FRACTAL ANALYSIS AND RELATED FORMS OF COMPLEXITY OF EMBRYO DEVELOPMENT. IS THERE A NEW TOOL FOR HUMAN EMBRYO SELECTION ?

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A total number of 1092 embryos, originated from 168 couples that underwent IVF cycles, were analyzed in this study. The embryos were cultivated in a continuous culture and monitored using the time lapse system EmbryoScope. The microscopic images of the fertilized eggs and the resulting embryos were thorough processed and analyzed using program ImageJ.The paper aims at presenting a noninvasive method, which is based on a thorough analysis of the microscopic image of fertilized eggs, using different forms of complexity (fractal dimension, lacunarity and succolarity), that may improve our ability to select embryos with the highest implantation potential.

Keywords: In-vitro fertilization (IVF), embryos transfer, fractal dimension, lacunarity, succolarity

1. Introduction

One of the key objectives of human assisted reproductive technology (ART) is the selection of a single embryo that may lead to the birth of a healthy newborn. Human embryos produced in vitro display diminished viability after transfer, this aspect being highlighted by the low success rates which show that not as much as half of all embryo transfers (ETs) result in live births, even in ladies under 35 years old [1]. Assembling more data around an embryo before transfer will enable embryologists and clinicians to choose a high quality embryo, thereby increasing the chances of achieving a pregnancy and preventing the transfer of multiple embryos and the complications associated with multiple pregnancy.

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Presently, the most broadly utilized indicator of quality to choose embryos for transfer is morphology (metabolomics and proteomics). Albeit moderately fruitful, the subjectivity of embryo evaluation and the physiological anomalies embryos with comparative morphological characteristics result in variable developmental potential [2,3]. As a result, morphokinetics by means of time-lapse imaging frameworks is becoming a useful tool in the embryology lab, and a current randomized controlled trial demonstrated a little yet noteworthy increment in implantation rates utilizing morphokinetics for embryo selection over customary morphology [2,4]. Nevertheless, there are studies which state that there is insufficient evidence to support that time lapse imaging is superior to conventional methods for incubation and selection of human embryo, more randomized studies and algorithm analysis being needed [5,6].

Another breakthrough that has enhanced embryo selection techniques and fundamentally influenced live-birth rates is the comprehensive chromosome screening (CCS) [7-9]. The CCS enables the analysis of the entire 23 set of embryonic chromosomes, so that the transfer of embryos with aneuploidy may be avoided and may facilitates elective single-embryo transfer [10].

Undoubtedly, aneuploidy is a major cause of pregnancy loss in human reproduction, advanced maternal age being the most noteworthy hazard factor. Just an exceptionally frail correlation has been seen between fetus morphology and chromosome constitution; in any case, to picture these chromosomes, an invasive procedure, called embryo biopsy should be performed.

In-vitro fertilization (IVF) treatment is a complex and costly process requiring decision support and future predictions in certain stages. Due to the difficulty faced in manual observation of multiple variables and the examination of nonlinear correlations between features, more advanced prediction models for the IVF process are required.

This paper aims at presenting a noninvasive method, which is based on a thorough analysis of the microscopic image of fertilized eggs, using different forms of complexity (fractal dimension, lacunarity and succolarity), that may improve our ability to select embryos with the highest implantation potential.

2. Materials and methods

A total number of 1092 embryos, originated from 168 couples that underwent IVF cycles, were analyzed in this study. Intracytoplasmic sperm injection (ICSI) was performed in 72% of the cycles, while IVF in 28%. Different protocols were used for ovarian stimulation, 87% of patients receiving antagonist protocol with gonadotropins, while long agonist protocol was used for 13% of patients.

The mean age of the female patients included in the study was 36.2 years, patients with good ovarian reserve and low ovarian reserved being in approximately equal proportions. However, patients from whom less 3 oocytes or more than 20 were retrieved were excluded from the study. Only day 5 blastocyst were transferred, single embryo transferred being performed.

In cases where the progesterone value exceeded 1.5 ng /dl in the day of the trigger administration, all the embryos were frozen (freeze all), frozen-thawed embryo transfer being performed afterwards.

Protocol IVF/ICSI/IMSI

Retrieval of cumulus-oocyte complexes (COC)

The ovarian stimulation was monitored by ultrasound and blood analysis, subsequently a hCG or agonist trigger being administrated 36 hours prior the retrieval of cumulus-oocyte complexes (COC). The follicular fluid was aspirated by ultrasound-guided transvaginal aspiration and analyzed in the stereomicroscope. After identification, the COC were transferred to Petri dishes which contained washing medium. For Intracytoplasmic sperm injection (ICSI) and Intracytoplasmic morphologically selected sperm injection (IMSI), which is a variation of ICSI that uses a higher-powered microscope to select sperm, oocytes were firstly denudated 37-40 hours after the trigger administration. Denudation was performed in petri with central well, by placing 5-6 COC and adding an appropriate amount of hyaluronidase. After removal of cumulus cells by repeated pipetting using a pipette whit top of properly diameters (135-200 μm), oocytes were evaluated in terms of their maturity. Further on, mature oocytes (MII) were used for ICSI/IMSI.

Sperm preparation for IVF/ICSI/IMSI

The semen sample was prepared using density gradient centrifugation. Depending on the semen volume, one or more sterile centrifugation tubes were prepared by loading 1,2-2.0 mL of 40%, respectively 80% gradient, in such manner to create a clear interface between the layers. The liquefied sperm sample was carefully pipetted on top of the gradient, centrifugation being performed at 350 g-400 g, for 10-20 min.

The resulting deposit after centrifugation was transferred into a tube with washing medium and centrifuged again for 5 minutes at 300 g. The deposit resulting after the second centrifugation was used for IVF/ICSI/IMSI.

Procedures FIV/ICSI/IMSI

For IVF, the insemination volume from the final sample was calculated so that the concentration would be about 200,000 spermatozoa per every insemination dose. Using a pipette, the predetermined volume of semen was

added in each petri containing 5-6 COC. The fertilization rate was assessed 16-18 hours after insemination.

For ICSI/IMSI, a small amount of the final semen sample was transferred in the petri dish for injection, while a small a drop was placed in advance in the PVP. Subsequently, the semen sample is transferred in the same petri with the mature oocytes that must be injected. The oocyte is immobilized using a holding micropipette. One mobile sperm, morphologically normal observed in the microscopic field (x400 for ICSI) is aspirated into the micropipette injection. The oocyte that must be injected is rotated so that the first polar body is positioned at 12 o'clock or 6 o'clock. After the oocyte is immobilized using the holding micropipette, the injection micropipette is inserted into the oocyte and the sperm can be easily introduced into the cytoplasm. IMSI procedure takes place in similar conditions, with the only difference that sperm selection is done at magnifications higher than x1000-X10000, using Nomarski differential contrast, thus enabling a more thorough analysis of the sperm morphology. After injection, all mature oocytes are transferred into the petri culture, the fertilization progress being evaluated after 16-18 hours.

All fertilized oocytes were transferred to petri dishes that contain Single Continuous Media CSCM from Irvine Scientific in individual microdrops. The resulting embryos were assessed daily over the next 5/6 days by registering cell number, degree of fragmentation and embryo morphology (form and shape of the blastomere).

The insights of this study involved the handling of several images, program ImageJ (IJ) 1.28 being used for the image processing and analysis. ImageJ is a public domain, Java-based picture handling program created at the National Institutes of Health. ImageJ was designed with an open architecture that gives extensibility through Java plugins and recordable macros. Custom acquisition, analysis and processing modules may be developed using ImageJ's built-in editor and a Java compiler. User-written modules make it conceivable to solve many image processing and analysis issues, from three-dimensional live-cell imaging to radiological picture processing, multiple imaging system data comparisons to automated hematology systems. ImageJ enables users to display, edit, analyze, process, save, and print 8-bit color and gray scale, 16-bit integer, and 32-bit floating point pictures. The program may read many picture file formats (TIFF, PNG, GIF, JPEG, BMP, DICOM, FITS), and also raw formats. ImageJ supports picture stacks, a progression of images that share a single window, and it is multithreaded, so tedious operations may be performed in parallel on multi-CPU hardware equipment. ImageJ may calculate area and pixel value statistics of user-defined selections and intensity-thresholded objects, may measure distances and angles, may determine density histograms and line profile plots. Additionally, the program supports standard picture processing functions,

for example, logical and arithmetical operations between pictures, manipulate contrast, convolution, Fourier analysis, sharpening, smoothing, edge detection, and median filtering. Further on, geometric changes, for example, scaling, rotation, and flips may also be performed. The program supports the processing of many pictures at the same time, the only restriction being related to the accessible memory. The microscopic images of the fertilized eggs and the resulting embryos were thorough analyzed using different forms of complexity (fractal dimension, lacunarity and succolarity).

3. Discussions

Modern time lapse monitoring system (e.g. EmbryoScope ™) has emerged as a method of ensuring continuous monitoring of the embryo development without removing embryos from the incubator, thereby ensuring a stable environment and avoiding changes in temperature and pH. Beside the automated annotation that are generated from the system, a series of morphokinetic parameters which are specific to different events that take place during the embryo development may be identified, therefore different research teams focused on developing a generally applicable morphokinetic algorithm which would predict the implantation potential of embryos [11-13].

Numerous studies have shown that morphology and morphokinetics of embryos are correlated with the embryo implantation rate, which is why a number of working groups have developed a series of embryonic evaluation and grading criteria, all of which aim to select the embryo with the embryo better implantation rate. Since embryonic evaluation by the classical method involves removing embryos from the incubator (temperature differences, CO_2, O_2 and pH concentrations) and subjecting them to increased intensity light, efforts have been made to produce a time-lapse device that allows embryos to be evaluated in their environment culture without the need to remove them from the incubator and with a much better examination time with images obtained at short intervals (10-20min).

A 2014 study published in Fertility and Sterility on the relationship between aneuploidy and morphological and morphokinetic characteristics in patients with PGD / PGS could not find a perfect correlation between the euploid status of the embryo and its morphological and morphokinetic characteristics [14]. However, as a conclusion of the study, morphological and morphokinetic assessment is the best and cost-effective method of assessing embryos in patients that do not undergo PGT.

According to the ESHRE Consortium (ESHRE PGT Consortium/Embryology Special Interest Group—best practice guidelines [15] for polar body and embryo biopsy for preimplantation genetic testing), the indications

for performing PGT-M are: patients at high risk of transmitting genetic abnormality to their children, which includes all monogenic defects (autosomal recessive, autosomal dominant and X-linked disorders) and carriers of balanced translocations, which are at high risk of implantation failure and recurrent abortions. On the other hand, PGT-A has been conducted for infertile patients undergoing IVF with the aim of increasing IVF pregnancy and delivery rates. Cited indications for PGT-A include advanced maternal age, repeated implantation failure, severe male factor and couples with normal karyotypes who have experienced repeated miscarriages.

Fractal analysis of the embryos was used in a study published in 2012 [16], however, the fractal dimension was calculated only on the images obtained on the 5th day of the embryonic development and not in the dynamics. This paper presents a new embryo grading algorithm based on calculating the fractal dimension of the embryos in dynamics, respectively at 72h and 110h.

The definition of a fractal is a self-repeating (self similar) pattern that shows similar properties in different scales. Fractal analysis has found applications in the detection of coding regions in DNA and measurement of the space-filling properties of tumors, blood vessels and neurons. Fractal concepts have also been usefully incorporated into models of biological processes, including epithelial cell growth, blood vessel growth, periodontal disease and viral infections. In other words, one can find various uses of fractal geometry in pathology: molecular biology, tumour, bone and vascular pathology, neuropathology, modeling of biological processes using fractals and other miscellaneous applications [17], or integrative models for fractal description of the particular structure parameters [18, 19].

Three aspects of texture are considered by the fractal geometry: Fractal dimension (FD), Lacunarity and Succolarity. Fractal dimension has been well studied; a great number of approaches have been presented to extract it from images. It can be computed from black and white to gray scale images. There are many approaches also, from the simple Box-dimension to the most complex Hausdorff dimension. The same does not happen with the other two measures. Although Lacunarity has been more and more used in works exploring its characteristics, Succolarity, until now, has not been even computed.

The three fractal characteristics (Fractal Dimension, Lacunarity and Succolarity) explore different aspects of the images in a complementary way. Two images could present the same Fractal Dimension but different Lacunarity or the same Lacunarity but different Succolarity therefore there may be other combination of results. Fractal dimension (FD) is a measure that characterizes how much an object occupies the space that contains it. FD is a measure that does not change with scale neither with translation nor rotation. Lacunarity measures the size and frequency of gaps in the image. Succolarity measures how much a

given „fluid" may flow through an image, considering as obstacles the set of pixels with a defined color (e.g. white) on 2D images analysis.

When the most important desideratum is the birth of a healthy baby, no effort on the part of those involved in assisted human reproduction is too small. Which embryo should be transferred or how many embryos should be transferred are essential questions that many medical teams face in the daily work. What if we would ask ourselves which is the embryo with the greatest chance of becoming a healthy newborn? In order to answer this question, methods of invasive and non-invasive selection of human embryos have been developed. Among the invasive methods we mentioned previously the morphokinetic time-lapse analysis, metabolomics, proteomics and genomics. Promising results are found in the exponential development of molecular genetics techniques and preimplantation genetic screening of embryos. Notwithstanding the improvements in embryo selection made possible by genomic testing, according to some studies, the embryo morphokinetics, especially the inner cell mass (ICM) grade is still one of the basic predictors of pregnancy outcome.

The preimplantation genetic testing (PGT) has the advantage of offering an objective, functional and applicable diagnosis, however, the embryo biopsy, which represents an invasive intervention may be responsible for inducing iatrogenic damage to a good potential embryo. For this reason, finding a mathematical analysis method for assessing human embryo development may ease the work of an embryologist and contribute to the standardization of embryo selection, implicitly increasing the success rate.

4. Results and conclusions

Fractal dimension was measured by means of 4 different methods: mass-radius, box-counting, correlation and pixel dilation using two different softwares: Fractalyse and FracLac for ImageJ. Lacunarity was calculated with FracLac for ImageJ program, while succolarity was calculated by a program built by our team: Succolarity_1F.exe (written in MS VC# 2010 Express Edition). Fractal analysis of the microscopic image of sperm and egg united in a culture dish revealed a decrease of the fractal dimension from 72 to 110 h evolving time (the difference exceeding the standard error) for embryos which after implantation led to pregnancy and a constant (linear) value for those who failed to implant (Fig. 1 a,b).

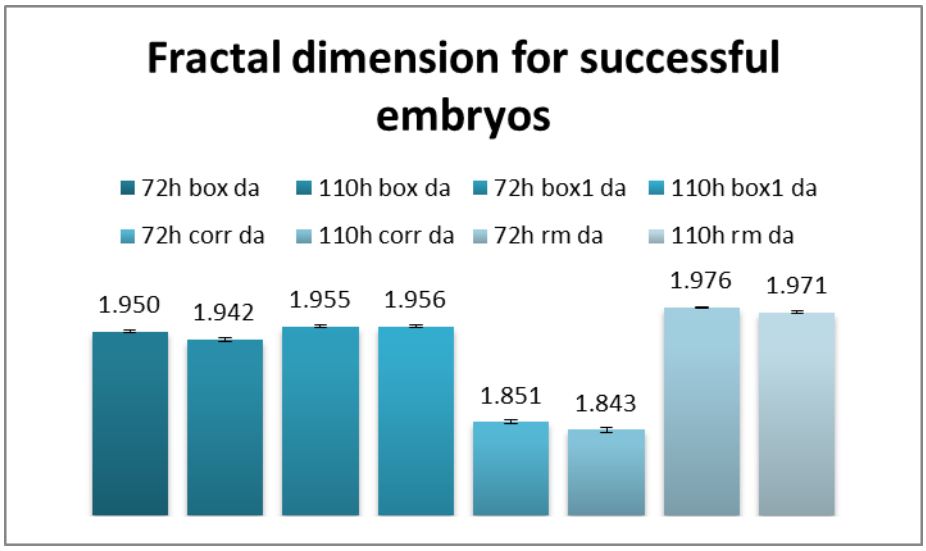

Fig 1a – Fractal dimension for embryos who where successfully implanted

For both measured samples, which resulted in pregnancy or not, the fractal dimension at 72 h was 1.933±0.002 and 1.932±0.001 respectively, while at 110 h the value was 1.928±0.002 and 1.930±0.002, respectively.

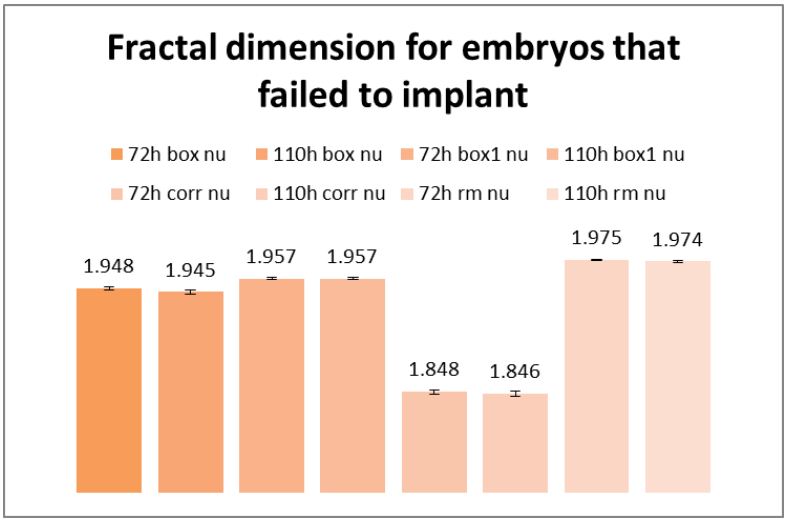

Fig 1b – Fractal dimension calculated for embryos who failed to implant

Fractal analysis of the microscopic image of sperm and egg united in a culture dish revealed the same decrease of lacunarity from 72 to 110 h evolving time, for both embryos which after implantation led to pregnancy in comparison to those

who failed to implant. Notwithstanding, the lacunarity slope from 72 to 110 h evolving time, decreased only for embryos which after implantation led to pregnancy and was almost constant for those who did not (Fig. 2a).

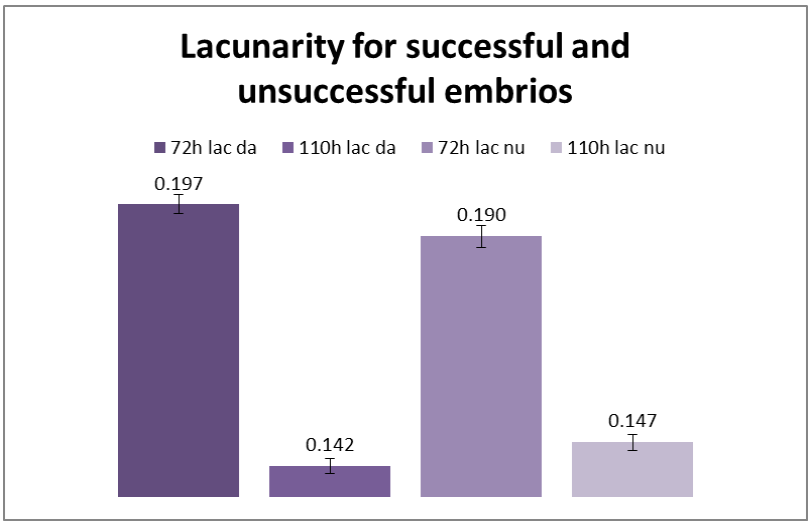

Fig 2a Lacunarity value plotted for both successful and unsuccessful embryos

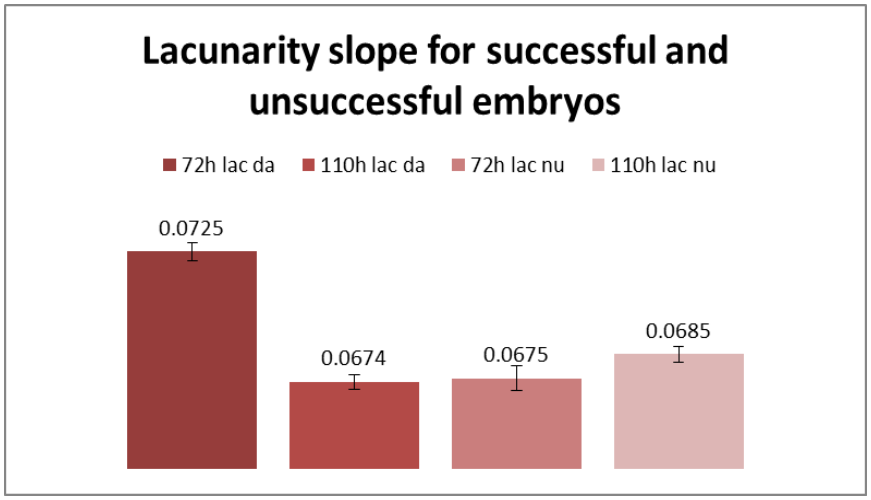

Fig 2b Lacunarity slope plotted for both successful and unsuccessful embryos

For both measured samples, which resulted in pregnancy or not, the lacunarity at 72 h was 0.197±0.002 and 0.190±0.002 respectively, and at 110 h was 0.142±0.002 and 0.147±0.002, respectively – no significant difference (Fig. 2a). However, for both samples, which resulted in pregnancy or not, the lacunarity slope at 72 h was 0.0725±0.0004 and 0.0675±0.0005 respectively, while at 110 h the values were 0.0674±0.0003, respectively 0.0685±0.0003, (Fig. 2b).

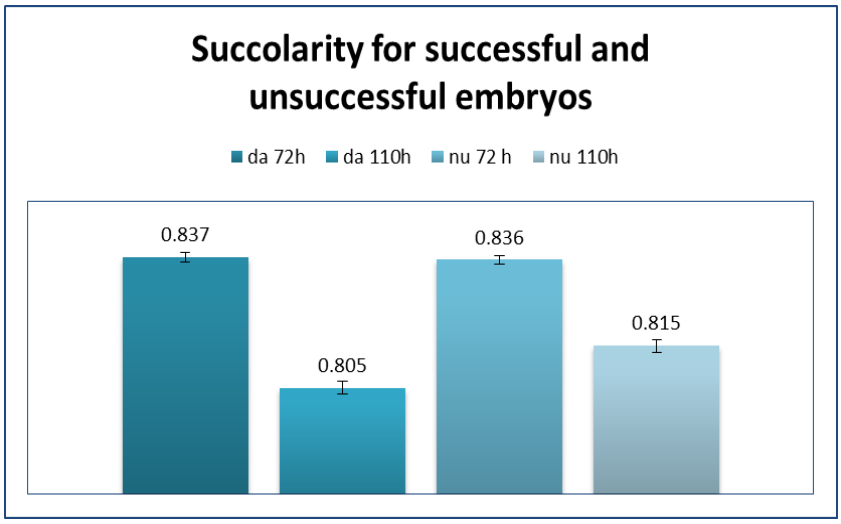

Fig 3 Succolarity value for both successful and unsuccessful embryos

Fractal analysis of the microscopic image of sperm and egg united in a culture dish revealed a higher decrease of succolarity, from 72 to 110 h evolving time, for embryos which after implantation led to pregnancy as compared to those who did not (Fig. 3).

For both measured samples, which resulted in pregnancy or not, the succolarity at 72 h was 0.837±0.001 and 0.836±0.001 respectively, and at 110 h was 0.805±0.002 and 0.815±0.002, respectively. Although the value for succolarity at 72 h was almost the same, the succolarity at 110 h was lower for successful embryos, suggesting there is a much more permeable environment in this case (Fig.3).

The results of our study may be the beginning of a new path in morphokinetic analysis by applying a fractal model in the succession of embryonic development to the blastocyst stage.

Validation of this method of selecting the human embryo with the maximum chance of obtaining pregnancy may be achieved by PGT (preimplantational genetic testing) using NGS (next generation sequencing). This research direction may be applied later in other studies complementary to our study.

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