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Pronuclear scoring as a predictor of embryo quality in *in vitro* fertilization program

Magdalena Depa-Martynow, Piotr Jedrzejczak, Leszek Pawelczyk

Division of Infertility and Reproductive Endocrinology, Department of Gynecology and Obstetrics, Karol Marcinkowski University of Medical Sciences in Poznań, Poland

Abstract: Many strategies have been proposed for the selection of viable embryos for transfer in human assisted reproduction. These have included morphological scoring criteria for 20, 28, 44 and 68 h after insemination. The embryo selection is based on morphology, degree of fragmentation and development to the 8-cell. All have shown some correlation with implantation. However, the overall success of these methods is still limited, with over 50% of all transferred embryos failing to implant. Pronuclear zygote morphology has gained much attention recently due to its positive value in predicting implantation and pregnancy. This prospective study involved 178 conventional IVF patients only. The key aspects of pronuclear scoring and namely the presence of a cytoplasmic halo were related to day 3 of development and morphology in a retrospective study. The Z-score and the presence/absence of a halo had significant effect on the rate of development on day 3 embryo. Low Z-score result in slow development and poor morphology. The absence of a halo also resulted in slow and poor development, low morphology, increased fragmentation.

Key words: Pronuclear scoring - Embryo quality - Assisted reproduction

Introduction

Precise selection of embryos and prediction of implantation is probably the most pressing issue in assisted reproduction. Various embryo scoring system have been described to assess the developmental potential of human day 2 or 3 preimplantation embryos. In the most commonly used systems the blastomeres cleavage rate (i.e. number of blastomeres), the shape and size of the blastomeres and the amount of anucleated fragments are estimated. Several studies have shown that regardless of minor fragmentation, the optimal cleavage rate would be the most important criteria when selecting embryos for transfer [1, 2]. Variation in zona thickness, embryo symmetry and the presence of multinucleated blastomeres has also been shown to affect implantation rates [1, 3, 4].

In recent years, there has been growing interest in the assessment of pronuclear morphology to select the most viable and competent embryos. In this regard in vitro fertilization (IVF) human pronucleate zygotes are scored on the basis of pronuclear alignment, size, number, equality and distribution of nucleoli, cytoplasmic heterogenity and presence or absence of cytoplasmic halos [5, 6, 7, 8]. This scoring system correlated positively with the implantation and delivery rates in pronuclear stage transfers. Many different pronuclear scoring systems have been proposed to select high- quality embryos. Unfortunately, there is no standard zygote grading system used thought assisted reproduction laboratories. The same holds true for other systems of evaluating embryo morphology. As a result, comparisons of the systems used correlations of embryo quality with success rates between different laboratories are vague. Two main systems for assessing pronuclear morphology were developed by Scott and Smith (1998) and Tessarik *et al.* (2000) [5, 9]. A pronuclear scoring system, as well as other embryo development markers and patient status, may be useful in determining the number and quality of embryos to transfer. The early scoring parameters currently used in clinical settings have a sound biological basis that reflects on the physiological process communication of the gametes and zygote forming.

Correspondence: Magdalena Depa-Martynow, Division of Infertility and Reproductive Endocrinology, Department of Gynecology and Obstetrics, University of Medical Science, 33 Polna St., 60-535 Poznań, Poland; tel.: (+4861) 8419604, fax: (+4861) 8419612, e-mail: magdzik_24@o2.pl

Materials and methods

A total of 787 zygotes from 178 IVF cycles were investigated in the Division of Infertility and Endocrinology of Reproduction in Poznań, between September 2005 and December 2006. All patient enrolled in this study were female factor of infertility and were treated by conventional IVF.

For ovarian stimulation 150-300 IU human menopausal gonadotrophin (Menopur, Ferring) injections were started daily beginning from day 3 and after verification of pituitary suppression with the gonadotrophin - releasing hormone agonist triptorelin (Decapeptyl 0.1 mg/day, Ferring). Triptorelin injections were started daily on day 21 of the menstrual cycle preceding the treatment cycle.

Final oocyte maturation was triggered with 10 000 IU of human chorionic gonadotrophin (HCG, Pregnyl) when the leading two follicles were >17 mm or the leading cohort measured 15-16 mm in diameter. Transvaginal ultrasound guided oocyte retrieval was performed under conscious sedation at 35-36h post - HCG. Oocytes were collected directly in to warmed (37°C) G-MOPS Plus medium (Vitrolife, Sweden) and placed in CO2 incubator (Heracell, Heraeus).

Men's ejaculate after liquefaction, were analyzed for sperm density, motility and morphology. Spermatozoa were purified by centrifugation through discontinuous SpermGrad (Vitrolife), and prepared with swim - up method.

For fertilization a conventional IVF procedure were used. All retrieved oocyte - corona-cumulus -complexes were placed in 4-well dishes with G-FERT Plus medium (Vitrolife, Sweden) and inseminated 100 000 - 300 000 motile sperm after 3 h. from oocyte retrieval. At about 16-18h after insemination, oocytes were examinated for the presence of pronuclei and polar bodies. Fertilization considered normal when two clearly distinct pronuclei were present.

The presence or absence of a cytoplasmic halo. About 16-18h after fertilization a differential cytoplasmic distribution for pronuclear embryos was reported. According to the appearance (presence) of a halo a halo-positive zygote or a halo-negative zygote were classified (Fig. 1).

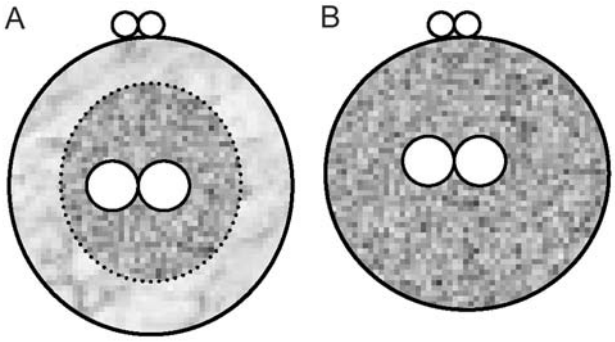

Fig. 1. The presence or absence od a cytoplasmic halo: Halo-positive zygote (**A**); Halo-negative zygote (**B**).

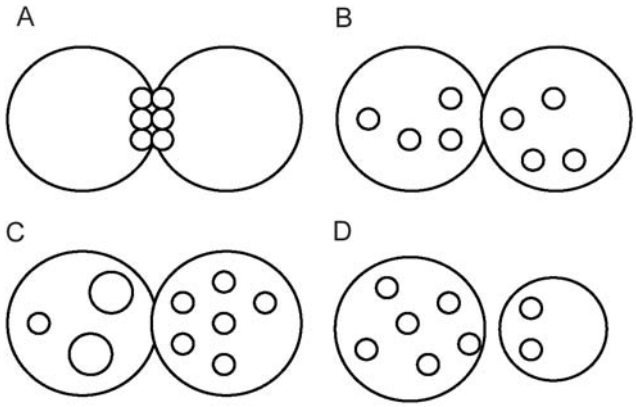

Fig. 2. S-score: Z1 zygote (**A**); Z2 zygote (**B**); Z3 zygote (**C**); Z4 zygote (**D**).

The Z-Score. The zygotes were scored according to the Z-scoring system [10]. The system took account of nuclear size and alignment and nucleoli (nucleolar precursor bodies, NPB) number and distribution.

Briefly, Z-1 zygotes had equal numbers of NPB aligned at the pronuclear junction. The absolute number was not counted but was between three and six. Z-2 zygotes had equal number and sizes of nucleoli (between three and six) which were equally scattered in the two nuclei. Z-3 zygotes had equal numbers of NBP of equal sizes in the same nuclei but with one nucleus having alignment at the pronuclear junction and the other with scattered nucleoli. Zygotes with unequal numbers (a difference of more than two nucleus) and /or sizes of nucleoli were also considered as Z-3. Z-4 zygotes were those with pronuclei that were separated, of very different sizes or periphery located (Fig. 2).

Day 3 embryo scoring. The morphology of an embryo was noted 68h (day3) after insemination. The embryos grade A-D according to degree of cytoplasm fragmentation and the number of blastomeres was scored. Grade A contained the best embryos: at least 7 blastomeres (7-9 blastomeres) and maximum 20% of cytoplasm fragmentation. Grade B embryos have 7-9 cells also but with over 20% of cytoplasmic fragmentation. Grade C has 4-6 cells embryos with maximum 20% fragmented cytoplasm and finally grade D, contained the worst (morphologically lower) embryos with 4-6 cells and over 20% of fragmentation (Fig. 3).

Statistical analysis. Results were expressed as percentages. Statistical analysis of the data was performed using Excel program and Student's test of the Statistica 5.0 software. The comparisons were made by using the $\chi 2$-test with continuity correction.

Results

A total of 787 pronuclear oocytes were obtained in our laboratory over a 16-month period. Each pronucleated oocyte was assessed individually for:

1. The presence or absence of a cytoplasmic halo,
2. Z-scoring.

Each of these two morphological parameters of zygote was correlated with grades of embryos: A, B, C. D.

The presence or absence of a cytoplasmic halo

In all, 787 pronuclear oocytes an accurate scoring of presence or absence of a cytoplasmic halo was checked. A total of 628 (79.8 %) zygotes was included in group of zygotes with halo positive effect and 159 (20.2%) in group of zygotes without cytoplasmic halo. The comparison of a grade A, B, C and D day 3 embryos and presence or absence of a cytoplasmic halo was evaluate.

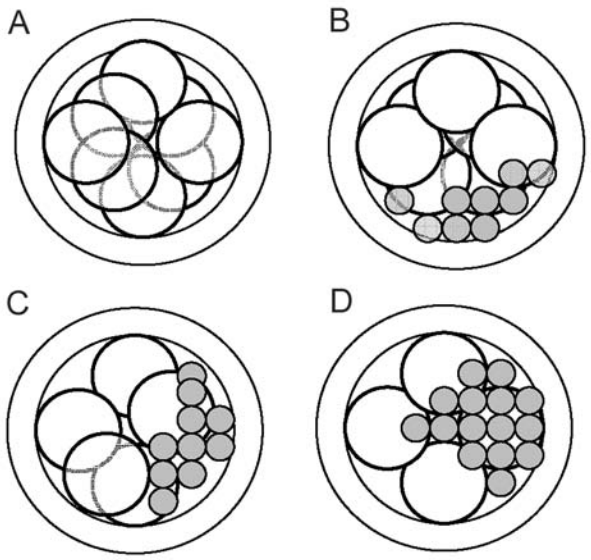

Figure 3. Embryos grade: grade A (**A**); grade B (**B**); grade C (**C**); grade D (**D**).

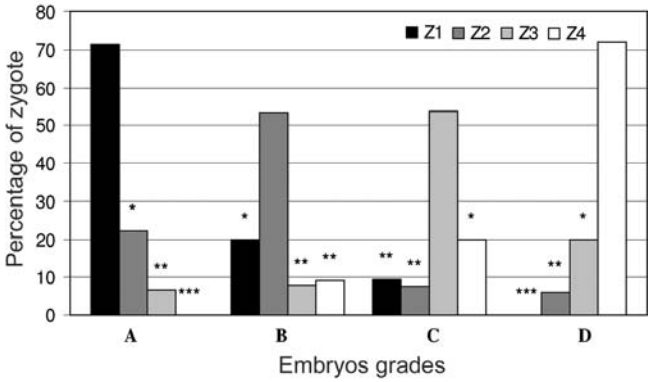

Figure 4. Percentages of embryo grades A, B, C and D obtained from zygote type Z1, Z2, Z3 and Z4. All embryos were scored for day 3 morphology regardless of Z-score. The score for each Z-score group was then analyzed. There was a significant difference in the day 3 morphology embryos between the four Z-groups. From Z1 and Z2 there was an increase in grades A and B embryos respectively. The Z3 and Z4 pronuclear embryos had poor day 3 morphology mostly (grade C and D embryos)
* - represents statistical significance for quality of embryo, P value <0,001
** - represents statistical significance for quality of embryo, P value <0,0001
***- represents statistical significance for quality of embryo, P value <0,00001

During the study period 358 (57.0%) grade A embryos, 191 (30.4%) grade B embryos, 79 (12.6%) grade C embryos and none (0%) grade D embryos derived from halo positive zygotes was observed. From a total of 159 halo negative zygotes 3 (1.9%) grade A embryos, 37 (23.0%) grade B embryos, 52 (32.7%) grade C embryos and 67 (42.1%) grade D embryos we obtained. The difference between these two groups for grade A, B, C, and D embryos was statistically different (P<0.001), (P<0.05), (P<0.001), (P<0.001) respectively. The percentages of embryo development from halo positive and halo negative zygotes are shown in Table 1.

Table 1. Percentages of embryos grade A, B, C and D obtained from zygotes with the presence or absence of a cytoplasmic halo. In all, 787 pronuclear oocytes an accurate scoring of presence or absence of a cytoplasmic halo was checked. A total of 628 (79.8%) zygotes was included in group of zygotes with halo positive effect and 159 (20.2%) in group of zygotes without cytoplasmic halo. The comparison of a grade A, B, C and D day 3 embryos and presence or absence of a cytoplasmic halo was evaluate.

Embryos	Halo		
	Positive	Negative	P value
A	57,0 %	1,9 %	^{a}P<0,001
B	30,4 %	23 %	^{b}P<0,05
C	12,6 %	32,7 %	^{c}P<0,001
D	0 %	42,1 %	^{d}P<0,001

The difference between these two groups was statistically different; ^{a}P, ^{b}P, ^{c}P, ^{d}P represents statistical significance for quality of embryo acquired from zygotes with presence and absence of a cytoplasmic halo.

Z- scoring

The day 3 embryos rates showed statistical differences between embryos formed from zygote types Z1, Z2, Z3 and Z4. The number of zygotes reached grade A embryos stage was greater in group Z1 (71,3%) than in group Z2 (22.2%), Z3 (6.5%) and Z4 (0%) (P<0.001), (P<0.0001), (P<0.00001) respectively. Statistical differences among groups were observed in grade B embryos in four types of zygotes. The percentage of grade B embryos was the highest in Z2 group (53.3%). The number of grade B embryos in Z1, Z3 and Z4 zygote group was significant lower (P<0.001), (P<0.0001), (P<0.0001) respectively. The presence grade C embryos was also observed. The most grade C embryos developed from Z3 zygotes. The statistical differences were observed between Z3 zygote group and Z1 zygote group (P<0.0001), Z2 zygote group (P<0.0001), and Z4 zygote group (P<0.001). The percentage of grade D embryos was significantly higher in Z4 zygotes group than in Z1 zygotes group (P<0.00001), Z2 zygotes group (P<0.0001) and Z3 zygotes group (P<0.001) (Fig. 4)

Discussion

Assessment of development and implantation potential of embryos is a great importance for selection of embryos for transfer, so as to achieve high pregnancy rates associated with a low number of multiple pregnancies. For that purpose, scoring of zygotes

and cleavage stage embryos has been proposed to identify and to select embryos for fresh transfer [7,11].

The finding of a positively correlation of appearance of a cytoplasmic halo and embryo quality is in accordance with a previous study [7,12,13]. The halo effect has been variously attributed to cytoplasmic rotation or movement or to the differential distribution of mitochondria [14].

Payne *et al.* (1997) were the first to report a subplasmalemmal zone of translucent cytoplasm immediately prior to formation of the male and female pronucleus [14]. Subsequently, this focal clearing within the cortical cytoplasm (cytoplasmic flare) often progress to involve the entire cytocortex (halo). This phenomenon is thought to be the manifestation of a microtubule - organized translocation of mitochondria and other cytoplasmic components to the centre of the oocyte, since virtually no detectable mitochondria were found in the cortical region of fertilized oocytes. More likely, mitochondria concentrate in the perinuclear cytoplasm in an ellipsoidal mass [15]. The physiological role of mitochondrial redistribution in human zygotes is unknown but it may be speculated that clustering of mitochondria to perinuclear regions may be involved in cell cycle regulation by means of calcium mobilization and ATP liberation [15-19]. In addition, location of mitochondria next to the pronuclei would allow immature mitochondria, as seen in zygotes, to complete maturation presuming that some input from the nucleus is needed [17, 20].

Another parameter evaluated in zygote morphology is the appearance of a nucleolar precursor bodies (NPB) during pronucleus formation. The data presented in this paper indicate that, the morphology of the pronuclear oocyte has a direct effect on continued in vitro development, namely increased quality of day 3 embryos, giving a greater chance for successful implantation and pregnancy.

Previous studies have also clearly demonstrated the effect of this parameter on developing embryo [7,12, 21-24]. Since the oocyte is ovulated with all the machinery in place for these initial cell cycles, it is unlikely that nuclear events will have much of an effect [25]. The one aspects: equal number of NPB between the nuclei is a necessary event in any mitotic cell. Unequal number of NPB results in abnormal cell cycles, which may be the foundation of cancer cells [12]. The asynchrony in the numbers and pattern of NPB between the nuclei leads also to the lack of polarization [15]. The coalescence and reformation of functional nucleoli has profound effect on the ability of cells to grow and function [23].

Embryos with equal number of NBP, Z1 and Z2, have normal development and good morphology. The lack of polarization of the NPB in the Z3 and Z4 embryos is a morphological indication of a lack of chromatin polarization, which will lead to abnormal, slower and much more poor development as recorded in this data.

Generally cell cycle events are controlled by checkpoints, or surveillance systems, that either block or allow cell cycle transitions. Incomplete, incorrect or damaged transitions or events are stopped by these mechanisms [26]. Some of these surveillance mechanisms operate to ensure that chromosome duplication and completion occur correctly, and that chromosome alignment on the spindle is both timely and spatially correct. If incorrect alignment is detected, a signal is transduced /initiated, which stops the system until the correct alignment is attained [27]. This could account for the slower development, more fragmentation and abnormal cleavage and finally fewer embryos continuing development to the 3 day stage [25]. When transferred, they result in fewer implantation [5, 10, 28-31].

The present study and the results of other published studies confirm that when there exists polarization of NPB in both pronuclei and the pronuclei are similar size (Z1) and additionally a halo is observed, embryo quality and development is improved. The pronuclear morphology scoring is an additional criterion for selecting embryos for extended culture. The Z-score and halo score in combination with day 3 embryo morphology is useful in determining the most suitable number of embryos for transfer, and achieving the optimal chance of conception while reducing the risk of high order multiple pregnancy.

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