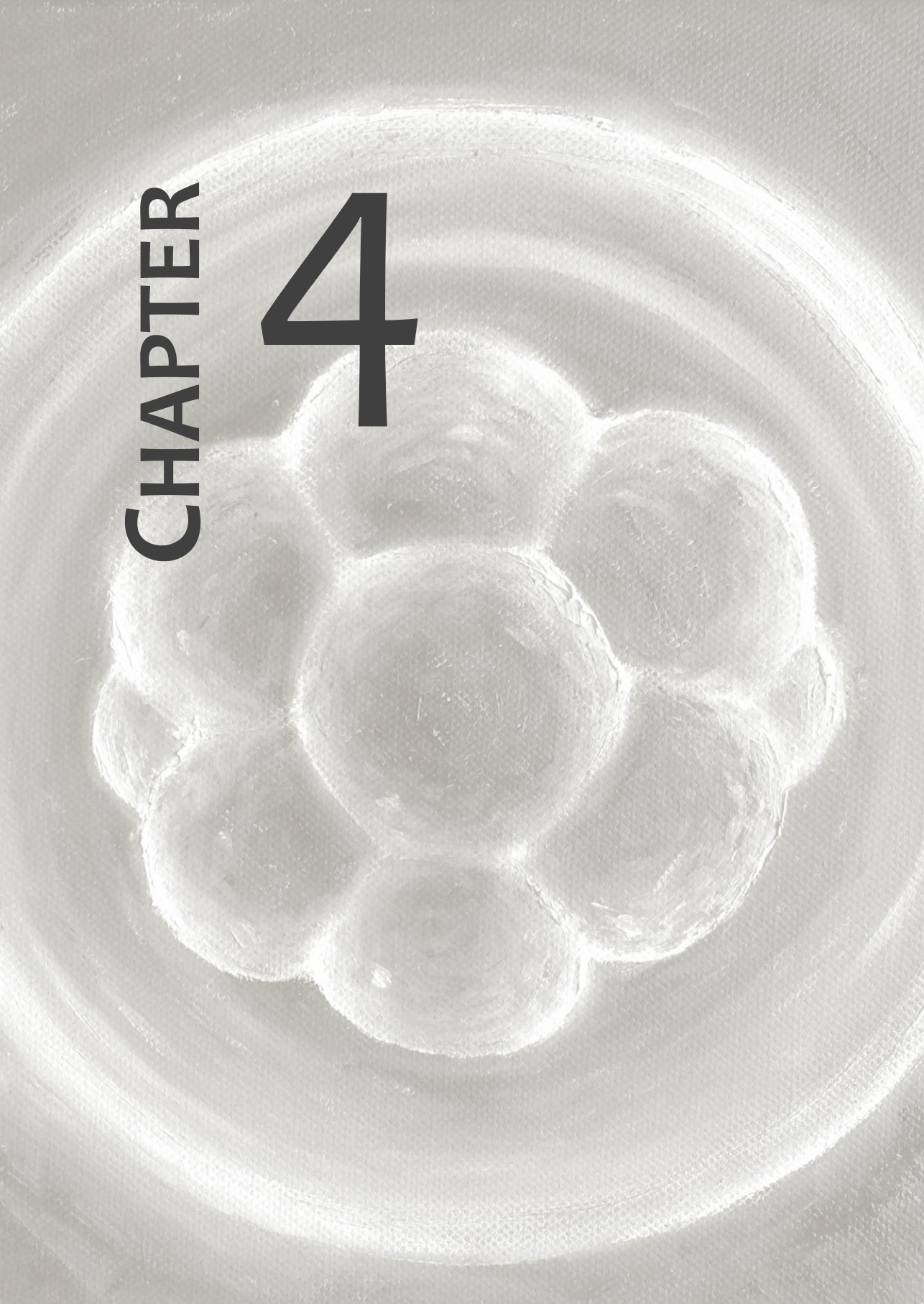
Chapter 4



NON-INVASIVE VIABILITY ASSESSMENT OF DAY 4 FROZEN-THAWED HUMAN EMBRYOS BY NEAR-INFRARED SPECTROSCOPY

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ABSTRACT

This study investigated if metabolomic profiling of culture media using near-infrared (NIR) spectroscopy was related to live birth rates after single embryo transfer (SET) of frozen-thawed embryos. Analysis of culture media of frozen-thawed embryos was performed by NIR spectroscopy. A viability score was calculated using a predictive multivariate algorithm of fresh day 5 embryos with known pregnancy outcomes. This algorithm generated with fresh day 5 embryos, could help to identify the live birth group from the no live birth group. Multivariable regression models that tested the predictive ability of the viability score for live birth, showed an odds ratio (OR) in the crude analysis of 1.50 (P=0.008), after adjustment for embryo morphology, 1.44 (P=0.022), and after adjustment for all variables, 1.71 (P=0.005); based on a 0.1 step increase in viability scores. In conclusion, higher viability scores resulted in higher live birth rates. An algorithm generated from fresh embryos might be used to predict viability of frozen-thawed embryos. Frozen-thawed embryos have different metabolic activity which is related to implantation potential. Therefore, this method might be useful to select the best embryo for transfer within a group of embryos with similar morphology.

INTRODUCTION

Although elective single embryo transfer (eSET) is frequently applied in fresh cycles, it is generally assumed that the implantation potential of cryopreserved embryos is lower than that of fresh embryos[1,2]. Therefore, more than one embryo is usually transferred in frozen-thawed embryo transfer (FET) cycles. Several studies report higher clinical pregnancy rates, implantation rates and/or delivery rates after double embryo transfer (DET) compared to single embryo transfer (SET) in FET cycles, but DET is also associated with elevated multiple pregnancy rates[3-5]. However, some reports suggest that eSET might be a feasible option in FET cycles when a good quality embryo is selected for transfer [3,4]. It is therefore of great importance to define parameters which are related to embryo viability post thawing[5].

Until recently, research of embryonic factors that influence the outcome of FET cycles has mainly focused on embryo survival rate and post-thawing morphology and kinetics. Important embryonic factors influencing implantation in FET cycles are the developmental stage before cryopreservation [1,6,7], the occurrence of cryopreservation related blastomere loss[1,4,8-12] and the resumption of cell division during post-thaw embryo culture[12-15]. Although very helpful, morphological assessment remains subjective and can be unreliable in predicting an embryo's viability[16-18]. Therefore, there is a strong need for other objective markers to aid the prediction of embryo viability. Conaghan et al.[19] and Gardner et al.[20] showed that differences in embryo metabolism of glucose and/or pyruvate are related to embryo viability. Others showed an association between amino-acid turnover and developmental or implantation potential using high-performance liquid chromatography (HPLC) in fresh[16,21] and frozen-thawed embryos[22]. These studies claim that embryos with a low or quiet amino-acid turnover have the highest developmental potential. Seli et al.[23] used proton nuclear magnetic resonance (H NMR) and found that embryos that resulted in clinical pregnancy and delivery had higher glutamate levels in the culture media.

Relatively new is the development of metabolomic profiling of spent embryo culture media by near-infrared (NIR) and Raman spectroscopy. These technologies measure modifications of the chemical composition of culture media made by embryos or oocytes and generate a value reflective of the implantation potential of an embryo[17,24-27] or nuclear maturity status and quality of oocytes[28]. Using this technology distinct differences were found in culture media composition of embryos with proven reproductive potential compared to embryos that did not implant[17,24-30]. These differences were seen regardless of day of transfer and were independent of embryo morphology[17,29,30]. Nagy et al.[28] showed significant differences in NIR profiles of mature

and immature oocytes. They also demonstrated that oocytes that resulted in implanted embryos had higher viability indices than oocytes that resulted in embryos that did not implant. Furthermore, using Fourier transform infrared (FT-IR) spectroscopy, differences in metabolomic footprints were found between viable and non-viable embryos[31].

Previous studies using metabolomic profiling of spent culture media by NIR and Raman spectroscopy have all focused on fresh embryo transfer cycles. There are no data available of metabolomic profiles analysed by NIR spectroscopy of frozen-thawed embryos. This study analysed if metabolomic profiling of spent culture media by NIR spectroscopy could also serve as a marker of embryo viability in frozen-thawed embryo transfer cycles.

MATERIALS AND METHODS

PATIENTS

From November 2007 to January 2009, spent embryo culture media was collected from 127 frozen-thawed single embryo transfers of 115 patients. The outcomes were retrospectively reviewed. The study had ethical approval of the Institutional Review Board (IRB reference non-WMO).

FRESH CYCLE PROTOCOL

Patients under the age of 38 years or with previous good response in an IVF or intracytoplasmic sperm injection (ICSI) treatment were treated as previously described[32,33]. In women older than 38 years or with a previous poor response, a short GnRH-agonist protocol was applied[32,33]. Ovarian response was monitored by vaginal ultrasonography and serum estradiol. Human chorionic gonadotropin (HCG) (Pregnyl [Organon, Oss, the Netherlands]) was given 36 h before ultrasonographically directed oocyte retrieval.

IVF/ ICSI PROCEDURE

IVF and ICSI were performed according to the laboratory's routine insemination procedures (day 0). On day 1, 16-18 h after insemination, fertilization was checked. Embryos were cultured individually in 25-µl pre-equilibrated medium drops (human tubal fluid, HTF; Lonza, Belgium) with 10% human serum albumin (HSA; GPO, Sanquin, the Netherlands) in incubators at 37°C, under 5% CO_2 and atmospheric O_2 concentration. Embryos were selected by their morphological appearance and developmental

potential prior to transfer. Fresh embryo transfer was performed on day 3. When good quality embryos were left over after transfer, they were assessed again on day 4 prior to freezing.

CRYOPRESERVATION PROCEDURE

Embryos with at least eight cells and < 20% fragmentation were frozen on day 4. Two or three embryos, depending on pre-freezing quality, were frozen in 0.5 ml straws (CBS, L'Aigle, France). The embryo freezing media consisted of HTF / Hepes (Lonza, Belgium) with 10% HSA and dimethyl sulphoxide (DMSO; Sigma Aldrich, Germany). Embryos were frozen using a standard slow protocol. Briefly, the embryos were placed in 40-µl of 0.75 mol/l DMSO media drops at room temperature. After 10 min, the embryos were transferred to 40-µl 1.5 mol/l DMSO media drops and subsequently loaded in straws. Cooling was performed by programmable freezers (Kryo 360, Planer Products, UK) starting at a rate of 2.0°C per min to -6.0°C. At -6.0°C the straws were soaked for 5 min after manual seeding. Hereafter, cooling continued at a rate of 0.3°C per min to -40.0°C and subsequently at -20.0°C per min to -140.0°C, after which they were placed in liquid nitrogen storage.

THAWING PROCEDURE

Embryos were thawed for 2-3 min at room temperature after removing the straws from the liquid nitrogen storage. Thawed embryos were incubated in a series of decreasing DMSO media solutions (1.25, 1.0, 0.75 and 0.375 mol/l) DMSO in HTF / Hepes with 10% HSA. The final step was a rinse in DMSO free HTF / Hepes / HSA media. Embryos were assessed after thawing by routine morphological criteria and subsequently cultured for 20-24h in individual 25-µl media drops. Prior to embryo transfer the embryos were assessed again by routine morphological criteria and the best morphology embryo was selected for transfer. Only embryos in the compaction stage or embryos with a blastocoelic cavity were selected for transfer. No assisted hatching was performed.

SET CYCLE AND PREGNANCY

Transfer of frozen-thawed embryos was performed by monitoring follicle growth during a natural cycle by vaginal ultrasonography and administering HCG when a follicle reached the size of 18 mm or more in diameter and the two endometrial layers had reached at least a thickness of 6 mm or more. SET was performed 6 days after HCG injection. Combined with the overnight culture, this equates to a day 5 transfer after the IVF/ICSI procedure. For each patient live birth was recorded.

SAMPLE ANALYSIS BY NIR SPECTROSCOPY

After embryo transfer, the media drops in which the thawed embryos were cultured were collected along with a parallel embryo-free control drop and were snap frozen in liquid nitrogen and stored at -80°C. Analysis was performed using the same methodology previously described[17,28,30]. Briefly, culture media samples of frozen-thawed embryos were thawed on ice for 30 min, vortexed and subsequently centrifuged at room temperature for 5 min at 9660g. Sample cells were filled with a 10-µl aliquot of the thawed media droplet and placed in a holder with a specified temperature (24.3°C). After at least 4 min in the holder, the sample cell was transferred into the NIR spectrometer (prototype ViaMetrics-E™; Molecular Biometrics Inc., New Haven, USA). NIR spectra were obtained from each sample. The measurement was repeated with the control media to account for any variations between culture conditions between embryos.

VIABILITY SCORE DETERMINATION OF THAWED EMBRYO MEDIA SAMPLES

A multivariate algorithm that generates a viability score was previously developed using NIR spectra with known fetal cardiac activity outcome of freshly transferred day 5 embryos. This algorithm was developed using proprietary methodology of Molecular Biometrics entailing a modification of previous publications[17,30]. The spectra generated were assessed blindly by this predictive algorithm and a viability score was calculated for each individual sample.

STATISTICAL ANALYSIS

Mean viability scores (mean ± SD) were compared between live birth and no live birth outcome sample populations. Quartile trends were plotted for increasing live birth rate versus viability scores of all data and then again for subsets of 67 compaction stage embryos and 60 blastocysts. Quartile trend significance was evaluated using the Cochran-Armitage test. Student's t-tests were used to compare mean viability scores between populations. Three regression analyses were performed to analyse the relationship between the viability score and live birth: 1) a crude analysis; 2) an analysis adjusted for embryo morphology where embryo morphology was added to the model as a dichotomous variable; and 3) an analysis further adjusted for resumption of mitosis, elective or non-elective SET, IVF or ICSI treatment in fresh cycle (all added to the model as dichotomous variables); embryo survival rate, stimulation protocol in fresh cycle, infertility indication (all added to the model as categorical variables); and infertility duration and age of patient at ovum pick-up (both added to the model as

continuous variables). Data were analysed using Statistical Package for Social Sciences version 18.0 (SPSS, Chicago, IL, USA).

RESULTS

The baseline characteristics of patients and embryos are presented in Table 1. A total of 127 embryos were transferred; 67 were compaction stage embryos and 60 had blastocoelic cavities at the time of transfer. Of the 127 SETs, 67 were performed when more than one embryo of sufficient quality was available (elective SET) and 60 when only one embryo was available for transfer (non-elective SET). A total of 120 embryos showed resumption of mitosis 20-24 h post thawing.

The live birth rate per embryo transfer was 30.7% (39/127), with 38 healthy babies delivered at term and one pre-term delivery at 25 weeks of gestational age due to premature rupture of the membranes. All were singletons. The 39 live deliveries were from 13 compaction stage embryos and 26 embryos had blastocoelic cavities. Of the 39 live deliveries, 37 were from embryos that showed resumption of mitosis 20-24 h post thawing.

The mean viability score from implanted embryos that gave rise to live birth (0.565 ± 0.129) was significantly higher than that of embryos which failed to give rise to a live birth (0.480 ± 0.165), P=0.002.

Table 2. Results of the three multivariable regression analyses that tested the predictive ability of the viability score for live birth.

	OR	95% CI	P-value
Crude	1.50	1.11-2.03	0.008
Adjusted 1	1.44	1.05-1.96	0.022
Adjusted 2	1.71	1.17-2.48	0.005

Odds ratios (OR) are based on 0.1 difference in viability score.

Adjusted 1= adjusted for embryo morphology.

Adjusted 2= further adjusted for resumption of mitosis, elective or non-elective single embryo transfer, embryo survival rate, IVF or intracytoplasmic sperm injection treatment in fresh cycle, stimulation protocol in fresh cycle, infertility duration, infertility indication, age at oocyte retrieval and embryo viability score.

CI = confidence intervals.

Table 1. Baseline patient and embryo characteristics.

Characteristic	Live birth	No live birth	Total
Embryo morphology			
Compaction stage	13 (33.3)	54 (61.4)	67 (52.8)
Blastocyst	26 (66.7)	34 (38.6)	60 (47.2)
Embryo viability score	0.565 ± 0.129	0.480 ± 0.165	0.506 ± 0.159
Resumption of mitosis			
Yes	37 (94.9)	83 (94.3)	120 (94.5)
No	2 (5.1)	5 (5.7)	7 (5.5)
Elective SET	19 (48.7)	48 (54.5)	60 (47.2)
Non-elective SET	20 (51.3)	40 (45.5)	67 (52.8)
Embryo survival rate			
Fully (100%)	29 (74.4)	67 (76.1)	96 (75.6)
Partially (75%-99%)	5 (12.8)	11 (12.5)	16 (12.6)
Partially (50%-74%)	5 (12.8)	10 (11.4)	15 (11.8)
IVF	18 (46.2)	38 (43.2)	56 (44.1)
ICSI	21 (53.8)	50 (56.8)	71 (55.9)
Stimulation protocol fresh cycle			
Short GnRH agonist	6 (15.4)	18 (20.5)	24 (18.9)
Long GnRH agonist	33 (84.6)	70 (79.5)	103 (81.1)
Infertility duration (years)	2.4 ± 1.3	3.3 ± 2.3	3.0 ± 2.1
Infertility indication			
Tubal	7 (17.9)	12 (13.6)	19 (15.0)
Endometriosis	3 (7.7)	7 (8.0)	10 (7.9)
Poor semen quality	21 (53.8)	43 (48.9)	64 (50.4)
Anovulation	2 (5.1)	6 (6.8)	8 (6.3)
Unexplained	4 (10.3)	17 (19.3)	21 (16.5)
Cervical	0 (0)	1 (1.1)	1 (0.8)
Other	2 (5.1)	2 (2.2)	4 (3.1)
Age at oocyte retrieval (years)	33.2 ± 4.4	33.4 ± 5.2	33.3 ± 4.9

Values are n (%) or mean ± SD.

GnRH = gonadotropin-releasing hormone; ICSI = intracytoplasmic sperm injection; SET = single embryo transfer.

Table 2 shows the results of the logistic regression analyses that tested the predictive ability of the viability score for live birth. In the crude analysis, an odds ratio (OR) of 1.50 (95% Confidence Interval [CI] 1.11-2.03; P=0.008) was found based on a 0.1 step increase in viability score. In the second analysis (adjusted for embryo morphology) an OR of 1.44 (95% CI 1.05-1.96; P=0.022) was found, and in the third analysis (adjusted for all variables), an OR of 1.71 (95% CI 1.17-2.48; P=0.005) was found.

Figure 1 shows viability scores determined in the blinded analysis of 127 frozen-thawed embryos analysed in quartiles. Individual embryos showed a positive relationship between increasing viability scores and increasing live birth rate (P=0.02). The viability scores within two separate groups of embryos with the same morphological score (compaction or blastocyst) showed a nonsignificant trend (P=0.08) toward an increasing percentage of live births in relation to increasing viability scores (Figure 2). The viability score from blastocysts (0.539 ± 0.144) was significantly higher than that of compaction stage embryos (0.467 ± 0.167), P=0.025).

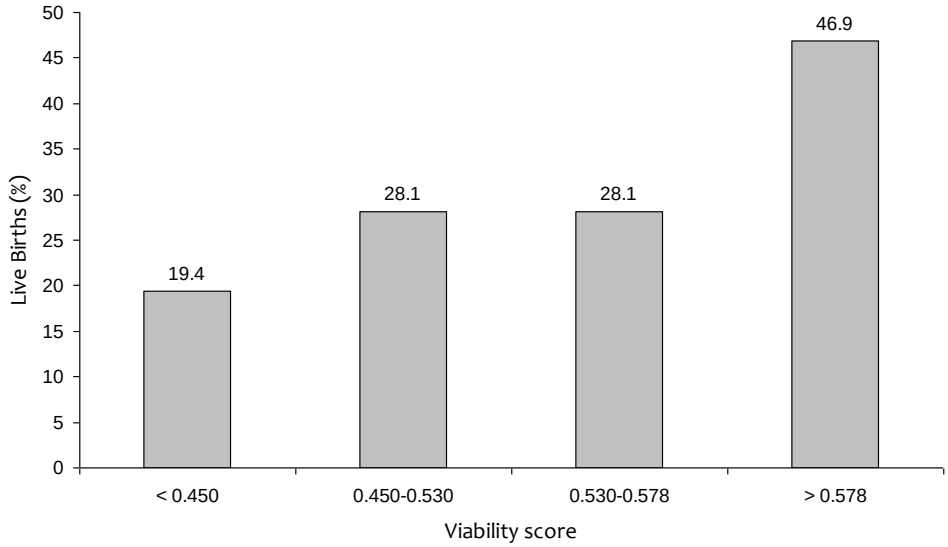

Figure 1. The relationship between the percentage of live births in relation to increasing viability scores. Viability scores were determined in the blinded analysis of 127 cryopreserved embryos. The viability scores are presented in quartiles of increasing viability scores and contain 31 patients in the first group and 32 patients in the last 3 groups.

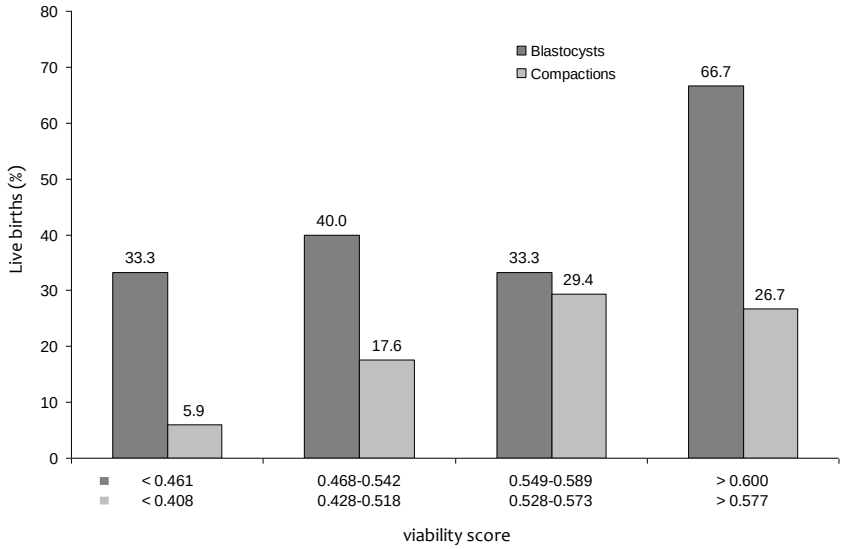

Figure 2. The relationship between the percentage of live births and increasing viability scores within two separate groups of embryos with the same morphological score (compaction and blastocyst). Viability scores determined in the blinded analysis of 66 compaction stage embryos were analysed in quartiles with 17 patients in the first 3 groups and 15 patients in the last group. Viability scores of 60 blastocysts determined in the blinded analysis were analysed in quartiles with 15 patients in each quartile.

DISCUSSION

This study analysed spent culture media of frozen-thawed embryos with known implantation potential after SET and related the results to live birth. When using a previously established predictive multivariate algorithm developed from fresh day 5 embryos, the data showed a positive relationship between increasing viability scores and live birth outcomes: i.e. a higher viability score indicated a higher viability of frozen-thawed embryos. Other studies in which the same viability assessment method was used for fresh embryos[17,24-30] and oocytes[28] found a similar relation. Brison et al.[31] also showed that metabolic footprints of viable embryos cluster together using Fourier transform infrared spectroscopy.

The blind evaluation of spent culture media of frozen-thawed embryos on predictive multivariate algorithm of fresh day 5 embryos with known pregnancy outcomes, could help to identify the live birth group from the no live birth group. This result strongly suggests that viable frozen-thawed embryos have the same areas in NIR spectra that

are predictive of implantation as viable fresh embryos. In other words, an algorithm generated by using fresh embryos may be used to predict viability of frozen-thawed embryos. More research is needed to make strong conclusions, but this might indicate that the metabolism of viable frozen-thawed embryos, which give rise to a live birth, is not altered significantly by the cryopreservation process. This is in agreement with a previous study by Stokes et al.[22], who showed that the amino-acid profiles obtained from frozen-thawed embryos were similar to those for fresh embryos, using high-performance liquid chromatography.

In future, it would be interesting to analyse the metabolites found in the spectral regions that are indicative of live birth. NIR spectra do not identify specific metabolites, but only contain information on vibrations of functional groups. Knowing the specific metabolites that are of importance in the metabolism of a viable (frozen-thawed) embryo, will help in understanding better the NIR spectroscopy technique and possibly even the implantation processes[27]. The algorithm used in the current prediction was largely based on four wavelet regions in the 1200-1430 nm range. The predominant weighting of the two major wavelets were in the 1340-1430 nm range. Between 1110 and 1460 nm, there is a mixed region which contains information from fatty acids, polysaccharides, nucleic acids and proteins[31].

A series of multivariable regression models that tested the predictive ability of the viability score for live birth, showed before and after adjustment for the variables embryo morphology, resumption of mitosis, elective or non-elective SET, embryo survival rate, IVF or ICSI treatment in fresh cycle, stimulation protocol in fresh cycle, infertility duration, infertility indication and age of patient at oocyte retrieval, an OR between 1.44 and 1.71 based on a 0.1 difference in viability scores. In other words, this means that a 0.1 step increase in the viability score was associated with a 1.44-1.71 times higher odds for live birth.

When the viability scores of the 127 embryos were separated into quartiles to test whether increasing viability scores give rise to higher live birth rates (Figure 1), a significant increasing live birth fraction with increasing viability scores was observed (P=0.02). Seli et al.[30] found comparable results for fresh day 2 embryos. The viability scores within two separate groups of frozen-thawed embryos with the same morphological grade (compaction and blastocyst) showed a trend towards a relationship with reproductive potential (Figure 2), although not to a statistically significant level, which may be due to the decreased number in patients per group. Previous studies using the same viability assessment method, also demonstrated higher viability scores for pregnant patients within each separate morphological grade of blastocysts[27] or cleavage stage embryos[30] after fresh embryo transfer. Another study

where the profiling of culture media could distinguish between frozen-thawed embryos of the same morphological grade was performed by Stokes et al.[22]. In that study the amino-acid profiles of frozen-thawed embryos of the same morphological grade could separate the arrested embryos from the embryos that developed into the blastocyst stage: the arrested embryos were metabolically more active.

Although blastocysts have significantly higher viability scores than compaction stage embryos, the results of the multivariable regression models and the quartile range results indicate that a higher viability score predicts a higher live birth rate independently from embryo morphology. Earlier studies in which the same viability assessment method was used for fresh embryos[17,29,30] didn't find a correlation between embryo morphology and embryo viability score either. However, regarding the assessment of the embryo morphology parameters, the current study was already looking at a very selected group: first, the embryos were selected to be cryopreserved, and second, they were again selected to be transferred after thawing. Nevertheless, metabolomic profiling of spent culture media by NIR spectroscopy might be used as an adjunct to morphology when selecting the most viable frozen-thawed embryo to transfer. It should be kept in mind that in most FET cycles, fewer embryos are available to choose from for transfer compared to embryo transfers from fresh cycles. Therefore, the practical implication of using the viability score or equivalent procedure might not be relevant for IVF clinics that cryopreserve one embryo per straw. The extra information provided by the viability score, however, might be used as information to perform elective SET instead of DET if more than one embryo is available to transfer.

These types of diagnostic technologies may also be used to compare and optimize freezing techniques such as vitrification and slow freezing, or different cryoprotectants. This would allow researchers to gain a more rapid evaluation of changes in freezing protocols. Furthermore, beside the evaluation of cryopreservation procedures, this technique may be used to pro-actively assess which embryos to freeze. Prior to broader acceptance of the use of spectroscopic methods to select (frozen-thawed) embryos for transfer, more research has to be performed to investigate the advantages and limitations of these methods, preferably by randomized controlled trials.

Variables such as resumption of cell division[12-15], woman's age[2,12], aetiology of infertility[2] and cryopreservation related blastomere survival rate[1,4,8-12] have all shown a significant correlation to pregnancy outcomes in other studies. This is in contrast to the current data but might be explained by the already selected group of patients. This study could not investigate if a better pre-freeze embryo quality increases post thawing survival[1,6,7], because the study centre freezes more than one embryo per straw. In the future, it will

be interesting to compare pre-freeze viability scores with post-freeze viability scores. Not recording the pre-freeze embryo quality may have biased the results, because an embryo with a lower pre-freeze quality will likely have a lower post freezing quality as well, especially since the study centre also freezes embryos with as few as eight cells on day 4 which can be considered as slower developing embryos. These slower developing embryos, however, have shown in the embryo culture system used in this study the potential to develop into blastocysts and have led to pregnancies and live births. But looking only at the data from blastocysts, the positive relationship between increasing viability scores and increasing live birth rate is even more distinctive. The time of FET equates to a fresh day 5 transfer, the day an embryo is preferably in the blastocyst stage. Blastocyst stage embryos are expected to have conquered eventual damage of the cryopreservation process and a lesser pre-freeze quality.

In conclusion, the metabolomic profiling data from frozen-thawed embryos showed similar findings to earlier work using this method with fresh embryos[17,24-30] and oocytes[28]: higher viability scores result in higher pregnancy outcomes. Frozen-thawed embryos (of the same morphological grade) have different metabolic activity which is related to implantation potential. This indicates that the use of morphological and metabolomic criteria can both help with the decision of which embryo to transfer after thawing.

New techniques in assessing embryo quality might improve pregnancy and delivery rates per embryo transfer and therefore encourage the implementation of SET[34]. Metabolomic profiling by NIR spectroscopy is a rapid, objective and non-invasive embryo assessment technique which may provide extra information about the implantation potential of frozen-thawed embryos at the time of transfer. This might reduce the number of embryos transferred in FET cycles and therefore the incidence of multiple pregnancies.

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