Pronuclear score improves prediction of implantation rate in ICSI cycles

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Abstract

**Background:** In assisted reproduction technology embryo competence is routinely evaluated on morphological criteria but their efficacy remains relatively low. Additional information could be obtained by evaluation of pronuclear (PN) morphology. Up to now controversial results have been reported about prognostic value of PN score. One of the main limitation of literature data is the use of different methods of PN classification. To this regard, in 2011 the ESHRE and Alpha Scientists in Reproductive Medicine defined three PN categories to standardize the zygote assessment. In this study we evaluated whether the consensus ESHRE-Alpha system for the pronuclear scoring could be an useful additional criterion to improve prediction of embryo implantation potential.

**Methods:** This is a retrospective, longitudinal cohort study. We included 3004 zygotes from 539 women who underwent ICSI treatment at our Center between January 2014 and June 2019. The pronuclear were categorized as score 1: symmetrical, 2: non-symmetrical, 3: abnormal. A subset of 110 zygotes did not cleaved. On day 2-3 1163 embryos were transferred, 232 arrested, and 9 were cryopreserved. Among the 1490 embryos cultured up to day 5-7, 516 became blastocysts: 123 were transferred on day 5 and 393 were cryopreserved. Relationship between pronuclear score and cleavage rate, quality of embryos, blastocyst development, and implantation rate was evaluated by chi-square test. Multivariate regression analyses corrected the results for putative confounders (age of patient; infertility cause; cleavage-stage embryo morphology grade; day of transfer).

**Results:** There was not significant difference in patients’ age, cleavage rate and embryo morphology among the three pronuclear score groups. No reduction of blastulation rates was found in score 2-3 (34%) groups respect to score 1 (35%). The pronuclear score 1-embryos had a higher implantation rate respect to score 2-3-ones (15% and 9%, respectively, P=0.0121; OR 0.46; 95% CI 0.25-0.76, P=0.004). Consistently, the pronuclear score remained predictive of implantation in top quality embryos (OR 0.51; 95% CI 0.28-0.87, P= 0.01).

**Conclusions:** The consensus pronuclear score may be routinely included among criteria for embryo evaluation to increase patient’s chance of becoming pregnant.

Introduction

In reproductive medicine identifying the embryo(s) with the highest implantation potential is still an unmet challenge, and it is a fundamental step toward single embryo transfer. During the years, several approaches have been proposed for embryo viability evaluation including embryo morphology, study of metabolic activity, extended culture, and both invasive and non-invasive preimplantation genetic testing [1, 2, 3, 4]. Various embryological parameters have some predictive value for implantation potential. However, the overall success of these markers is still limited, with over 50% of transferred embryos failing to implant. Even invasive preimplantation genetic testing failed to improve overall pregnancy outcomes in a randomized controlled trial [5]. Thus, the search for new, more reliable markers of embryo viability
continues. For instance, it has been proposed that additional information on embryo viability potential could be obtained by evaluation of pronuclear (PN) morphology based on zygote features 16–18 hours after fertilization. Female pronucleus originates near the second polar body, whereas the male pronucleus appears at the center of the cytoplasm. Following their formation, the female pronucleus migrates towards the male one until they become in close apposition. Spherical bodies called nucleolar precursor bodies (NPB) are randomly allocated within the pronuclei. They appear shortly after fertilization and persist throughout the first cell cycles [6]. Differently from initial suggestions [7], the NPB are not precursors of nucleoli and they structurally support building of functional nucleoli when transcription resumes in early embryos [6]. Nucleoli are sites of pre-ribosomal RNA (rRNA) synthesis and the newly synthesized rRNAs are necessary for the translational process when the embryonic genome becomes fully activated [8]. Progressively, polarization of NPB occurs, and this phenomenon controls the design of the embryonic axis, a fundamental step for cell determination in the developing embryo [9]. Alterations of these strictly related events may have abnormal consequences, including fertilization failure and uneven cleavage.

Since the preliminary observations on PN formation and morphology, several scoring systems were proposed mainly taking into account the PN size, the NPB position and alignment [10]. Although the scores have been correlated with improved embryo development as well as with increased pregnancy and implantation, to date clinical data about the relationship between zygote morphology and IVF outcomes are conflicting [10]. One of the main limitation of literature data is the use of different zygote grading systems. To this regard, in 2011 the ESHRE-Alpha consensus defined three PN categories to standardize the zygote assessment: symmetrical, non-symmetrical and abnormal [1]. The symmetrical category includes zygotes showing two polar bodies, two centrally located and juxtaposed pronuclei, equal size and equivalent numbers and size of NPB equatorially aligned at the membrane juxtaposition. All the zygotes differing from this ideal configuration are included in the non-symmetrical category. The abnormal category includes zygotes with no or one NPB. No studies verified the efficacy of such classification so far.

The aim of this retrospective study was to assess whether the consensus ESHRE system for the PN scoring could be an useful additional criterion to improve prediction of embryo implantation potential.

**Methods**

**Study design, size, duration.** We included 3004 zygotes from 539 women (mean age: 35.6 years; range: 21–43) enrolled at our center between January 2014 and June 2019. Selection of the zygotes to be included in the study was done on the basis of knowledge of their outcome: when 2 embryos from zygotes with different PN scores were transferred and only one implanted, these zygotes were excluded from the study. We included only homolog cycles using fresh eggs and ejaculated sperm. Another exclusion criterion was standard IVF, in order to standardize the fertilization check timing.
Embryo transfers (ET) were routinely performed on day 2–3 and, when available, two cleavage-stage embryos were transferred. Surplus embryos were cultured up to day 5–7 and those that developed up to blastocyst stage were cryopreserved. The ET were performed on day 5 in those cases with at least four good quality cleavage-stage embryos owing to the benefit from further observation in selecting the best embryos to transfer. On day 5 only elective single-embryo transfer were performed. In the study we included: i) cleavage-stage ET; ii) blastocyst-stage ET. Implantation rate was defined as fetal cardiac activities at 12 weeks of gestation divided by number of embryo transfers; miscarriages were excluded.

**Outcomes measures.** The primary outcomes were cleavage rate, quality of embryos, blastocyst development in relation to the PN score, and their predictive factors. The secondary outcome was implantation rate in relation to the PN score of i) cleavage-stage embryos; ii) blastocyst-stage embryos, and their predictive factors. We also evaluated the outcome of the newborns collecting their birthweights (expressed as percentile and SD-score for gestational age, according the World Health Organization reference curves).

**Patients treatment.** Standard controlled ovarian stimulation protocols were used. Pituitary suppression was achieved with either Gonadotropin Releasing Hormone agonists or antagonists. Stimulation with gonadotropins was monitored by measuring serum estradiol levels and follicle growth. The trigger was either recombinant or urinary human chorionic gonadotropin or agonist trigger. Cumulus-oocyte complexes were collected 36 hours later, washed in Sydney IVF Gamete buffer (Cook Medical, Sydney, Australia) and immediately incubated in Sydney IVF Fertilization medium (Cook Medical) at 37 °C in a humidified atmosphere of 6% CO₂, 5% O₂ (Galaxy 48R incubators, New Brunswick Scientific, Edison, NJ, USA).

**Standard embryo culture.** After 2 hours of incubation, the oocytes were denuded in HEPES-buffered medium (Sydney IVF Gamete medium, Cook Medical) containing 20 IU/ml of Hyaluronidase (Origio, Målov, Denmark). ICSI was performed immediately after denudation. Sperm samples were treated with a two-layer density gradient system (Sydney IVF Sperm Gradient, Cook Medical) or via Swim-up using Sydney IVF Gamete Buffer (Cook Medical). Incubations were performed at 37 °C in a humidified atmosphere of 6% CO₂, 5% O₂ (Galaxy 48R incubator; New Brunswick Scientific). Fertilization was assessed 16–18 hours after injection (PN score 1: symmetrical, score 2: non-symmetrical, score 3: abnormal) [1], and embryos with two pronuclei were individually cultured from day 1 to day 3 into Sydney IVF Cleavage medium (Cook Medical) and from day 3 to day 5–7 in Sydney IVF Blastocyst medium (Cook Medical).

Standard day 2–3 embryo and blastocyst morphological assessment was carried out according to the current consensus system [1]. Arrested embryos were non-viable embryos in which development arrested for at least 24 hours, or in which all the cells degenerated or fragmented.

**Statistical analyses.** Categorical variables were presented as percentages with 95% CI. Continuous variables were presented as mean ± SD and range. In univariate analysis the relationship between the PN
score and cleavage rate, quality of embryos, blastocyst development, implantation rate was evaluated by chi-square test. Logistic regression analyses corrected the results for putative confounders upon the outcomes under investigation: age of patient; infertility cause; cleavage-stage embryo morphology grade; day of ET. All the possible predictors detected as significant at univariate analysis were considered as independent variables for the multivariate regression analysis models. Analyses were carried out by MedCalc® software (Mariakerke, Belgium) and R software, v. 3.5.2. A P value < 0.05 was considered significant.

Results

Among the embryos originated from the 3004 zygotes enrolled in the study, 1163 embryos were transferred into uterus at their cleavage-stage, 1490 were cultured up to day 5–7, 232 arrested on day 3, 9 were cryopreserved on day 2–3; 110 zygotes did not cleaved. Among the 1490 embryos whose culture was extended up to day 5–7, 516 became blastocysts: 123 were transferred into uterus on day 5 and 393 were cryopreserved (226 on day 5, 156 on day 6, and 11 on day 7).

Relationship between PN score and embryo quality at cleavage stage. A total of 2280 (76%) score 1, 645 (21%) score 2, and 79 (3%) score 3 zygotes were obtained. There was not a significant difference in patients' age, cleavage rate and day 2–3 embryo morphology among the three PN score groups (Table 1). Moreover, the PN score did not always correlate with the embryo grade: only 58% top quality zygotes formed grade 1 embryos, and 53% poor quality zygotes (score 3) also became high quality embryos (Fig. 1).

Table 1
Relationship between PN score and embryo quality parameters.

| PN score | 1 | 2           | 3           |
|----------|---|-------------|-------------|
| N. zygotes (%) | 2280 (76%) | 645 (21%)   | 79 (3%)     |
| Patients' age (mean ± SD, years) | 35.7 ± 4.4 | 35.3 ± 4.8 | 34.4 ± 4.1 |
| Cleavage rate | 97% | 96%         | 94%         |
| N. grade 1 embryos (%) | 1325 (58%) | 320 (52%)  | 42 (53%)   |
| N. grade 2 embryos (%) | 578 (25%) | 173 (27%)  | 18 (23%)   |
| N. grade 3 embryos (%) | 139 (6%) | 70 (11%)   | 3 (4%)     |
| N. arrested cleavage-stage embryos (%) | 161 (6%) | 55 (8%)    | 11 (14%)   |
| Blastulation rate | 35% (384/1106) | 33% (114/346) | 47% (18/38) |

Relationship between PN score and blastocyst development. Blastocyst development rate was similar among the three PN score-derived embryo groups, without any statistically significant differences (Table 1). A total of 1106 PN score 1-, 346 score 2-, and 38 score 3-derived embryos were placed in
extended culture. We did not find any reduction of blastulation rates in score 2–3 (132/384, 34%) groups respect to score 1 (384/1106, 35%) (Table 1). Notably, a single NPB was observed in the majority of the abnormal score 3 PN category (75/79 cases) and 45% of cases (17/38) developed up to blastocyst stage when prolonged culture was performed.

First, we compared PN score and timing of blastulation. Among the 384 PN score 1-derived embryos that reached the blastocyst stage, 191 (50%) showed blastocele expansion (grade ≥ 3) on day 5; 77 (20%) embryos were early blastocysts on day 5, and 116 (30%) full blastocysts later on day 6. Similarly, among the 132 PN score 2- and 3-derived embryos that reached the blastocyst stage, 59 (45%) showed blastocele expansion on day 5, 25 (19%) were early blastocysts on day 5 (n = 22) or day 6 (n = 3), and 48 (36%) full blastocysts later on day 6 (Fig. 2A).

Second, we stratified embryos according to ICM and TE morphological grade. From PN score 1-derived embryos there were 232/384 (60%) ICM grade A blastocysts (153 on day 5, 79 on day 6–7), 68/384 (18%) ICM grade B blastocysts (35 on day 5, 33 on day 6–7), and 7/384 (2%) ICM grade C blastocysts (3 on day 5, 4 on day 6). Similarly, from PN score 2 and 3-derived embryos there were 77/132 (58%) ICM grade A blastocysts (42 on day 5, 35 on day 6–7), 26/132 (20%) ICM grade B blastocysts (15 on day 5, 11 on day 6–7), and 4/132 (3%) ICM grade C blastocysts (2 on day 5 and 2 on day 6–7) (Fig. 2B).

From PN score 1-derived embryos there were 127/384 (33%) TE grade A blastocysts (89 on day 5, 38 on day 6–7), 154/384 (40%) TE grade B blastocysts (94 on day 5, 60 on day 6–7), and 26/384 (7%) TE grade C blastocysts (8 on day 5, 18 on day 6–7). From PN score 2- and 3-derived embryos there were 51/132 (39%) TE grade A blastocysts (32 on day 5, 19 on day 6), 49/132 (37%) TE grade B blastocysts (24 on day 5, 25 on day 6–7), and 7/132 (5%) TE grade C blastocysts (3 on day 5, 4 on day 6–7) (Fig. 2C).

**Relationship between PN score and clinical outcome.** We analyzed 1286 transferred embryos from 463 patients, in which the outcome for all embryos was known. Due to the paucity of score 3 PN zygotes, we merged score 2 and score 3 PN-derived embryos in the subsequent analyses. Univariate analysis disclosed a higher implantation rate for PN score 1- respect to PN score 2-3-derived embryos (15% and 9%, respectively, P = 0.0121). This behavior was observed for both ET types, with statistically significant differences reached for the cleavage-stage group (15% vs. 7%, P = 0.0043), due to its numerousness (Fig. 3). Three out of 21 transferred abnormal score 3 PN zygotes - specifically with a single NPB - were observed in the implanted embryo group.

The multivariate logistic regression analysis model showed that embryos from score 2–3 zygotes had a significant lower implantation rate than those from top quality zygotes (OR 0.46; 95% CI 0.25–0.76, P = 0.004) (Table 2).
Table 2
Multivariable logistic analysis: predictors of implantation of all embryos (n = 1286)

| Predictor                    | OR of implantation (95% CI) | P-value |
|------------------------------|-----------------------------|---------|
| PN score                     |                             |         |
| 1                            | 1                           |         |
| 2–3                          | 0.53 (0.32–0.82)            | 0.006   |
| Cleavage stage morphology    |                             |         |
| 1                            | 1                           |         |
| 2                            | 0.43 (0.24–0.70)            | 0.001   |
| 3                            | 0.16 (0.03–0.54)            | 0.01    |
| Age                          |                             |         |
| <35                          | 1                           |         |
| ≥35                          | 0.35 (0.24–0.48)            | <0.001  |
| ET day                       |                             |         |
| Day 2–3                      | 1                           |         |
| Day 5                        | 1.68 (1.02–2.72)            | 0.03    |

A significant prediction of implantation was also found analyzing only embryos from PN score 1 zygotes versus PN score 2 ones (OR 0.49; 95% CI 0.27–0.83, P = 0.01), demonstrating that the few PN score 3 zygotes do not influence the results (see Additional file 1).

Consistently, the PN score remained predictive of implantation in the subgroup of embryos with top quality morphology grade on day 2–3 (OR 0.51; 95% CI 0.28–0.87, P = 0.01), even when controlled with the other significant variables identified (Table 3).

Table 3
Multivariable logistic analysis: predictors of implantation for top quality embryos on day 2–3 (n = 955)

| Predictor | OR of implantation (95% CI) | P-value |
|-----------|-----------------------------|---------|
| PN score  |                             |         |
| 1         | 1                           |         |
| 2–3       | 0.51 (0.28–0.87)            | 0.01    |
| Age       |                             |         |
| <35       | 1                           |         |
| ≥35       | 0.34 (0.23–0.49)            | <0.001  |
| ET day    |                             |         |
| Day 2–3   | 1                           |         |
| Day 5     | 1.55 (0.901–2.62)           | NS      |

Perinatal characteristics of newborns. We excluded from this analysis outcome data of ET of two embryos with discordant PN scores that resulted in delivery of only one baby. A total of 104 neonatal outcomes from PN score 1 embryos (of which 15 from twin gestations) and 4 from PN2 ones (all from...
single pregnancies) were available. As detailed in Additional file 2 no statistical analyses could be performed due to the small number of evaluable newborns in PN2 score group. We observed absence of twin pregnancies in PN2 score, that accounts for the higher birthweights and longer gestational period in PN2 respect to PN1 score group. In PN1 score group 8 pregnancies started as dichorionic-diamniotic twin pregnancies resulted in the live birth of only one of the fetuses: in 7 cases there was a spontaneous first trimester abortion of one twin and in one case the patient adopted for an elective pregnancy termination for a congenital megabladder. Among singleton pregnancies, there was another elective termination for a major central nervous system malformation. No stillbirths as well as no malformations were recorded among the newborns of both score groups.

Discussion

To date, literature data about the correlation among zygote morphology, biological and clinical outcomes are inconclusive, mainly due to different methods used for PN scoring system, the time of PN observation, and the insemination procedure [10].

This is the first study that evaluated the prognostic effect of the ESHRE consensus PN scoring system by a multivariate analysis. We did not find any relationship among PN score, embryo quality at cleavage and blastocyst stages, and blastulation rate, according to some previous studies [11, 12, 13, 14, 15, 16, 17]. Intriguingly, half embryos from a single NPB zygote developed up to blastocyst stage and three successfully implanted. These data suggest that embryos derived from abnormal PN zygotes have some development potential, as observed after time-lapse imaging [18].

We demonstrated that the PN score improves prediction of implantation of cleavage-stage good morphology embryos, regardless of the day of transfer. Moreover, PN scores 2 and 3 were associated with a lower implantation rate, even though the morphology of the embryos was good. Based on these findings we argue that PN score may provide a non-invasive, early criterion helpful for selecting the best embryo(s) for transfer, particularly when similarly quality embryos are available.

The 2 and 3 PN patterns may be characterized by asynchrony in the formation and polarization of pronuclei. Such alteration of normally sequential, linked events can be at the origin of chromosomal abnormalities, whose consequences may appear at the implantation phase, after the embryonic genome activation [19, 20, 21]. This proposition would explain why we found a significant positive association between PN score 1 and implantation, without any evident effect of PN morphology on in vitro embryo developmental potential.

Most of embryos at pronuclear stage check are at S or G2 phase when chromosomes are interconnected via nucleoli. As it has been already reported by some authors [22, 23, 24], if zygotes have different PN size and non-synchronous NPB there is an increased risk of embryo aneuploidy, due to disturbance in chromosome duplication and division. Therefore, the PN score may help selection of those top quality embryos that have a chromosomal euploid set, in absence of a genetic embryo assessment.
As observed in this study, top quality zygotes can become low quality embryos as well as top quality embryos can develop from low quality zygotes. In other words, neither zygote, nor embryo morphology alone are fully predictive of IVF outcome. Therefore, we believe that a combination of assessments and scores, including the PN score, may be helpful in non-invasive embryo selection.

Time-lapse monitoring of embryo development showed that PN morphology changes within a short time, at 16–20 h after ICSI, mostly from an asymmetry of NPB towards a symmetric or perfectly aligned distribution [18]. Therefore, a single microscopy observation may be misleading and such changes may explain in part the contradictory data in the literature, where based on static observation some authors reported no benefit when PN scoring was applied [14, 25, 26]. Despite the awareness of the dynamicity of the PN formation, our findings could be useful for the majority of laboratories that do not have availability of time-lapse technology. Certainly a critical issue for PN scoring remains the standardization in the observation timing that should take into consideration the method used for insemination. In fact, pronucleus development has an average delay of 4 hours after conventional IVF as compared to ICSI, likely because the need for the spermatozoon to pass through the cumulus and corona cells and the zona pellucida [27].

**Conclusions**

This is the first study of correlation between PN morphology and implantation rate by applying the PN score system proposed by ESHRE and a multivariate analysis which evaluated various potential confounding factors. Although validation in randomized perspective studies is needed, our findings suggest that the PN score could represent the earliest point at which the quality of the fertilized oocyte can be non-invasively evaluated and that the PN score may be routinely included among criteria for embryo evaluation. In this way, a selection based on combination both of zygote and cleavage-stage morphology could assist in selecting the top quality embryo(s) with the highest chances of implantation. This could be of great value for all laboratories performing clinical IVF without any pre-implantation genetic testing means.

**Abbreviations**

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), embryo transfers (ET), European Society of Human Reproduction and Embryology (ESHRE), inner cell mass (ICM), intra cytoplasmic sperm injection (ICSI), in vitro fertilization (IVF), nucleolar precursor bodies (NPB), pronuclear (PN), ribosomal RNA (rRNA), standard deviation (SD), Trophectoderm (TE).

**Declarations**

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Ethical Committee of Regione Liguria (approval n. 356/2019), and each couple signed a written informed consent.

**CONSENT FOR PUBLICATION**

Not applicable.

**AVAILABILITY OF DATA AND MATERIALS**

The datasets analyzed during the current study are available from the corresponding author upon request.

**COMPETING INTERESTS**

The authors declare that they have no competing interests.

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**AUTHORS’ CONTRIBUTIONS**

SS collected biological data and contributed to critical discussion. CM collected clinical data, performed statistical analyses, and contributed to critical discussion. IC performed ICSI cycles as embryologist. FS and PA performed patient recruitment and treatments. VR, PA, AC contributed to critical discussion. PS performed ICSI cycles as embryologist, designed the study, analyzed and interpreted the data, drafted the manuscript, and contributed to critical discussion. All authors read and approved the final article.

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