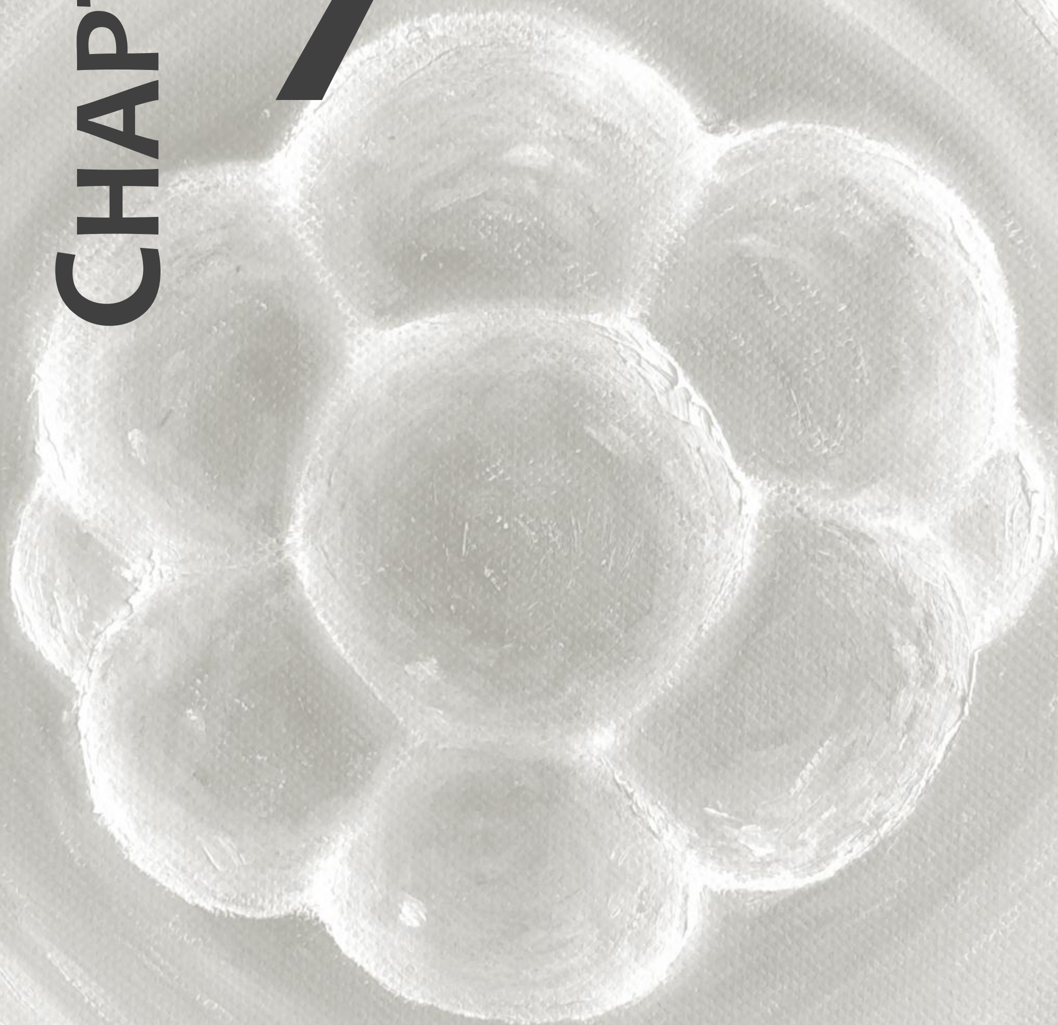
CHAPTER 7



THE ASSOCIATION OF THE BLASTOMERE VOLUME INDEX (BVI), THE BLASTOMERE SYMMETRY INDEX (BSI) AND THE MEAN OVALITY (MO) WITH ONGOING IMPLANTATION AFTER SINGLE EMBRYO TRANSFER

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ABSTRACT

Purpose: To generate novel, objective variables that resemble embryo quality and relate them to ongoing implantation, using multilevel imaging of single-transferred embryos.

Methods: Retrospective analysis of multilevel images of 659 day 3 single-transferred embryos. Each embryo was photographed on seven different levels, in order to measure the largest diameter of every blastomere within an embryo. The volume of each blastomere was calculated using the equation $1/6\pi*\text{diameter}^3$. The blastomere volume Index (BVI) represented the ratio between the total blastomeric volume of an embryo and the mean cytoplasmic volume of an oocyte on day 0. The blastomere symmetry index (BSI) represented the ratio between the greatest blastomere volume and the smallest blastomere volume within an embryo. The mean ovality (MO) represented the presence of non-spherical blastomeres. Analyses were performed to compare the BVI, BSI and MO between patients with and without an ongoing implantation.

Results: The mean BVI was significantly higher for embryos in the ongoing implantation group compared to the no ongoing implantation group. The mean BSI was associated with ongoing implantation for unevenly cleaved embryos. The MO of blastomeres within an embryo was similar for embryos in the ongoing implantation group compared to the no ongoing implantation group. The association of the BVI and BSI with ongoing implantation was confounded, because only female age and cleavage rate were significantly associated with ongoing implantation in multiple logistic regression analyses.

Conclusions: The BVI, BSI and MO are objective variables that resemble embryo quality, but they are not suitable to use as embryo selection tools.

INTRODUCTION

IVF usually results in a cohort of embryos from which embryologists have to choose the most viable embryo(s) to transfer. The main purpose of embryo selection is to improve success rates and reduce the number of transferred embryos to diminish the incidence of multiple pregnancies. Currently, routine embryo selection is mainly focused on morphological criteria and cleavage rate during embryo development, assessed at several predetermined microscopic evaluations[1].

These standard morphological evaluations have limitations. First, culture dishes with embryos have to be removed from the incubator for microscopy. Therefore, the evaluation time has to be kept as short as possible to avoid detrimental effects of changes in temperature and culture medium pH[2,3]. Second, there are several reports of moderate or considerable intra- and inter-observer variability among experienced embryologists in embryo grading[4,5]. Third, the lack of objective and standardized methods to assess especially embryonic fragmentation results in a rough estimate of a very important embryo quality parameter[6,7]. It is suggested that the use of computer-controlled systems for multilevel morphometric analysis of embryo morphology can overcome most of these limitations[2].

The group of Ziebe[6,7] was the first in reporting data of computer-assisted multilevel morphometric analysis to quantify the degree of fragmentation within an embryo. Sequences of digital images focusing at intervals through zygotes and embryos allowed them to calculate the degree of fragmentation by analysing the reduction of cytoplasmic volume from the zygote stage to the combined volume of the individual blastomeres. Paternot et al.[2] showed that a computer-assisted scoring system (CAS) using multilevel digital images of embryos, was better in predicting implantation and live birth rates than a standard scoring system (SSS). They suggested that the CAS provided a more accurate and mathematical way to assess embryo morphology, possibly resulting in a reduction of the intra- and inter-observer variability[2].

Multilevel analysis of blastomere size may function as a new biomarker for embryo quality[7]. The aim of our study was to generate objective variables that resemble embryo quality and relate them to ongoing implantation, using multilevel imaging of single-transferred embryos. We explore the possibilities and shortcomings of this novel strategy of identifying the embryos with the highest potential to result in an ongoing implantation.

MATERIALS AND METHODS

PATIENTS AND PROCEDURES

This study included multilevel images of 659 embryos from 659 patients transferred in IVF or ICSI single embryo transfer cycles between 2005 and 2007. The study protocol was approved by the institutional review board. Standard long or short GnRH agonist (triptoreline [Decapeptyl®; Ferring, Denmark]) protocols were used. Ovarian stimulation was performed with recombinant FSH (Gonal-F®; Merck Serono, Germany or Puregon®; MSD, USA). On the day of oocyte retrieval (day 0), IVF or ICSI was performed according to the laboratory's routine insemination procedures. Fertilization was checked 16-18 h after insemination or injection. Embryos were cultured individually in 25-µl pre-equilibrated medium drops under oil in incubators at 37°C, under 5% CO_2 and atmospheric O_2 concentration. Human tubal fluid (HTF; Lonza, Belgium) with a protein solution (GPO; Sanquin, the Netherlands) containing 4 mg/ml human serum albumin (HSA) was used as culture medium. Embryonic development and morphology, combining the number and regularity of blastomeres and the degree of fragmentation, was recorded at 44-48 h and 68-72 h after insemination.

Prior to transfer on day 3 in the afternoon (73-75 h after insemination), embryo development and morphology were checked again. The morphological choice of which embryo to transfer was based on the laboratory's routine procedures.

RECORDING OF DIGITAL MULTILEVEL IMAGES

Multilevel images were recorded of all transferred embryos routinely. The embryo selected for transfer was placed under a computer controlled Zeiss PLAS DIC modulation microscope, with a Zeiss digital camera connected. Sequences of seven digital images per embryo were taken at 400 times magnification with 12 µm intervals (Figure 1). Images were taken in one plane, without rolling the embryo. Imaging took about 15 seconds per embryo. Therefore, the amount of time that the embryo was out of the incubator was within normal range.

MULTILEVEL IMAGE PROCESSING AND EVALUATION

Blastomere sizes were analysed in a semiautomatic manner. Images were loaded in a software program called Axiovision (Zeiss). Axiovision converts pixels into micrometres and has several measurement tools. With Axiovision, embryologists were able to draw a line that marked the greatest diameter of a blastomere on the image displaying the middle section. Axiovision subsequently calculated the length of the line. Axiovision

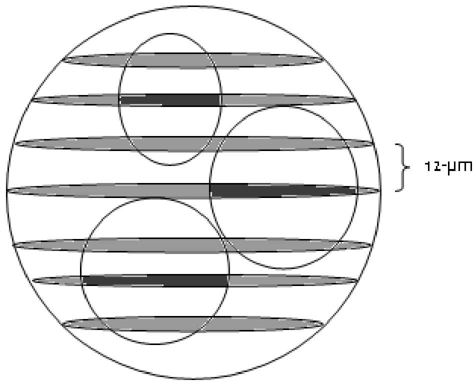

Figure 1. The photographed levels within an embryo, with 12 μm intervals. The middle section of each blastomere, displayed in dark grey, was used for the calculation of the diameters.

was calibrated with a micrometre slide: an image was taken of the micrometre slide, a line was drawn and the system used the scale-slide to calculate the physical distance between two pixels.

For each cell, two lines were drawn perpendicularly and the mean of these two measurements was considered as the actual diameter. The differentiation between a blastomere and a fragment was based on criteria defined by Johansson et al.[8] who reported that a blastomere should have a diameter of >40 μm on day 3. The volume of each blastomere was calculated using the equation $1/6\pi*\text{diameter}^3$. As the system cannot make a precise evaluation of the highness of the blastomeres, this calculation was based on a theoretical assumption. It took approximately two minutes to measure all blastomeres of one embryo.

CONVERTING MEASUREMENTS INTO NEW VARIABLES

To quantify fragmentation or cytoplasmic reduction, we introduced a new variable: the blastomere volume index (BVI). The BVI is the ratio between the total blastomeric volume of an embryo and the cytoplasmic volume of an oocyte, calculated by dividing the total volume of all blastomeres within an embryo by the mean cytoplasmic volume of an oocyte. We used 71 randomly selected oocytes of 58 randomly selected patients to compute the mean oocyte volume ($0.825*10^6$ μm^3 ± 0.098 [SD]) which we used in the BVI calculations. Furthermore, we compared the volumes of the smallest and largest blastomere volume within embryos to analyse (dis)similarities between blastomeres within an embryo and calculated the blastomere symmetry index (BSI): the volume of the smallest blastomere divided by the volume of the largest blastomere

within an embryo. We analysed the presence of non-spherical blastomeres by the mean ovality (MO): the sum of the relative difference between the X-axis and Y-axis of a blastomere diameter of all blastomeres within the embryo divided by the number of blastomeres of the embryo.

STATISTICS

We used Student's t-tests (continuous variables) and chi-square tests (binary and categorical variables) to analyse if the ongoing implantation rate was affected by patient and treatment baseline characteristics (female age, the number of previous IVF cycles, indication and duration of infertility, parity, fertilization method [IVF or ICSI], number of oocytes at ovum pick up, the number of blastomeres of the transferred embryo and the degree of fragmentation of the transferred embryo as assessed by the laboratory technicians). We defined ongoing implantation as a vital pregnancy at 12 weeks of gestational age.

The Student's t-test was used to compare mean values of the BVI, BSI and MO between the ongoing implantation group and the no ongoing implantation group. Because embryos should have an asynchronous division process during early embryo development[9], we split the embryos into two groups for the BSI analyses: a group of unevenly cleaved embryos (3,5,6,7,9,10,11 and 12 blastomeres) and a group of evenly cleaved embryos (2,4 and 8 blastomeres).

We entered possible confounding factors (female age, the number of previous IVF cycles, indication and duration of infertility, parity, fertilization method [IVF or ICSI], number of oocytes at ovum pick up, the number of blastomeres of the transferred embryo, cleavage rate [slow i.e. embryos with ≤ five blastomeres or normal i.e. embryos with ≥ six blastomeres] and the BVI, BSI and MO) into multiple logistic regression analyses to analyse their association with ongoing implantation.

The coefficient of variation (CV=100*[SD/mean]) was calculated to measure the inter-observer variability (comparison of measuring blastomere sizes by different embryologists). For this purpose, three embryologists measured the BVI of 31 day 3 embryos.

A two-sided P-value of 0.05 or less was considered statistically significant.

RESULTS

Overall, 176 of the 659 transferred embryos (26.7%) generated an ongoing implantation. Patient and treatment baseline characteristics are presented in Table 1. Female age, duration of infertility, number of blastomeres of the transferred embryo

Table 1. Patient and treatment baseline characteristics and the mean blastomere volume index (BVI), blastomere symmetry index (BSI) and mean ovality (MO) of embryos that generated an ongoing implantation and embryos that did not generate an ongoing implantation.

Variable	Ongoing implantation n=176	No ongoing implantation n=483	P-value
Female age (years)	32.8 ± 3.7	34.0 ± 4.6	<0.01
Duration of infertility (years)	3.0 ± 1.8 n=170	3.5 ± 2.2 n=472	0.01
Primary infertility	97 (57.4)	264 (56.1)	0.76
Number of previous IVF attempts	1.5 ± 0.9 n=173	1.6 ± 1.1 n=475	0.34
Medical cause of infertility			
Male	81 (46.6)	223 (46.9)	
Tubal	38 (21.8)	77 (16.2)	
Unexplained	22 (12.6)	81 (17.1)	
Endometriosis	14 (8.0)	44 (9.9)	
Other	19 (10.9)	47 (9.9)	0.35
Fertilization method			
IVF	96 (54.5)	258 (53.4)	
ICSI	78 (44.3)	222 (46.0)	0.76
Number of oocytes retrieved	11.2 ± 6.0	10.5 ± 6.6	0.25
Number of cells of ET embryo			
2	0	6 (1.2)	
3	1 (0.6)	7 (1.4)	
4	2 (1.1)	20 (4.1)	
5	5 (2.8)	43 (8.9)	
6	21 (11.9)	60 (12.4)	
7	40 (22.7)	109 (22.6)	
8	85 (48.3)	174 (36.0)	
9	15 (8.5)	37 (7.7)	
10	4 (2.3)	22 (4.6)	
11	3 (1.7)	3 (0.6)	
12	0 (0)	2 (0.4)	0.01
Fragmentation of ET embryo assessed by laboratory technician			
0-10%	107 (30.8)	240 (69.2)	
11-25%	63 (23.2)	209 (76.8)	
26-50%	2 (8.3)	22 (91.7)	0.01
BVI	0.79 ± 0.14	0.74 ± 0.17	<0.01
BSI unevenly cleaved embryos	0.47 ± 0.12 n=89	0.43 ± 0.14 n=283	0.04
BSI evenly cleaved embryos	0.52 ± 0.12 n=87	0.51 ± 0.14 n=200	0.56
MO	77.6μm ± 28.9μm n=85	71.0μm ± 32.6μm n=417	0.09

For continuous variables mean ± SD is presented; for categorical variables n (%) is presented.

and the degree of fragmentation of the transferred embryo as assessed by laboratory technicians significantly affected implantation rates. Table 1 also shows the mean BVI, mean BSI and MO of embryos that generated an ongoing implantation and embryos that did not generate an ongoing implantation. The mean BVI was significantly higher in the group of embryos that generated an ongoing implantation (0.79±0.14) compared to the group of embryos that did not generate an ongoing implantation (0.74±0.17).

The mean BSI was significantly higher for evenly cleaved embryos compared to unevenly cleaved embryos (0.52±0.14 vs. 0.44±0.13 respectively; $P<0.001$). The mean BSI for embryos with uneven cell divisions was significantly higher in the ongoing implantation group (0.47±0.12) compared to the no ongoing implantation group (0.43±0.13). Within the group of embryos with even cell divisions, the mean BSI was not significantly different between the ongoing implantation group (0.52±0.12) and the no ongoing implantation group (0.51±0.14).

The MO of blastomeres within an embryo was not significantly different for embryos in the ongoing implantation group compared to the embryos in the no ongoing implantation group ($P=0.09$).

Female age and cleavage rate were significantly related to ongoing implantation in multiple logistic regression analyses with possible confounding factors female age, the number of previous IVF cycles, indication and duration of infertility, parity, fertilization method [IVF or ICSI], number of oocytes at ovum pick up, the number of blastomeres of the transferred embryo, cleavage rate [slow or normal] and the BVI, BSI and MO ($R^2=4.0\%$).

The inter-observer variability in terms of mean CV of the BVI is 5.4%.

DISCUSSION

This study shows that the blastomere volume index (BVI) by analysis of multilevel images of embryos is associated with ongoing implantation. The blastomere symmetry index (BSI) is associated with ongoing implantation for unevenly cleaved embryos but not for evenly cleaved embryos. The mean ovality (MO) of blastomeres within an embryo did not affect ongoing implantation. However, it appeared that the association of the BVI and BSI with ongoing implantation was confounded, because only female age and cleavage rate were significantly associated with ongoing implantation in multiple logistic regression analyses. As a consequence, the BVI, BSI and MO are not suitable to use as embryo selection tools.

Our study is large in number and it is one of the first to objectively measure fragmentation, blastomere symmetry and ovality and relate these parameters to

ongoing implantation. Previous studies have shown that the combined volume of the individual blastomeres of a cleaved embryo compared to the ooplasm of the oocyte or zygote may be used to quantify the degree of fragmentation[6,7]. Using this precondition, we introduced a new variable to quantify fragmentation, the BVI. The BVI is the ratio between the total blastomeric volume of an embryo and the cytoplasmic volume of an oocyte. A higher BVI means a higher total blastomeric volume and subsequently less fragmentation.

Standard assessment of embryo fragmentation is dependent on the embryologist and not based on objective measurements[2]. It is therefore important to differentiate between blastomeres and fragments. Johansson et al.[8] showed that cells smaller than 40 μm on day 3 were always anucleate and should thus be considered as fragments. With this definition, it is possible to distinguish objectively between blastomeres and fragments using diameter measurements in multilevel images. This way we actually measure the fragmentation instead of giving a rough estimation. Therefore, we believe that the BVI displays the loss of cytoplasmic volume to fragmentation in a more reliable way compared to the standard estimation of fragmentation. This idea is supported by results from the study of Paternot et al.[2], who demonstrated that multilevel images combined with a computer-assisted scoring system predicted ongoing implantation based on the number and size of blastomeres in a more accurate and mathematical way than a standard scoring system. On top of the more objective analysis of an embryo using multilevel imaging compared to standard morphology assessment, there is no limitation in analysis time, which allows a more detailed evaluation of the embryo[2].

A limitation of our study might be the number of levels photographed within an embryo. In our study, we used sequences of seven images per embryo with 12 μm intervals. Because images were taken in one plane, without rolling the embryo, the spatial variation of the third axis was not taken into account. This might have had an influence on the measurements. Hnida et al.[7], Hnida and Ziebe[8] and Paternot et al.[2] photographed with 5 μm intervals, displaying a more accurate representation of the blastomeric volume. Fewer photos per embryo allowed us to limit the exposure time of the embryo to suboptimal conditions, a factor known to compromise embryo development[3,10]. Just like the study of Paternot et al.[2], we did not use a fully automatic system to measure the diameters of blastomeres. This is still creating a low inter-observer variability (coefficient of variation was only 5.4%). A fully automatic detection and recognition of the blastomeres by a software program can overcome this problem. This will also reduce the time needed to analyse the multilevel images[2].

Several studies have shown that unequally sized blastomeres are associated with a higher degree of aneuploidy and multinuclear rate which have a negative impact on implantation rates[11,12]. But from a biological point of view, during the division from one to eight cells, some sort of asynchrony should be expected in three, five, six and seven cell embryos[9]. Therefore, the significant higher mean BSI in the evenly cleaved embryos compared to the unevenly cleaved embryos was expected. Goyanes et al.[13] also showed that asymmetrical embryos displayed a higher ratio between the largest and smallest blastomeres. The BSI proved only to be associated with ongoing implantation for unevenly cleaved embryos. The BSI for unevenly cleaved embryos was significantly higher in the ongoing implantation group, indicating that unevenly cleaved embryos that implant have less difference in volume between the largest and smallest blastomere within an embryo. We speculate that there is probably a limit to the amount of asynchrony within viable, unevenly cleaved embryos.
Numerous blastomeres are not perfectly spherical shaped. Therefore, to obtain a more realistic measurement of the blastomere diameter, we manually drew two lines perpendicularly. This also allowed us to assess the ovality of blastomeres and its relation to ongoing implantation, a factor that rarely has been studied. We found that the mean ovality of blastomeres within an embryo did not significantly affect ongoing implantation.
Multiple logistic regression analyses showed a significant influence of female age and cleavage rate on ongoing implantation. However, the very poor R^2 (4.0%) in this model indicates that only a very small portion of the variation in ongoing implantation rates is explained by the model. The BVI, BSI and MO were not significant variables in the model, indicating that the single relationship between ongoing implantation and the BVI and BSI for unevenly cleaved embryos respectively was confounded. We assume that the cleavage rate of embryos was a considerable influence here: the chances of implantation of slow cleaving embryos are most likely more related to their cleavage rate than to their volume or symmetry. Besides, regarding the BVI, several studies have shown that not all fragmentation is detrimental to embryos. The majority of embryos transferred in the current study showed less than 25% fragmentation. This might have caused the lack of association of the BVI with implantation shown in the multiple regression analyses. At present, it is generally known that the presence of large amounts of fragments significantly reduce implantation rates[14]. The presence of minimal fragmentation, however, might even be part of normal embryo development and does not have to impair implantation[8,14]. Several studies report a threshold under which embryo fragmentation does not influence pregnancy outcomes. This threshold varies from <10% fragmentation[15] to <15% fragmentation[16] and <20% fragmentation[17,18].

Furthermore, the pattern of fragmentation can also be of influence on implantation potential. Large, asymmetrical fragments that cause loss of large volumes of cytoplasm may lead to a considerable loss of implantation potential[16]. Others have indicated that fragmentation can even be attributed to the type of culture media used and the environment that the embryo is exposed to during early embryo development[19].
One of the biggest challenges in an IVF laboratory is the selection of the most viable embryo to transfer, especially from a cohort of good quality embryos. Worldwide, years and years of research have been done on this subject, but it has not (yet) led to a novel tool, uniformly used in IVF laboratories, that (non-invasively) identifies the best embryo to transfer more accurately than standard morphology. For example, pre-implantation genetic screening (PGS) and metabolomic profiling of embryo culture media by near-infrared spectroscopy were (recently developed) tools that did not redeem the promise of better pregnancy outcomes[20-22]. In the current study we tried to objectify familiar parameters of embryo morphology to analyse whether this relatively simple novel method of measuring instead of estimating important embryo quality parameters could refine the way of selecting the most viable embryo within a cohort of good quality embryos and hence lead to a higher implantation rate. Unfortunately, multiple logistic regression analyses showed that the BVI, BSI and MO are not suitable to use as embryo selection tools.
In conclusion, the BVI, a novel objective parameter to measure, instead of estimate fragmentation is associated with ongoing implantation. The BSI, a novel objective tool to measure (a)synchrony between blastomeres is only associated with ongoing implantation in unevenly cleaved embryos. Non-spherical blastomeres do not influence embryo implantation. However, multiple logistic regression analyses showed that the association of the BVI and BSI with ongoing implantation was confounded. As a consequence, the BVI, BSI and MO are not suitable to use as embryo selection tools. Therefore, they are unable to contribute to the solution of finding a non-invasive, uniformly used tool that is more accurate in predicting embryo viability than standard morphology.

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