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Objective variables obtained by image analysis of double transfers of top quality embryos in women under 36 years old. Smaller circular embryos with thinner ZP are more likely to implant.

Objective: To demonstrate the usefulness of image analysis in designing objective embryonic morphometric variables.

Background: Multiple pregnancies carry a significant risk for maternal-fetal health. It is essential to develop new non-subjective tools which allow the selection of embryos with the highest implantation potential.

Design: Retrospective study of 214 top quality day-2 embryo photographs coming of 50 double embryo transfers (DET) without pregnancy (Group 0) and 57 with twin pregnancy (Group 1).

Setting: Human Reproduction Unit.

Patient(s): Study of 107 IVF-ICSI cycles in women under 36 years of age with DET of top quality embryos. Only the first cycle of IVF-ICSI was included.

Intervention(s): Standard IVF-ICSI protocols.

Main Outcome Measure(s): The embryo photographs were analyzed using the *ImageJ* program. The effects of the embryo variables and the clinical variables on embryo implantation were evaluated using a stepwise dichotomous logistic regression.

Results: Significant differences were observed due to the women´s ages, internal perimeter (IP), roundness factor (RF) and ZP thickness (ZPT). Embryos with smaller IP, circular shape and smaller ZPT are more likely to implant.

Conclusions: Morphometric variables lower the subjectivity of the current embryo grading systems. These variables are non-subjective factors to consider when predicting implantation. Embryo image analysis is an accurate tool which can improve IVF-ICSI outcomes and reduce twin pregnancies.

Keywords: embryo selection; embryo score, morphological and morphometric embryo variables; images analysis; embryo implantation, embryo grading systems.

INTRODUCTION

One of the major issues in Assisted Reproduction Technologies (ART) is multiple pregnancies which carry a significant risk to maternal-fetal health (1-3). The woman's age and the number and quality of transferred embryos are correlated to high multiple pregnancy rates (Wright 2006,Strandell 200)

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These risks can be reduced by lowering the number of transferred embryos (4, 5). The ideal approach to studying the morphologic determinants of a single embryo's implantation would be to analyze exclusively single-embryo transfers. However, in most single-embryo transfer programs only 'top' quality embryos are transferred so an optimal span of variables for statistical evaluation cannot be reached. The progressive implementation of a top quality single-embryo transfer (SET) produces an important decrease in multiple pregnancies without a significant reduction in pregnancy rates (6-10). Nevertheless, the linear implementation of SET produces an unacceptable low pregnancy rate, particularly in older patients and those with poor embryo quality (10). Thus, it is important to increase knowledge of the implantation potential of each individual embryo in order to select the top quality embryos for transfer.

Until now, embryo selection has been routinely based on embryo development and morphologic characteristics by using different classification and scoring systems in order to evaluate the embryo quality (6-8).

Embryo quality assessment based on morphologic criteria of transferred embryos is highly subjective with inter-observer variability in the evaluation of morphological parameters (9-11). The absence of a clearly defined standard method to measure specific characteristics determines a loss of essential information.

The evaluation in real-time of all the morphologic characteristics is time consuming. The embryo evaluation time has to be as short as possible to prevent embryo exposure to suboptimal culture conditions. Fluctuations in pH and temperature have deleterious effects on the embryo development, quality and implantation (12).

Time-lapse imaging has been proposed as a method for embryo selection by adding new dynamic predictors of viability to the assessment. The time lapse embryo assessment allows the evaluation of ~~also~~ embryonic morphokinetic parameters (13-15). However, the high costs of these technologies do not allow their implementation in many laboratories.

The increasing international recognition of the value of performing elective simple or double-embryo transfer in order to prevent higher-order multiple pregnancies, ~~is showing~~ demonstrating ~~the~~ importance in selecting embryos which result in a live birth. Therefore, it is essential to develop new objective tools for selecting the embryos.

Work has been performed linking morphometric embryo variables to embryo quality parameters such as embryo fragmentation and multinuclearity, as well as embryonic segmentation and three-dimensional reconstruction (3D), (13-17). However, there are few studies which compare the embryo morphometric parameters ~~which depend on~~ with embryo implantation. Recently, Partenot et al (11, 15) have demonstrated a better prediction of implantation rate based on blastomere number and size. They have also shown correlations between total embryo volume and clinical pregnancy in day 3 embryos.

In our previous study (18), morphometric embryo variables were demonstrated to be more objective and powerful in predicting embryo implantation. However, the low number of successfully implanted embryos (27 embryos) did not allow conclusive results.

The aim of this study is to demonstrate the usefulness of the Image J program for image analysis in the design of objective embryonic morphometric variables. These were obtained from a selected population of women less than 36 years of age with double embryo transfer of morphologically selected top quality embryos successfully implanted.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board of the hospital. All procedures in the Methods section were compliant with ethical guidelines, i.e. approved by the Ethical Committee.

1. Patients and embryos.

Retrospective study of 214 transferred embryos coming from 100 ICSI cycles and 7 IVF-ICSI cycles performed from January 2008 to December 2010 in a selected population of women under 36 years of age, with double embryo transfer of top quality day-2 embryos of the same morphological characteristics. An IVF-ICSI cycle was only performed when more than 12 oocytes were retrieved. Half of the oocyte were micro injected and the best quality embryos were transferred regardless which insemination technique was used (IVF or ICSI). Only 2 embryos corresponding to 2 different IVF-ICSI cycles were derived from IVF insemination technique which resulted in no pregnancy.

All the embryos had 4-cells with equal, symmetrical and mononucleated un-fragmented blastomeres. The stimulation protocol used in this study has been previously published (19).

2. Experimental design

The embryos were distributed in the following groups**:** Group 0 (0% implantation): 50 double embryo transfers without pregnancy and Group 1 (100% implantation): 57 double embryo transfers with a twin pregnancy (2 gestational sacs).

Only the first cycle of IVF-ICSI was included. Cycles with endometriosis, low response, uterine malformations, recurrent abortions or with donor gametes were excluded.

All the embryos were morphologically top quality day-2 embryos and all the women had a good reproductive prognosis. Therefore, the differences between implanted and no implanted embryos should have been due to the morphometrically evaluated embryo variables which could not have been evaluated by only a simple observation through the inverted microscope.

3. ART procedure.

After oocyte retrieval, the oocytes were placed separately in 200 microliter drops of culture medium (IVF medium, Medicult, Denmark) under mineral oil (Mineral oil Medicult). Semen samples for the IVF-ICSI cycles were prepared using standard swim-up procedures. They were diluted and centrifuged twice at 300g for 10 min. Standard IVF/ICSI procedures were performed between 2–6 hours after oocyte retrieval. In the IVF procedure, oocytes were inseminated with 100.000-300.000 progressively motile sperm per oocyte.

In the ICSI cycles, injected oocytes were incubated together in a 20 microliter drops of culture medium (IVF medium, Medicult, Denmark) under mineral oil (Mineral oil Medicult). On Day 1 (16–20 hours after insemination/injection) fertilization was evaluated. Only normally fertilized oocytes (2PN) were cultured individually in a 20 microliter droplet of culture medium (IVF medium; Medicult) covered with mineral oil.

On day 2 (41–44 hours after insemination/injection) the embryo evaluation was based on the assessment of cell number, size and the degree of fragmentation. Only replacements of two top quality embryos were considered. All the embryos were photographed immediately before transfer. Photographs were taken using “Cronus 3” software (*Research Instruments LTD*) implemented in a phase contrast inverted microscope (Nikon Eclipse) with a 20 x optic magnifications and Hoffman modulation contrast.

An ongoing twin pregnancy was defined as the presence of two intrauterine gestational sacs after 6-8 weeks of pregnancy.

4. Patients and IVF/ICSI cycles characteristics.

The following clinical variables which could influence the embryo implantation: female age (FA), body mass index (BMI, Kg/m2), estradiol levels on hCG day (E2, pg/ml), endometrial thickness (ET, mm), total number of retrieved oocytes (TNO) and sub-fertility causes (tubal factor, ovulation cause, male factor and normal reproductive function) were studied in the two groups.

5. Morphometric characteristics of the embryos.

The morphometric variables: external area (EA), external perimeter (EP), internal area (IA), internal perimeter (IP)**,** roundness factor (RF), zona pellucida thickness calculated (ZPTC) and zona pellucida thickness deduced (ZPTD) were evaluated from the photographs taken immediately before the transfer.

The images were analyzed by using *ImageJ,* a public program and the available tools developed by Wayne Rasband (http://rsb.info.nih.gov/ij/).

Prior to the analysis, a change of units from pixels (graphic measurement units) to micrometers (linear measurement embryo units) had to be made. This was done by taking a photograph of a micrometer (whose units are known) with the same magnifications as the embryo when photographed.

-*Embryo area and perimeter*: To measure both the external and internal area and perimeter variables, the tool "*Elliptical or brush selection*" was used. To increase measurement accuracy, the ellipse described by the program was adjusted to the embryo boundary, so that both shapes coincided in as many points as possible. The internal perimeter was evaluated without including ZP.

The ZP thickness (ZPT) was evaluated through 2 different methods:

ZP calculated thickness (ZPTC). To measure this variable the tool "*Straight*" was used and the thickness variation of the ZP at three different points for each embryo was measured. This tool measures the width of the ZP thickness in different parts of the embryo cover.

ZP deduced thickness (ZPTD). In this case, the ZP thickness was evaluated as the difference between the average radius of the external and internal area by obtaining each radius from its respective areas (internal and external), as shown in the next formula:

$r_i = \sqrt{Area_i/\pi}$.

This variable could include, in some cases, a part of the area of the embryo perivtelline space.

-*Circularity factor or roundness factor (RF)* is a rate defined as $4\pi(\text{Area})/(\text{Perimeter})^2$, with a value of 1.0 indicating a perfect circle. As the value approached 0.0, it indicated an increasingly elongated shape (41). For easer understanding this factor was analyzed and expressed in a 0-100 scale instead of a 0-1 scale

All morphometric variables were expressed in microns**.**

6. Statistics.

To study the 214 transferred embryos, a stepwise dichotomous logistic regression analysis was performed (20). Each embryo was considered one experimental unit and the corresponding embryo implantation (0 = not implanted; 1= implanted) the response variable. Both, the clinical variables of the couple and the morphometric characteristics of each embryo were considered potential explanatory variables of embryo implantation

The statistical model used expressed the probability of embryo implantation (p) depending on the 16 explanatory variables (X_i):

$$\text{p} = \frac{e^{\beta_0 + \sum_i \beta_i X_i}}{1 + e^{\beta_0 + \sum_i \beta_i X_i}}$$

β_0 is the independent term and β_i is the regression coefficient associated with the explanatory variables $X_{i.}$

RESULTS

The results of the stepwise dichotomous logistic regression analysis showing regression coefficients (RC), standard errors (SE), p-values ($p< 0.05$), odd-ratios (OR) and their lower and upper 95% confidence intervals (LCI and UCI) for the morphometric embryo variables and the clinical variables of the couple with significant coefficients for group 0 (0% implantation) and Group 1 (100% implantation) are presented in Table 1.

The model obtained from the logistic analysis had also an adequate goodness of fit (Pearson test with a p-value of 0.307 and deviance test with a p-value of 0.487). Therefore, no significant differences were observed between the model predictions and the observations in the sample.

A woman´s age showed a highly significant effect (p value 0.000) on embryo implantation. The negative regression coefficient indicated a decrease in embryo implantation when a woman´s age was increased (Group 0: 32.88 ± 0.26 years; Group 1: 31.67 ± 0.28 years). The odd-ratio obtained for this variable (0.785) indicated that the ratio between the probability of implanted embryo and non implanted embryo $\frac{p}{1-p}$ decreased approximately 22% when age was increased by one year.

Regarding the embryonic characteristics, the analysis detects highly significant coefficients for the morphometric variables, IP (p-value of 0.000), ZPTC (p-value of 0.000) and RF (p-value of 0.013). The first two coefficients had a negative mark, which indicated that an increase in the internal perimeter or in the ZP thickness adversely affected embryo implantation. The odd-ratio associated with these variables (IP: 0.9544; ZPTC: 0.774) indicated that this ratio decreased approximately 5% and 23% respectively when the IP or ZPTC increased 1 micron.

In contrast, RF showed a positive coefficient indicating that embryos with more circular shape (greater RF) were more likely to implant. The odd-ratio obtained for this variable (1.277) indicated that this ratio increased approximately 28% when the RF increased a percentage unit.

The average values for IP were: 430.35 ± 2.31 for Group 0 and 414.25 ± 1.48 for Group 1. In relation to ZPTC, the average values were: 18.09 ± 0.36 for Group 0 and 15.91 ±0.18 for Group 1. For the RF the average values were: 0.954 ± 0.003 for Group 0 and 0.970 ± 0.001 for Group 1.

For the RF the average values were: 0.945 ± 0.1 for Group 0; 0.968 ± 0.0 for Group 0 and 0.970 ± 0.0 for Group 1.

Figure 1 shows the detail of the distribution values for the variables with significant coefficients: FA and for the morphometric variables IP, ZPTC and RF for group 0 (0% implantation) and Group 1 (100% implantation). The distribution moves towards higher values for RF variable with increasing implantation. By contrast, the distribution for the morphometric variables IP and ZPTC moves towards lower values with increasing implantation.

DISCUSSION

Although there is general agreement among embryologists as to what morphological features are characteristic of a top quality embryo in the cleavage stage, evidence is still lacking for the ranking of implantation potential of non-top quality embryos. The need to increase knowledge about non-subjective variables of embryo implantation, thereby constructing reliable scoring systems, is becoming evident.

A woman's age and embryo quality are the most influential variables in the implantation rate (8, 21-23). The first variable is unchangeable, but when there are a sufficient number of available embryos we can select those embryos with the greatest implantation potential according to morphological criteria for transfer (8, 24-30). Thus, it is important to increase our knowledge of the characteristics of embryos with a high implantation potential as well as that of non-top embryos.

Conventional evaluation techniques are static and do not allow accurate timing of embryo morphokinetics. The introduction of time-lapse technology into the IVF laboratories allows a more precise definition of embryo development dynamics and a given algorithm to select the embryo with the highest implantation potential (16,17 AÑADIR CITAS).

Time-lapse markers, which are defined by time-lapse imaging and correlated with clinical outcomes, may provide embryologists with new opportunities for improving embryo selection (14,15).

~~Nevertheless, despite the great advantage of the embryo morphokinetics parameters evaluation, time lapse technology neither allows us to assess objectively the morphologic embryo variables cell symmetry and fragmentation nor the ZP appearance~~.

An embryonic classification system based on the use of objective parameters of embryo morphology should be developed. That is, measurements objectively should be taken directly from the embryos.

Computer-assisted scoring systems (CASS) in combination with automation of embryo visualization can improve embryo assessment (11, 15). These systems give additional information on embryo characteristics that cannot be evaluated by a manual scoring. However, the CASS system (FertiMorph, Image House, Copenhagen,Denmark) allows the evaluation of only three embryo characteristics: the number and size of blastomeres and fragmentation degree.

In our previous study (18,19), morphometric embryo variables have shown to be more objective and powerful in predicting embryo implantation. However, the low number of embryos successfully implanted (27 embryos), did not produce conclusive results.

In the present study embryonic objective morphometric variables were designed using the Image J program in a selected population of women under 36 years of age with double embryo transfer of top quality day-2 embryos evaluated demonstrates by the manual morphologically scoring method.

Although our study only included women less than 36 years of age, implantation was significantly affected. The woman's age and the embryo quality are the most influential factors in the implantation rate (8, 23). The first factor is unchangeable but when there are a sufficient number of embryos available, we can select ~~the~~ embryos with the greatest implantation potential for transfer (26, 29, 30, 31).

In relation to the variables that could define the embryo size, the only one with significant differences was the internal perimeter. Embryo implantation decreased ~~progressively and~~ significantly as IP increased. The embryos that were successfully implanted had a small internal perimeter. Because only four cell embryos were transferred in this study, the changes in the embryo perimeter should not have been influenced by the cell number of the transferred embryos.

Recently, Partenot et al (11, 15) have demonstrated a better prediction of implantation rate based on the blastomere number and size. Correlations between total embryo volume and clinical pregnancy in day 3 embryos were shown. In these studies, the coefficients of diversity (ratio of the largest/smallest blastomeres) of implanted embryos top quality embryos with 100% implantation tended to be lower than for non-implanted embryos, although the difference was only significant for 6-cell stage embryos. Moreover, an association between total embryo volume and pregnancy was observed and both higher and lower volumes were associated with a lower probability of successful pregnancy (15).

These results, suggest that implanted embryos tend to have more uniform blastomere size than non-implanted embryos and that embryos with a total volume beyond the optimal range have a lower chance of resulting in an ongoing pregnancy.

The biological relevance of the embryo volume as a characteristic which is important for implantation can be explained by the fact that a failure in volume regulation can cause the arrest of the embryonic development (32).

Symmetric embryos are related to good implantation prognosis contrary to the asymmetrical ones which significantly affect the implantation rate. However, good cleavage pattern predicts implantation outcome even for non synchronized cleaving cells (33)

We do not know the biological explanation for decreasing embryo implantation with increasing internal perimeter. Perhaps, four cell embryos, with equally symmetrical sized blastomeres, should be more regular if they have a better cell organization inside the perivitelline space.

Some authors have evaluated the impact of the zona pellucida thickness variation of human embryos on IVF outcomes indicating that this influences the embryo's ability to both develop and implant (34, 34)

In this study, the implantation decreases ~~progressively~~ and significantly with increasing the calculated ZP thickness. The other variable used to evaluate the ZP appearance was the deduced ZP thickness which could include in some cases a part of the area of the embryo perivtelline space. This variable did not reach statistical significance in the model. This could means that the volume of the perivitelline space does not affect the embryo implantation potential

From the results obtained in this work, embryos with lower ZP thickness have more chances to successfully implant. These results coincide with those proposed by the research group that studied morphometrically the ZP thickness and their relationship to embryo implantation (34). The ZP thickness influences the embryo's ability to both develop and implant (36-38).

Embryo implantation presents high significant coefficients with RF. This is difficult to explain due to the minimal differences between the embryos in each group, indicating that embryo implantation potential decreases when embryos becomes more elliptical. It could be a phenomenon related to a correct cells distribution inside the embryo.

Partenot et al (15), observed that blastomeres started as irregular spheres at the 2-cell stage embryo and became ellipsoid by the 8-cell stage. Moreover, Goyanes et al 1990 (38) demonstrated that no significant changes occurred during successive cleavages in the coefficient of implanted/non-implanted embryos. Based on previous studies and our results, it could be hypothesized that 4-cell embryos with equally symmetrical cells might be responsible for the circularity shape of the successfully implanted day-2 embryos. Both (15, 39) indicated that tetrahedral embryos are more likely to implant than planar embryos. Our results agree with those obtained by both authors i.e. embryo circularity factor could be a measure of embryo regularity. Therefore, it is reasonable to believe that the tetrahedral embryos tend to be circular in their maximum projection. This coincides with the results of our study indicating that the circularity factor influences embryo implantation. However further studies are needed to understand the importance of embryo regularity in the implantation process.

Unfortunately, the explanatory variables are continuous therefore it is not possible to give cutoff ranges which guarantee implantation. The model indicates that embryo implantation increases as RF increases and FA, ZPT and IP variables decrease. Therefore, the implantation increase depends on the explanatory variables which reach statistical significance in the model.

In order to determine the values for IP and ZPTC for a good implantation, predictions from the model have been made and can assert that:

- For FA = 32.2 , RF = 96.3 and ZPTC = 16.9 (mean values of these variables in the sample) a 75% of probability to implantation is reached for a value of IP = 399.2 (with a confidence interval of 95% ranging from 376.3 to 408.9 units).

- For FA = 32.2, RF = 96.3 and IP = 421.8 (mean values of these variables in the sample) a 75% of probability to implantation is reached for a value of ZPTC = 13.1 (with a confidence interval of 95% ranging from 8.8 to 14.7 units).

The morphometric variables internal perimeter, ZP thickness and embryonic roundness provide information about embryo size, shape and ZP thickness. This cannot be appreciated from the morphologic variables by simple visual observation. Embryos with smaller internal perimeter, circular shape and smaller ZP thickness are more likely to implant. We believe that this is the first study evaluating the impact of the embryo size, shape and ZP appearance of embryo implantation. However this evaluation assessment has some limitations:

1-The ability to manipulate and move the embryo in a culture media droplet is very limited and this determines that the images you can get from photographs are not always optimal for measuring the cells of the lower plane. This limitation can be overcome by using 20x magnifications in order to see the contours of all the embryo cells clearly. Moreover, photographs should be considered as the projection of a sphere on the plane. For this reason, the contours of the equatorial area will be more marked than the caps area. This will facilitate the measurement implementation.
2. The image capture is crucial for obtaining accurate linear dimensions. One must be extremely careful with the image capture to avoid bias in the linear measurements.

3-A major limitation when comparing this evaluation assessment to time lapse technology is that the embryos must be removed from the incubator in order to take photographs, therefore altering the embryo culture conditions.

Time-lapse imaging has been proposed as a method to refine embryo selection by adding new dynamic predictors of viability to the assessment. Also, its integration in routine processes yield several advantages, such as kinetic embryo parameter evaluation and better embryo culture conditions (14,15 AÑADIR CITAS). However, these technologies do not allow an objective evaluation of embryo variable such as cell symmetry and fragmentation. In addition, neither the features regarding the shape and size of the embryo nor the ZP thickness can be evaluated with time lapse methodology

Despite the limitations above indicated, the morphometric embryo scoring has some advantages. Firstly, it could prevent the subjectivity of currently used grading systems and the images can be evaluated directly from the embryo photographs without any time restriction. This can decrease the exposure time to sub-optimal culture conditions. The embryo characteristics can also be analyzed more accurately by reducing the inter-observer variability during embryo scoring.

Secondly, the use of image analysis programs such as *image J*, have a large number of tools which not only allows objective embryo characterization but also reduce the time for computer-assisted embryo analysis. The above mentioned program is used professionals in other scientific fields.

Another advantage is the low economic cost of Image J. There is also the possibility for it to develop a useful standard which would allow extrapolate the results of all the scoring systems used in different IVF laboratories. Moreover, the philosophy of free distribution of this program can be used in any ART laboratory.

In future works day 2 embryos of different qualities, based on cell number, blastomere size and fragmentation should be included in order to know the morphometric characteristics of non top quality implanted embryos. These studies are essential to assess if morphometric variables could be used as a factor in the decision-making process.

~~A Nikon digital camera of 14 megabits with a graphic tablet should also be attached to the microscope covering the upper to the lower plane of the embryo. A three-dimensional reconstruction of each embryo and the evaluation of new morphometric variables related to the embryo volume would be the result.~~

CONCLUSIONS

In conclusion, the results of this study show that assessment of morphometric characteristics of early stage embryos using images analysis can reduce the intra and inter observer agreement. The morphometric variables related to the embryo size, shape and ZP thickness which cannot be assessed by only optical observations may assit in determining which embryo has the highest implantation potential. Furthermore, this method is a very cheap, quick and accurate tool which can improve IVF outcomes and help reduce twin pregnancies. This approach should be considered in the future for evaluation of embryo implantation.

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