Association between Number of Formed Embryos, Embryo Morphology and Clinical Pregnancy Rate after Intracytoplasmic Sperm Injection

Associação entre número de embriões formados, morfologia embrionária e taxa de gravidez clínica após injeção intracitoplasmática de espermatozoides

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Abstract

Introduction   Infertility has a high prevalence in the general population, affecting ∼5 to 15% of couples in reproductive age. The assisted reproduction techniques (ART) include in vitro manipulation of gametes and embryos and are an important treatment indicated to these couples. It is well accepted that the implantation rate is positively influenced by the morphology of transferred embryos. However, we question if, apart from the assessment of embryo morphology, the number of produced embryos per cycle is also related to pregnancy rates in the first fresh transfer cycle.

Purpose   To evaluate the clinical pregnancy rate according to the number of formed embryos and the transfer of top quality embryos (TQEs).

Methods   In a retrospective cohort study, between January 2011 and December 2012, we evaluated women who underwent intracytoplasmic sperm injection (ICSI), aged < 40 years, and with at least 1 formed embryo fresh transferred in cleavage stage. These women were stratified into 3 groups according to the number of formed embryos (1 embryo, 2–3 and ≥ 4 embryos). Each group was divided into 2 subgroups according to the presence or not of at least 1 transferred TQE (1 with TQE; 1 without TQE; 2–3 with TQE, 2–3 without TQE; ≥ 4 with TQE; ≥ 4 without TQE). The clinical pregnancy rates were compared in each subgroup based on the presence or absence of at least one transferred TQE.

Keywords
► number of embryos
► morphological evaluation
► embryo quality
► clinical pregnancy
► ICSI

Results   During the study period, 636 women had at least one embryo to be transferred in the first fresh cycle (17.8% had 1 formed embryo [32.7% with TQE versus 67.3% without TQE], 42.1% of women had 2–3 formed embryos [55.6% with TQE versus 44.4% without TQE], and 40.1% of patients had ≥ 4 formed embryos [73.7% with TQE versus 26.3% without TQE]). The clinical pregnancy rate was significantly higher in the subgroup with ≥ 4 formed embryos with at least 1 transfered TQE (45.2%) compared with the subgroup without TQE (28.4%).

Conclusions   Having at least two available embryos and at least one TQE for embryo transfer are predictors of the pregnancy rates.

Resumo

Introdução   A infertilidade tem uma alta prevalência na população geral, afetando ~ 5 a 15% dos casais em idade reprodutiva. As técnicas de reprodução assistida (TRA) incluem a manipulação *in vitro* de gametas e embriões e são um importante tratamento indicado para esses casais. Sabe-se que a taxa de implantação é positivamente influenciada pela morfologia dos embriões transferidos. No entanto, questiona-se, se além da avaliação da morfologia do embrião, o número de embriões produzidos por ciclo também está relacionado com as taxas de gravidez do primeiro ciclo de transferência fresco.

Objetivo   Avaliar a taxa de gravidez clínica de acordo com o número de embriões formados e a transferência de embrião com ótima morfologia (EOM).

Métodos   Em um estudo de coorte retrospectivo, entre janeiro de 2011 e dezembro de 2012, avaliamos mulheres submetidas a ICSI com idade < 40 anos e com pelo menos um embrião formado e transferido a fresco em estágio de clivagem. Estas mulheres foram estratificadas em 3 grupos de acordo com o número de embriões formados (1 embrião, 2–3 e ≥ 4 embriões). Cada grupo foi subdividido em 2 subgrupos de acordo com a presença ou não de EOM transferido (1 com EOM; 1 sem EOM; 2–3 com EOM; 2–3 sem EOM; ≥ 4 com EOM; ≥ 4 sem EOM). As taxas de gravidez clínica foram comparadas em cada subgrupo segundo a presença ou não de pelo menos um EOM transferido.

Resultados   Durante o período do estudo, 636 mulheres tiveram pelo menos 1 embrião para ser transferido no primeiro ciclo a fresco (17,8% possuíram 1 embrião formado [32,7% com EOM versus 67,3% sem EOM], 42,1% das mulheres apresentaram 2–3 embriões formados [55,6% com EOM versus 44,4% sem EOM], e 40,1% das pacientes formaram ≥ 4 embriões [73,7% com EOM versus 26,3% sem EOM]). A taxa de gravidez clínica foi significativamente maior no subgrupo com ≥ 4 embriões formados com transferência de pelo menos 1 EOM (45,2%) comparando-se ao subgrupo sem EOM (28,4%).

Conclusões   Ter pelo menos dois embriões e pelo menos um EOM para transferência são fatores preditivos da taxa de gravidez.

Palavras-chave
► número de embriões
► avaliação morfológica
► qualidade embrionária
► gravidez clínica
► ICSI

Introduction

Infertility, defined as the absence of conception after a year of unprotected sexual activity, has a high prevalence in the general population, affecting ~ 5 to 15% of couples in reproductive age.[1,2] The assisted reproduction techniques (ART) are procedures that include in vitro manipulation of gametes (eggs and sperm) and embryos for the purpose of establishing a pregnancy.[3,4] In developed countries, ~ 5% of all births are resultant of ART, which is an important treatment indicated to infertile couples.[5,6] For ART, women are submitted to controlled ovarian stimulation (COS) with artificial hormones.[7–9] This strategy aims to obtain a greater number of oocytes and embryos, enabling an increase of cumulative pregnancy rate per cycle.[10,11] However, we question whether the total number of formed embryos has an influence in the pregnancy rate in the first fresh transfer cycle.

The conventional morphological evaluation is used as an indirect method for checking the quality and the potential of embryonic development; however, the analyzed parameters still diverge.[12,13] To homogenize the conventional morphological evaluation, experts in the issue created the Istanbul Consensus.[13] Based on this consensus, a top quality embryo (TQE) on the second day of development (D2) has 4 cells Grade 1 (symmetrical blastomeres, with less than 10% of fragmentation and without multinucleation), and on the third day of development (D3), has 8 cells Grade 1.[13] Some studies suggest that the implantation rate is positively influenced by the morphology of transferred embryos: the TQEs, for example, present greater potential for implantation and to generate live births compared with embryos with worse morphology.[12–14]

Thus, we question if, apart from the assessment of embryo morphology, the number of produced embryos per cycle is also related to pregnancy rates in the first fresh transfer cycle. Elucidating the impact of the number of formed embryos together with embryonic morphology can help determine the gestational outcomes, adjusting the expectations of patients and physicians. Therefore, the aim of this study

was to compare the clinical pregnancy rate in the first fresh transfer cycle of women who formed and transferred at least 1 embryo, divided into 6 groups according to the number of formed embryos (1, 2–3 and $\geq$ 4) and transfer or not of at least 1 TQE in D2 or D3.

Methods

This retrospective cohort study evaluated all women who underwent COS for intracytoplasmic sperm injection (ICSI) at the fertility clinic of the Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Brazil, between January 2011 and December 2012. All data were obtained from medical records by one author (MACN). The study protocol was approved by the Institutional Review Board (# 9682/2012), which waived the requirement for additional informed consent due to the retrospective nature of the study.

Women younger than 40 years submitted to COS for ICSI who had at least 1 embryo formed and transferred in cleavage stage in the first fresh cycle were considered eligible. If the patient had been subjected to more than one COS cycle during this period, only data from the first cycle were included in the analysis. All women were followed-up until a negative pregnancy test or until the first ultrasound performed two to three weeks after the positive pregnancy test.

Patients were stratified into 3 groups according to the number of formed embryos in cleavage stage (1 embryo; 2–3 embryos and $\geq$ 4 embryos). Each group was subdivided into two subgroups (with or without TQE), transferred in the first fresh cycle (1 with TQE; 1 without TQE; 2–3 with TQE, 2–3 without TQE; $\geq$ 4 with TQE; $\geq$ 4 without TQE). Patients who had formed two or more embryos transferred only two embryos in the fresh cycle.

All included patients had menstruation programmed with oral combined contraceptives, starting during the previous menstrual cycle. The COS was started five days after the discontinuation of oral contraceptives. The transvaginal ultrasound was performed on the first day of the COS to assess the endometrial pattern and rule out the presence of ovarian cysts. These may interfere in the response to exogenous gonadotropins or in the monitoring of follicle growth by ultrasound.[15]

Three COS protocols could be used: in the standard long protocol, the use of gonadotropin-releasing hormone (GnRH) agonists (leuprolide acetate 0.5 mg/day) was initiated during the luteal phase of the previous cycle, followed by gonadotropin (150–300 UI/day) during the first days of COS. Subsequently, the daily dose of gonadotropin was adjusted according to follicular growth.

In the flexible antagonist protocol, gonadotropin (150–300 IU/day) was administrated during the first 6 days of COS, with a daily dose adjusted according to follicular growth. Gonadotropin-releasing hormone antagonists (cetrorelix or ganirelix 0.25 mg/day) were administered on the day that the average diameter of the largest follicle was $\geq$ 14 mm.

The minimal stimulation protocol (clomiphene citrate [CC] plus gonadotropins and GnRH antagonist) was offered to some women with low antral follicle count (AFC; AFC $\leq$ 6).[15] The CC (100 mg/day) was administered during the first 5 days of COS, and gonadotropins (150 UI/day) were administered on days 2 and 4, and daily from day 6 on. The GnRH antagonist (cetrorelix or ganirelix 0.25 mg/day) was administered on the day that the average diameter of the largest follicle was $\geq$ 14 mm.

The recombinant human chorionic gonadotrophin (hCG [250 mcg, Ovidrel®, Serono, Brazil]), or urinary hCG (10,000 IU Choriomon®, Meizler, Brazil) was administered when at least 1 follicle with a mean diameter of 18 mm was present. Oocytes were obtained 34–36 hours after administration of the recombinant hCG, and the luteal phase was maintained by administration of micronized progesterone (600 mg/day).

The mature oocytes were subjected to ICSI and cultured individually. Fertilization was defined as the presence of two pronuclei and two polar bodies. Embryo quality was evaluated $\sim$ 43 and 45 hours after ICSI (second day of embryonic development), based on the number and symmetry of blastomeres' percentage of fragmentation and presence or absence of multinucleation. If the embryo transfer was not performed in D2, embryo quality was analyzed $\sim$ 67 to 69 hours after ICSI (D3).[13]

The D2 embryos with 4 symmetrical blastomeres, normal sized, with < 10% fragmentation, and without multinucleation were considered top quality.[13] Embryos on the third day of development and with 8 symmetrical blastomeres, normal sized, with < 10% of fragmentation, and without multinucleation were also considered top quality.[13]

The clinical pregnancy rate per cycle was defined as the number of patients that presented an ultrasound (4 to 5 weeks after transfer) with embryo heartbeat divided by the number of cycles $\times$ 100.

The primary endpoint of this study was the rate of clinical pregnancy. The following parameters were assessed: age, weight, height, body mass index (BMI), duration and etiology of infertility, antral follicle count (AFC), protocol used for COS, number of retrieved oocytes, number of captured metaphase II (MII) oocytes, number of formed embryos, of transferred embryos and of TQEs. All data were obtained from medical records.

Statistical Analyses

The study size was defined as the total number of eligible patients (women younger than 40 years submitted to COS for ICSI who had at least 1 embryo formed and transferred in cleavage stage in the first fresh cycle) during the study period.

First, women were stratified into 3 groups according to the number of cleavage stage formed embryos (1 embryo; 2–3 embryos and $\geq$ 4 embryos). Then, each group was divided into two subgroups according to the presence or absence of at least one transferred TQE; clinical pregnancy rates and other variables were compared in each group as described below.

The normal distribution of continuous variables was analyzed using the Kolmogorov-Smirnov test. Continuous variables with normal distribution were presented as mean $\pm$ standard deviation (SD) and compared between the groups with and without TQE by unpaired t-test. Continuous variables without normal distribution were presented as

Table 1 Characteristics of the included participants depending on the number of available embryos and on the presence of embryo(s) considered as having top morphologic criteria

Groups	1 embryo			2–3 embryos			≥ 4 embryos		
Subgroups	Without TQE	With TQE	p	Without TQE	With TQE	p	Without TQE	With TQE	p
N	76	37		119	149		67	188	
Parameter									
Age	35.7 ± 4.4	34.7 ± 4.7	0.27	34.6 ± 4.1	34.0 ± 4.5	0.26	33.4 ± 3.7	33.4 ± 4.1	0.91
Weight	69.1 ± 11.8	70.7 ± 15.7	0.60	64.8 ± 12.1	67.1 ± 12.8	0.15	65.2 ± 12.0	65.2 ± 10.7	0.98
Height	1.65 ± 0.06	1.65 ± 0.06	0.70	1.63 ± 0.07	1.64 ± 0.06	0.42	1.64 ± 0.06	1.63 ± 0.06	0.16
BMI	25.5 ± 4.3	25.4 ± 5.2	0.73	24.4 ± 4.1	25.1 ± 4.4	0.23	24.3 ± 4.1	24.6 ± 3.7	0.53
AFC	6 [4–11]	7 [4–14]	0.49	11 [7–15]	9 [6–16]	0.54	16 [11–25]	16 [11–21]	0.31
Duration of infertility	70 [43–105]	58 [41–87]	0.33	58 [36–88]	54 [28–77]	0.23	62 [37–91]	51 [32–77]	0.14
Cause of infertility			0.15			0.68			0.68
Ovulatory	4 (5%)	1 (3%)		8 (7%)	11 (7%)		1 (1%)	7 (4%)	
Endometriosis	13 (17%)	5 (14%)		13 (11%)	24 (16%)		9 (13%)	21 (11%)	
Male	21 (28%)	4 (11%)		34 (29%)	39 (26%)		15 (22%)	58 (31%)	
Tubal	5 (7%)	2 (5%)		15 (13%)	14 (9%)		5 (7%)	14 (7%)	
Combined	25 (33%)	22 (59%)		39 (33%)	53 (36%)		27 (40%)	69 (37%)	
Unexplained	8 (11%)	3 (8%)		10 (8%)	8 (5%)		10 (15%)	20 (11%)	
Ovarian stimulation protocol			0.96			0.79			0.10
FSH + Ant	45 (59%)	22 (59%)		79 (66%)	101 (68%)		51 (76%)	149 (79%)	
FSH + Ago	12 (16%)	5 (14%)		30 (25%)	33 (22%)		13 (19%)	38 (20%)	
CC + FSH + Ant	19 (25%)	10 (27%)		10 (8%)	15 (10%)		3 (4%)	1 (1%)	

Abbreviations: AFC, antral follicle count; BMI, body mass index; CC, clomiphene citrate; FSH$^+$Ago, agonist protocol with follicle-stimulating hormone; FSH$^+$Ant, antagonist protocol with follicle-stimulating hormone; N, number of women analyzed; TQE, top quality embryo.
Note: Data presented as mean ± SD, median [interquartile range], or number (proportion).

median (interquartile range), and comparisons were made by Mann-Whitney test. Binary data were presented as a ratio (%) and compared using Fisher's exact test. The level of significance was defined as $p < 0.05$. All statistical analyzes were performed using the SPSS software (version 18.0, SPSS Inc., Chicago, IL, US). For the additional analysis, we determined the power of the study to detect a difference of 10% in clinical pregnancy rates among women with one embryo; 2–3 embryos and ≥ 4 embryos.

Results

Within the study period, 792 women were subjected to ICSI. Among these women, 130 patients had no available embryos after oocyte retrieval, 19 did not transfer fresh embryos, 1 patient had the embryo culture extended to blastocyst, and 6 had no full records of morphological evaluation in the medical records. The 636 remaining patients (who had at least one embryo to be transferred in the same cycle) were followed until a negative pregnancy test or until the transvaginal ultrasound (TVU), four to five weeks after transfer.

Of the 636 patients included in the study, 17.8% had 1 available embryo (32.7% with TQE versus 67.3% without TQE), 42.1% of women had 2–3 available embryos (55.6% with TQE versus 44.4% without TQE), and 1% of patients

had ≥ 4 available embryos (73.7% with TQE versus 26.3% without TQE).

Within each group (1, 2–3 and ≥ 4), there were no statistically significant differences in the patients' age, weight, height, BMI, cause of infertility, duration of infertility, AFC, COS protocols (►**Table 1**), number of retrieved oocytes and MII oocytes compared with women who had transferred or not at least 1 TQE. However, the number of embryos was significantly higher in patients in groups 2–3 and ≥ 4 embryos with TQE compared with the groups without TQE (►**Table 2**).

Among women who had and transferred only 1 embryo, there was no difference in the pregnancy rate compared with women with TQE (16.2%) and without TQE (9.2%). Among those who had 2–3 available embryos, 33.5% of women with TQE and 30.2% of women without TQE became pregnant, also showing no significant difference between groups. Of the patients who had 4 or more available embryos, 45.2% of women with TQE and 28.3% of women without TQE became pregnant. The clinical pregnancy rate was significantly higher in the subgroup with ≥ 4 available embryos with at least 1 transferred TQE (≥ 4 with TQE) compared to the subgroup without TQE (≥ 4 without TQE) (►**Table 2**).

The present study presented a test power of ∼ 70% to detect a difference of 20% in clinical pregnancy rates between

Table 2 Main outcomes depending on the number of available embryos and on the presence of embryo(s) considered as having top morphologic criteria

Groups	1 embryo			2–3 embryos			≥ 4 embryos		
Subgroups	Without TQE	With TQE	p	Without TQE	With TQE	p	Without TQE	With TQE	p
N	76	37		119	149		67	188	
Parameter									
Retrieved oocytes	2 [1–3]	2 [1–4]	0.86	5 [4–7]	5 [4–7]	0.68	10 [7–12]	10 [8–14]	0.25
MII retrieved	2 [1–3]	1 [1–3]	0.45	4 [3–6]	4 [3–5]	0.42	7 [6–10]	9 [6–11]	0.07
Formed embryos	1 [1–1]	1 [1–1]	1.00	2 [2–2]	2 [2–3]	< 0.01*	4 [4–6]	5 [4–7]	< 0.01*
Transferred embryos	1 [1–1]	1 [1–1]	1.00	2 [2–2]	2 [2–2]	0.94	2 [2–2]	2 [2–2]	0.64
Clinical pregnancy	7 (9.2%)	6 (16.2%)	0.35	36 (30.2%)	50 (33.5%)	0.60	19 (28.3%)	85 (45.2%)	0.02*

Abbreviations: MII, metaphase II; N, number of women analyzed; p, *p-value*; TQE, top quality embryo. * *p* statistically significant.
Note: Data presented as median [interquartile range], or number (proportion).

patients with and without transferred TQE and only 1 available embryo; 40% for the subgroups that had 2–3 available embryos; and 30% for the subgroups that had ≥ 4 available embryos.

Discussion

Our study evidenced that, during ART, the embryonic morphological evaluation, in the second or third day of development, is an important factor involved in the prognosis of clinical pregnancy, especially in women who had four or more embryos. In the group with ≥ 4 available embryos, there was an increase of ∼ 17% in clinical pregnancy rates in the subgroup of patients that transferred at least 1 TQE in the first fresh transfer cycle (45%) compared with those women who had no TQE (28%) transferred. Among the many plausible conditions to influence pregnancy rates after ART, the number of retrieved oocytes, the number of available embryos and the number of transferred TQEs seem to be the most important factors of reproductive prognosis.[16–20] However, there are few studies evaluating the relationship between pregnancy rate and live birth rate and the number and morphological quality of the embryos concomitantly. In women over 40 years, Opsahl et al[21] found that patients who had less than 4 embryos presented lower chances of pregnancy than those who formed ≥ 4; however; this study did not take into account the morphology of the embryos.[21] Corroborating our findings, Van Loendersloot et al[22] suggested that the number of available embryos and their morphological quality in the third day of culture should be important factors in the development of a model to predict the probability of pregnancy after in vitro fertilization (IVF).[22]

In the group with 1 embryo, the difference in the clinical pregnancy rates among the subgroups with TQE (16.2%) and without TQE (9.2%) was 7%. And in the group with 2–3 available embryos, the difference in the clinical pregnancy rates among subgroups with TQE (33.5%) and without TQE (30.2%) was 3.3%. Despite the fact that these differences were not statistically significant, this study did not have the adequate test power to confirm the findings, and studies with larger sample sizes are necessary to evaluate whether patients who present less than 4 embryos have their pregnancy rates influenced or not by the morphology of transferred embryos.

Among several analyzed secondary outcomes, in groups with the formation of 2–3 embryos and ≥ 4 embryos, the number of embryos differed between groups with and without TQEs. In the group with ≥ 4 embryos, 73.7% of women had TQEs, and just 26.3% did not. These findings suggest that women who generate a greater number of embryos have a greater potential to form TQEs.[23] The other evaluated secondary endpoints showed no statistically significant difference between the subgroups, further reinforcing the findings of the study, excluding the interference of these parameters on the primary endpoint (clinical pregnancy rate).

Previous researches showed that the quality and size of the embryo cohort allow the selection of TQEs for transfer in the fresh cycle and, subsequently, in frozen cycles, thus influencing the cumulative pregnancy rate.[21,24] However, both factors, number and quality of embryos together, can be a valuable tool in predicting the probability of pregnancy after ICSI, adjusting the expectations of patients and physicians.

Since this is a retrospective study, the information was obtained exclusively from medical records. Another important point is the relatively small sample size in each subgroup, so that larger studies are important to confirm our findings and investigate the influence of embryo morphology on the embryos' cleavage stage in women that had fewer than 4 embryos per cycle of IVF/ICSI. Since in our analysis we included only data from the first cycle of each patient, it is not possible to extrapolate whether the cumulative clinical pregnancy rate, per cycle or time, is different in women with or without TQEs.

This study allows to more accurately establish the chances of clinical pregnancy per cycle for patients under 40 years who have at least 1 embryo in the first fresh transfer cycle. This information is useful when counseling couples in order to determine the gestational outcomes, adjusting the expectations of doctors and patients.

In conclusion, among women undergoing COS for ICSI, women that had four or more embryos and transferred at least one TQE in the first fresh transfer cycle have higher rates of clinical pregnancy compared with those of women without TQEs. Thus, we found that the number of embryos per cycle and the quality of embryos transferred are important prognostic factors in ART.

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