugnezditve pri zarodkih dobre kakovosti 27,0 % (20/74), pri zarodkih slabe kakovosti pa 9,8 % (4/41). Napovedna vrednost za ugnezditev je bila 3,43 (95-odstotni IZ = 1,08–10,84).

Zaključki: Kakovost zarodka, ki smo jo ovrednotili na osnovi fragmentacije blastomer in dinamike delitve blastomer hkrati, se je pokazala kot dober napovedni dejavnik za ugnezditev. Zarodki dobre kakovosti so imeli 3,43-krat večje obete za ugnezditev kot zarodki slabe kakovosti.

Introduction

Embryo quality is a key factor influencing the outcome of the *in vitro* fertilization (IVF) procedure. It is therefore very important how embryos for embryo transfer (ET) are selected. It would be ideal if it were possible to select embryos with the highest implantation potential. However, there are still no definitive selection criteria to determine the best quality embryo. Prolonged cultivation to the blastocyst stage allows following the embryo to the final preimplantation stage and selecting the most vital embryos more effectively.[1-2] The dilemma remains as to whether culture conditions in cultivation to day 5 really are optimal for the development of all embryos or whether it would be better, for the time being, to stick to the transfer of embryos to the uterus on day 2 or day 3. Embryo selection in a shorter cultivation period could only be equally effective if we could find sufficiently good criteria to determine the quality of the embryo or its implantation capacity.

The quality of the early preimplantation embryo is usually evaluated on the basis of three morphologic characteristics: blastomere shape and size, blastomere fragmentation and embryo cleavage rate or number of cells.[3-9] Most authors studied the impact of individual or several criteria on the outcome of IVF procedure. Only few authors discussed embryo quality assessed on the basis of several criteria at the same time: shape and fragmentation of blastomeres as well as the cleavage rate,[4,6-7,10] cell fragmentation and number, the number of multinucleated blastomeres,[11] cell number, fragmentation pattern and embryo morphology.[12]

Assessment of the impact of embryo quality on the implantation rate is mostly based on stimulated cycles in which several embryos are intended for ET. In these cases it is impossible to know the fate of individual embryos of a certain quality. A precise analysis of the relationship between embryo quality and implantation is only possible in cases when the number of implanted embryos equals that of embryos transferred. There are some reports showing the correlations between embryo characteristics and implantation potential: the study of Ziebe et al.[8] included only the transfer of embryos of equal quality, Van Royen et al.[11] only studied those ET where both transferred embryos had also implanted, and Giorgetti et al.[7] set the embryo score on the basis of the analysis of single embryo ETs from stimulated cycles.

A good model for this are also natural or unstimulated IVF/ICSI (intracytoplasmic sperm injection) cycles where aspiration of the dominant follicle gives only one oocyte and embryo development can be followed from fertilization to its implantation into the uterine wall.

The aim of our study was to analyse the embryo quality in unstimulated cycles in order to determine which criteria for embryo quality assessment were the most important predictive factors of successful implantation. As far as we know from the literature, the present study is the first to discuss the impact of embryo quality–defined on the basis of blastomere fragmentation, blastomere number and blastomere cleavage dynamics in the embryo cultivated to day 3–on implantation on the model of an unstimulated IVF/ICSI cycle.

Material and methods

In our study, 115 natural IVF/ICSI cycles (73 IVF and 42 ICSI), terminated by ET, were analysed retrospectively. The study included those couples from the list of candidates for a stimulated IVF/ICSI procedure who had decided to make use of the offered possibility of taking part in natural IVF/ICSI cycles. Patients were recruited consecutively, in 24 months. The classical IVF was only carried out in those couples in which isolation of spermatozoa from semen samples could be followed by the application of the swim-up technique to prepare semen samples with a 5–10 x 10^6/ml concentration of motile spermatozoa. If the concentration of spermatozoa in the rinsed semen sample was lower, or if the male partner had severe oligozoospermia, the ICSI procedure was used.

The mean age of women was 32.5 ± 4.0 years (range 24–41 years). In all women follicular growth was followed ultrasonographically and by assessing serum estradiol levels

Table I: Frequency distribution for day 2 and day 3 according to the fragmentation of all 24 embryos that implanted.

Fragmentation	Day 2 embryos (*n*)	Day 3 embryos (*n*)
Grade 1 (0–20 %)	20	21
Grade 2 (20–50 %)	4	3
Grade 3 (> 50 %)	0	0

according to the same protocol.[13] Ovulation was stimulated with 5000 IU hCG when the follicular diameter reached 16 mm and the estradiol level ≥ 0.50 nmol/L. Thirty-six hours later follicular aspiration of the dominant follicle served to obtain the oocyte – cumulus complex which was transferred to 0.7 ml of Universal IVF Medium (IVF medium) (MediCult, Jyllinge, Denmark) covered with paraffin oil. After one to two hours of cultivation in the incubator at 37 °C and an atmosphere with 5 % CO_2, the oocyte – cumulus complexes designed for the classical IVF were inseminated with 30ml of the corresponding semen sample with 5–10 x 10^6/ml motile spermatozoa, rinsed in Sperm Preparation Medium (SPM) (MediCult) and prepared by the swim-up technique. The oocytes for ICSI, however, were transferred from the IVF medium to hyaluronidase (MediCult) for one minute maximum in order to separate them from cumulus cells. The oocytes were then rinsed in Flushing Medium (FM) (MediCult) and with a thin glass pipette the corona radiata cells were removed. The denuded oocytes were transferred to 15ml drops of IVF medium covered with paraffin oil. ICSI was carried out under an IMT-2 invert microscope (Olympus, Tokyo, Japan), equipped with two MO-188 micromanipulators (Narishige, Tokyo, Japan). The purified spermatozoa from the ejaculates were transferred to polyvinylpyrrolidone (PVP) (MediCult). The injection pipette (Hunter, Saffron Walden, Great Britain) was drawn over the tail of each sperm in order to damage the plasma membrane. Thus immobilized spermatozoa were then used for ICSI.

The embryos were cultivated in IVF medium until day 2 post follicular aspiration. When evaluating fertilization on the first day, the oocyte was transferred to fresh medium. On day 2 post aspiration, the embryo was transferred to M3 complex medium (MediCult) and cultivation was prolonged to day 3. On day 3, ET to the uterus was carried out. Prior to transfer, embryo quality was evaluated on the basis of three criteria: (i) blastomere fragmentation, (ii) blastomere number and (iii) blastomere cleavage dynamics (the increase in the number of blastomeres from day 2 to day 3). The significance of individual criteria and combinations of various criteria on the assessment of embryo quality was established.

On the basis of the assessed morphological characteristics of implanted embryos, embryo quality was evaluated as follows (Table III): With respect to blastomere fragmentation, three groups of embryos were distinguished, graded 1 to 3 (grade 1 = up to 20 % fragmentation; grade 2 = 20–50 % fragmentation and grade 3 ≥ 50 % fragmentation). Based on the second criterion of embryo quality, i.e. blastomere number on the day of ET, the embryos were divided into groups of those with six or more blastomeres and those with less than six blastomeres. If seven or eight blastomeres had been taken as the lower limit, we would have left out 41.7 % or 50.0 % of embryos with a smaller number of blastomeres on day 3. Therefore, 6 embryos were set as the lower limit, and consequently, the majority of embryos were included (75.0 %). The third criterion for assessing embryo quality was the blastomere cleavage dynamics. The dynamics of development was adequate if the number of blastomeres increased by two or more from day 2 to day 3; or it was inadequate if the number of blastomeres increased by less than two.

With respect to individual criteria, a good quality embryo was one with < 20 % fragmented blastomeres, or one whose blastomere number on the day of ET was 6 or more, or whose blastomere number increased by at least two from day 2 to day 3. The rest were evaluated as poor quality embryos. In combinations of several criteria, a good quality embryo was the one meeting the above stated requirements for all criteria.

Implantation was confirmed by determining serum beta human chorionic gonadotropin (β-hCG) in the woman 16 days post oocyte pick-up.

Table II: Frequency distribution for day 2 and day 3 according to the number of blastomeres of all 24 embryos that implanted.

Blastomeres (n)	Day 2 embryos (n)	Day 3 embryos (n)
2	7	
3	2	
4	13	3
5	0	3
6	1	4
7	1	2
8		10
10		1
12		1

The relationship of different criteria to implantation was analysed statistically by logistic regression. Statistical analysis of data was done by the Statistica programme (StatSoft). Values of $P < 0.05$ were considered statistically significant.

Results

In order to characterize the embryos with optimal implantation capacity, we examined those which had implanted. Of 115 embryos 24 had implanted. Their morphological characteristics are shown in Tables I and II. As is evident from Table I, fragmentation did not change essentially from day 2 to day 3. Most embryos (87.5 %) had 20 % fragmentation or less. Of the embryos which had implanted, none had a fragmentation > 50 %.

Table II shows the frequency distribution for day 2 and day 3 according to the number of blastomeres. On day 2 the majority (thirteen) of embryos had four blastomeres, while a considerable number (seven) had only two blastomeres. Only two embryos had three blastomeres, one had six and another one had seven. On day 3 the majority of embryos (ten) had eight blastomeres, but the distribution of embryos was more dispersed.

Individual criteria for embryo quality evaluation such as fragmentation, number of blastomeres and blastomere cleavage dynamics were not significant in predicting implantation (Table III). Embryo quality only proved to be a good predictive factor of implantation in cases when the embryo was evaluated concurrently on the basis of blastomere fragmentation and the adequacy of blastomere cleavage dynamics ($\chi^2 = 5.23$; $P < 0.05$). In such cases, the implantation rate in good quality embryos amounted to 27.0 % (20/74) and in poor quality embryos to 9.8 % (4/41). The odds for implantation (i.e. the relationship between the probability that implantation would occur and the probability that implantation would not occur) in good quality embryos were 3.43 times higher (OR = 3.43; 95 % CI = 1.08–10.84) than in poor quality embryos.

According to the blastomere fragmentation and blastomere cleavage dynamics (i.e. a + c criterion in the Table III), the percentage of good quality embryos was 79.2 % (19/24) and 60.4 % (55/91) for embryos which had implanted, and embryos which had not implanted, respectively ($\chi^2 = 2.90$, $p > 0.05$). However, there was a significant difference in good embryo quality according to the IVF and ICSI procedure (71.2 % (52/73) vs. 52.4 % (22/42); $\chi^2 = 4.13$, $p = 0.042$). The mean patients' age was similar for women with embryo which had implanted (31.8 ± 3.5), compared to those with embryo which had not implanted (32.7 ± 4.1) ($p > 0.05$). Similarly, we could not confirm any significant difference in the mean age for women with good quality and poor quality embryos (32.7 ± 4.0 vs. 32.1 ± 4.1; $p > 0.05$).

Discussion and Conclusions

The question arises, which parameters will describe the embryo in such a way that the information will be an adequate predictive factor of implantation. In the study carried out in natural IVF cycles, Zayed et al.[14] evaluated embryo quality merely on the basis of its morphologic characteristics. Apart from fragmentation and shape of blastomeres, most authors also take into consideration their number on the day of ET.[4,6-8,10-11] Their studies show that embryo quality has a significant impact on the outcome of IVF.

Giorgetti's study[7] included a large number of embryos (957), in embryo scoring taking into consideration cleavage, cell shape, blastomere fragmentation and cell number

Table II1: The influence of embryo quality on implantation, assessed on the basis of different criteria. For each individual independent variable (criterion), logistic regression was used to calculate the regression coefficient (b), its standard error (SE) and the significance of individual explanatory independent variables for implantation (*p*).

Embryo quality criteria			Logistic regression (implantation / no implantation)		
	No. embryos	Implantation (%)	b	SE	*p*
a = fragmentation			1.030	0.659	0.121
Grade 1 (0–20 %)	86	21 (24.4)			
Grade 2 (20–50 %)	18	3 (16.7)			
Grade 3 (> 50 %)	11	0 (0.0)			
b = No. blastomeres			0.438	0.521	0.402
≥ 6	78	18 (23.1)			
< 6	37	6 (16.2)			
c = Cleavage dynamics			1.255	0.778	0.110
≥ 2 cells from D2/D3	91	22 (24.2)			
< 2 cells from D2/D3	24	2 (8.3)			
a + c			1.231	0.588	0.038*
Good quality	74	20 (27.0)			
Poor quality	41	4 (9.8)			
a + b			0.450	0.482	0.352
Good quality	67	16 (23.9)			
Poor quality	48	8 (16.7)			
b + c			0.487	0.520	0.351
Good quality	77	18 (23.4)			
Poor quality	38	6 (15.8)			
a + b + c			0.495	0.482	0.306
Good quality	66	16 (24.2)			
Poor quality	49	8 (16.3)			

** = statistically significant relationship between criterion and implantation (P < 0.05)*

on day 2. But this study was done on a specific embryo population: 97.3 % of cycles had only one available embryo, and only a smaller percentage of the couples had decided on single-embryo transfer.

Another study on embryo characterization was also done by Van Royen et al.[11] They retrospectively examined 23 double transfers resulting in ongoing twins in order to define 'top quality' embryos with high implantation potential. These embryos had 4 or 5 blastomeres on day 2 and at least 7 blastomeres on day 3 after fertilization, no multinucleated blastomeres and < 20 % of fragments on day 3 after fertilization.

When comparing different studies, we should keep in mind that the final results are always influenced by several factors. Apart from cultivation conditions, embryo development *in vitro* is also affected by patient

age, the stimulation protocol, the response to stimulation with gonadotropins and by the quality of germ cells. At the same time we should not ignore the importance of statistical data processing. In statistical analysis it is very important how individual variables (e. g. embryo quality) are defined and how their values, if the variables are attributive, are categorized. For a precise comparison of different studies, the groups of patients should be coordinated according to their age, indications, number of oocytes, fertilization rate and culture conditions, and embryo quality should be equally defined and categorized into equally defined categories.

In our study we analysed embryo quality in unstimulated cycles. In all patients included in our study, ET was carried out on day 3. Therefore, apart from assessing blastomere fragmentation and cell number, additional information regarding embryo vitality – blastomere cleavage dynamics from day 2 to day 3 – could be used in embryo quality assessment. Multinucleation was not evaluated, as the embryos with multinucleated blastomeres on day 2 were not used for ET.

It turned out that individual criteria, such as fragmentation stage, blastomere number and blastomere cleavage dynamics, were not sufficient to describe embryo quality. Embryo quality was considered a good predictive factor of implantation only in cases when it was defined by blastomere fragmentation and blastomere cleavage dynamics simultaneously. No other combination of individual criteria, merged into a joint embryo quality score, showed any correlation with the outcome of IVF (Table III). That is actually also what Van Royen et al.[11] have shown: namely, important is not the individual static characteristic, but rather the combination of several characteristics. Unexpected was our finding that blastomere number was an insignificant parameter in embryo quality assessment. For the blastomere number we could not show any significance in embryo quality assessment, neither as an individual parameter nor as a combination of observed parameters.

With respect to blastomere number on day 3, embryos with a minimum of 6 cells were considered good quality embryos. Van Royen et al.[11] set a lower limit of seven blastomeres on day 3. But if we look at the frequency distribution of the number of blastomeres shown in Table II, we see that in our case the frequencies were more dispersed and more day 3 embryos that had implanted had a smaller number of cells (4, 5 or 6). Which is not surprising as there were also more embryos on day 2 with only 2 or 3 blastomeres. Besides, it should be taken into account that embryo development differs in different culture conditions. In our previous study we found that the cleavage rate was lower and the fragmentation rate higher in embryos cultivated up to day 2 in IVF medium than in those cultivated in BlastAssist Medium M1 (MediCult).[15] We might infer from our results that for good developmental competence of the embryo it is important that cleavage of blastomeres of embryos in the early preimplantation period is continuous, irrespective of when the first zygote cleavage occurred and irrespective of the length of cell cycles, i.e. independently of the blastomere number one or two days post fertilization.

We can conclude that with the data given in our study, no prediction of implantation could be demonstrated for the individual criteria for embryo quality assessment. The only good predictive factor of pregnancy was embryo quality, defined concurrently by blastomere fragmentation and blastomere cleavage dynamics.

Acknowledgement

The authors wish to thank Marijana Gajšek-Marchetti, translator from the Medical Research Department, for her contribution in preparing the manuscript.

References

1. Gardner DK, Schoolcraft WB, Wagley L, Schlenker T, Stevens J, Hesla J. A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. Hum Reprod 1998; 13: 3434–40.
2. Milki AA, Hinckley MD, Fisch JD, Dasig D, Behr B. Comparison of blastocyst transfer with day 3 embryo transfer in similar patient populations. Fertil Steril 2000; 73: 126–9.
3. Cummins JM, Breen TM, Harrison KL, Shaw JM, Wilson LM, Hennessey JF. A formula for scoring

human embryo growth rates in *in vitro* fertilization: its value in predicting pregnancy and in comparison with visual estimates of embryo quality. J *In Vitro* Fertil Embryo Transfer 1986; 3: 284–95.
4. Puissant F, Van Rysselberge M, Barlow P, Deweze J, Leroy F. Embryo scoring as a prognostic tool in IVF treatment. Hum Reprod 1987; 2: 705–8.
5. Claman P, Randall Armant D, Seibel MM, Wang TA, Oskowitz SP, Taymor ML. The impact of embryo quality and quantity on implantation and the establishment of viable pregnancies. J *In Vitro* Fertil Embryo Transfer 1987; 4: 218–22.
6. Steer CV, Mills CL, Tan SL, Campbell S, Edwards RG. The cumulative embryo score: a predictive embryo scoring technique to select the optimal number of embryos to transfer in an in-vitro fertilization and embryo transfer programme. Hum Reprod 1992; 7: 117–9.
7. Giorgetti C, Terriou P, Auquier P, Hans E, Spach JL, Salzmann J, et al. Embryo score to predict implantation after in-vitro fertilization: based on 957 single embryo transfers. Hum Reprod 1995; 10: 2427–31.
8. Ziebe S, Petersen K, Lindenberg S, Andersen AG, Gabrielsen A, Nyboe Andersen A. Embryo morphology or cleavage stage: how to select the best embryos for transfer after in-vitro fertilization. Hum Reprod 1997; 12: 1545–49.
9. Hsu MI, Mayer J, Aronshon M, Lanzendorf S, Muasher S, Kolm P, et al. Embryo implantation in *in vitro* fertilization and intracytoplasmic sperm injection: impact of cleavage status, morphology grade, and number of embryos transferred. Fertil Steril 1999; 72: 679–85.
10. Minaretzis D, Harris D, Alper MM, Mortola JF, Berger MJ, Power D. Multivariate analysis of factors predictive of successful live births in *in vitro* fertilization (IVF) suggests strategies to improve IVF outcome. J Assist Reprod Genet 1998; 15: 365–71.
11. Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G, et al. Characterization of a top quality embryo, a step towards single-embryo transfer. Hum Reprod 1999; 14: 2345–9.
12. Desai NN, Goldstein J, Rowland DY, Goldfarb JM. Morphological evaluation of human embryos and derivation of an embryo quality scoring system specific for day 3 embryos: a preliminary study. Hum Reprod 2000; 15: 2190–6.
13. Reljič M, Vlaisavljević V. The preovulatory serum estradiol pattern in natural IVF/ICSI cycles. J Assist Reprod Genet 1999; 16: 535–9.
14. Zayed F, Lenton EA, Cooke ID. Natural cycle in-vitro fertilization in couples with unexplained infertility: impact of various factors on outcome. Hum Reprod 1997; 12: 2402–7.
15. Kovačič B, Vlaisavljević V, Reljič M, Gavrić Lovrec V. Clinical outcome of day 2 versus day 5 transfer in cycles with one or two developed embryos. Fertil Steril 2002; 77: 529–36.