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Selection of Blastocysts for Transfer Using a Quantitative Bioassay of Blastocoel Expansion Rather than Standard Morphology Grading

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Abstract

Recent evidence has shown a sustained singleton pregnancy benefit using trophectoderm biopsy and preimplantation genetic testing for euploidy. However, these results have utilized conventional standard grading morphology for the selection of the blastocysts actually selected for transfer. This approach is subjective and difficult to standardize. We describe here a new selection method using a non-invasive, physiological assay of blastocyst expansion using time-lapse imaging. Using this technique, blastocysts can be objectively ranked within cohorts for transfer based upon objective and quantitative measurements of expansion rates which is completely independent of standard grading. Results described here show that prospective selection of first blastocysts for transfer using this approach results in identical and high live birth pregnancy rates from either biopsied or unbiopsied blastocysts in younger patients <37yrs (73.6% vs 74.5%, respectively). For patients >37yrs, similar pregnancy rate parity was achieved between single euploid blastocysts using the two highest ranked unbiopsied blastocysts and with little twin risk (62.1% vs 60.1%). Unexpectedly, the results in the older age group show evidence for a significant reduction in pregnancy rate using biopsy on an intention to treat basis. This reduction (from 62.1% to 29.0%) may be explained by a masking of some blastocyst's normal totipotentiality by biopsy results that do not reflect true meiotic whole chromosome aneuploidy (e.g. segmental and/or mosaic results). Such cases were more prevalent in patients having smaller blastocyst cohort sizes (<4). While results from subsequent transfers from cohorts have yet to be assessed, this new prospective approach to using blastocyst expansion rate assessment represents a rational tool for the identification of individual blastocysts with the highest likelihood for euploidy without the need invasive biopsy and genetic analysis.

INTRODUCTION

Culture of human embryos to the blastocyst stage challenges the IVF laboratory to find discriminative markers useful to rank order individual embryos for transfer. For the past several decades, selection of blastocysts has largely relied upon the broad classification system of standard morphology grading [SG, 1-2]. However SG offers no guidance useful to discriminate between embryos that have very similar morphology grades. From the perspective of classification and prediction modeling, this limitation has contributed to the increasing application of trophectoderm biopsy with genetic screening, which has revealed considerable genetic heterogeneity in this tissue due to chromosome instability, even among embryos with high morphology grades [3-6].

Despite PGT-A's unassailable rationale to identify ploidy, this screening approach also has several practical limitations. This includes possible detrimental effects to the embryo from the biopsy, the problematic interpretation of certain screening results (such as mosaicism, sub-chromosomal abnormalities, and calls with no results), possible differences in the different molecular genetic platforms used for screening, as well as its costs and universal availability. Moreover, even after biopsy, single euploid blastocyst selection resorts back to standard

grading to select a given single euploid when more than one is available [7-9].

Time lapse imaging offers a variety of more objective and quantitative solutions potentially useful to improve ranking for transfer through annotations of the timing of defined developmental milestones, although its efficacy currently remains controversial [10-18]. Although time-lapse enables the evaluation of a multiplicity of developmental events throughout the entire preimplantation period, our group has taken a novel approach that narrowly focuses on the dynamics of the early blastocyst expansion period using a quantitative 10hr standardized expansion assay [qSEA, 19, 20]. In contrast to SG and other time-lapse approaches, it represents a bioassay that quantitatively measures how productively the trophectoderm functions as a newly differentiating epithelial tissue. [21,22]. Thus, qSEA analysis represents a narrowly focused physiological perspective, rather than a morphological one. The assay measures actual work performed by a social network of cells to drive expansion of a dynamically dividing epithelial architecture against its zona pellucida [23,24].

We have hypothesized that quantitative expansion dynamics may reflect implantation potential to the extent that expansion metrics are surrogate measures that integrate both known and unknown features beneficial for endometrial invasion, including cellularity and euploidy [25,26]. Initial support for this hypothesis came from the qSEA analysis of blastocysts from PGT-A cycles, which showed that averaged euploid blastocysts expand more productively than averaged aneuploids [19]. This is also consistent with basic studies describing the cell biology of aneuploidy [27]. From this perspective, qSEA measurements represent an objective, non-invasive assay capable of identifying embryos enriched for euploidy using the continuous, dynamic variable of expansion size. This predicts that qSEA selection of the highest ranked single unbiopsied blastocysts within a cohort should also have the greatest likelihood of resulting in a sustained implantation comparable to euploids that are selected in the same way.

Here we describe and compare pregnancy outcomes from such transfers over a four year period, where the first biopsied euploid or unbiopsied blastocysts from cohorts were prospectively selected for transfer using the qSEA assay, with the subsequent application of morphology only as confirming information. In patients <37ys, professional guidelines recommend single blastocyst transfers, with double unbiopsied blastocyst transfers acceptable in patients ≥37yr [28]. This non-randomized study was not designed to powerfully address any relative inferiority or superiority for the PGT-A pathway; rather, it was intended as a first step to validate the performance of this defined blastocyst selection algorithm – which is both simple and transparent-- across a broad spectrum of patient ages. The results presented here argue that qSEA selection can identify unbiopsied blastocysts that have comparable sustained singleton pregnancy rates as biopsied euploids in these first from cohort transfers. Perhaps a more important implication of these findings is that this defined assay offers an auditable pathway that can standardize blastocyst selection by all personnel both within and between laboratories using time-lapse imaging.

MATERIALS AND METHODS

This retrospective case control study consisted of 179 sequential, first vitrified blastocyst transfers from cohorts from 2018-2021 at a single program (Advanced Reproductive Center of Hawaii). This study received University of Hawaii's institutional review board approval (CHS no. 23407). Prior to IVF treatment, patients had failed multiple rounds of artificial insemination with low dose gonadotropin stimulations. Treatment plans were determined after counseling and without randomization to any given treatment pathways. Ovarian stimulation utilized a standardized protocol using gonadotrophin-releasing hormone antagonist with egg retrievals performed 34-36 hours after a trigger using human chorionic gonadotrophin or gonadotrophin releasing hormone agonist as described by Huang et al. [19]. Oocytes were all autologous to each patient with the exception of 2 and 5 egg donor cases in the <37yo and ≥37yo patient groups, respectively.

Following oocyte retrieval using transvaginal ultrasound guidance, all fertilization of mature oocytes used intracytoplasmic sperm injection (ICSI). Oocytes were then placed immediately into an Embryoscope (Unisense, Denmark) and cultured for up to 6 days in Global Total medium (Cooper Surgical, USA) as described by Huang et al., [19]. Regardless of age, Day 3 vitrification of all embryos was sometimes performed in more poorly responding patients that had no more than 3 normally cleaving embryos or where the treatment plan was to transfer all available embryos in a future hormone replacement cycle. When any selection would likely be required, culture was extended to the blastocysts stage. Such Day 3 cryopreservations were performed in 7 cycles in patients aged <37ys and 33 cycles in patients aged ≥37yrs (Figure 1, Tables 1 and 2)).

For biopsy cases, embryos were cultured further to Days 5-6. Embryoslides were briefly removed early on Day 4 for zonae ablation as described by Huang et al. [19], using a Lykos Laser (Hamilton Thorne, USA). Biopsy was then performed on Days

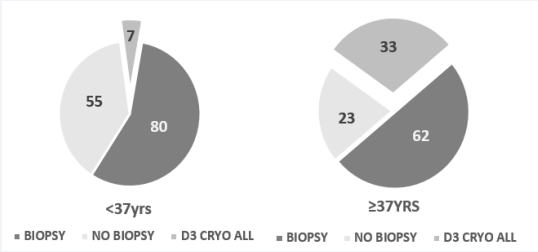

Figure 1 Treatment Pathway Distribution of Patients. For patients <37ys, only 7 cases (4.9%) required no further embryo selection before transfer and had cryopreservation of all cohort embryos on Day 3. Of the remaining cases having extended culture, 80 cases (56.3%) had biopsy and 55 (38.7%) had no biopsy. For patients ≥37yrs, 33 cases (27.9%) had Day 3 cryopreservation of all cleaving embryos due to their more limited response. Of the remaining having extended culture, 62 cases (52.5%) had a biopsy and 23 cases (19.5%) had no biopsy.

Table 1: Comparison of Patients and Pregnancy Outcomes from Single Euploid and Single Unbiopsied Blastocysts in Patients <37yrs

<37 yrs	GROUP A (BIOPSY)	GROUP B (NO BIOPSY)	P VALUE
# Patients (ITBx)	72 (80)	55	
Mean Age (Range) yrs	32.4 (23-36)	30.7 (22-36)	0.006
Mean # (Range) Vitrified	8.0 (1-17)	6.3 (1-20)	0.011
Mean # Euploid (% Biopsied)	3.9 (49.4%)		
# Cases (%) with No Euploids	8 (10%)		
HCG Doubling (%/ET)	62/72 (86.1%)	49/55 (89.1%)	0.62
HB+ (%/ET)	56/72 (77.8%)	42.55 (76.4%)	0.85
HB+ (%/ITBx)	56/80 (70.0%)	41/55 (74.5%)	0.42
Live Birth (%/ET)	53/72 (73.6%)	41/55 (74.5%)	0.41
Live Birth (%/ITBx)	53/80 (66.3%)	41.55 (74.5%)	0.30

Table 2: Comparison of Patients and Pregnancy Outcomes from Single Euploid and Double Unbiopsied Blastocyst Transfers in Patients ≥37yrs

≥37 yrs	GROUP C (BIOPSY)	GROUP D (NO BIOPSY)	P VALUE
# Patients (ITBx)	29 (62)	23	
Mean Age (Range) yrs	38.9 (37-42)	40.2 (37-44)	0.02
Mean # (Range) Vitrified	5.0 (1-17)	5.2 (2-15)	0.58
Mean # Euploid (% Biopsied)	1.8 (36.0%)		
# Cases (%) with No Euploids	33 (53.2%)		
HCG Doubling (%/ET)	23/29 (79.3%)	17/23 (73.9%)	065
HB+ (%/ET)	19/29 (65.5%)	15/23 (65.2%)	0.98
HB+ (%/ITBx)	19/62 (30.6%)	15/23 (65.2%)	0.004
Live Birth (%/ET)	18/29 (62.1%)	14/23 (60.1%)	0.93
Live Birth (%/ITBx)	18/62 (29.0%)	14/23 (60.1%)	0.007

5-6 and analyzed by Cooper Genomics (Livingston, New Jersey, USA). For biopsy cases, only outcomes from the transfer of single blastocysts with "normal euploid" calls were analyzed in this study; thus, transfers of blastocysts from cohorts having only mosaic or segmental classes were excluded from Group analysis. However, these and any other biopsy cases having no normal euploid calls were later added back, statistically, for "intention to treat after biopsy" (ITBx) analysis. Thus, there were 8 patients in biopsy Group A that had at least two blastocysts biopsied but with no normal euploid calls (8/80= 10.0%) and 33 such patients in biopsy Group C (33/62 = 53.2%, see Tables 1, 2).

A quantitative standardized expansion assay qSEA was used to select blastocysts was performed as described by Huang et al. [19]. This assay measures the total area contained within the blastocoel cavity in successive time-lapse image over the blastocyst's first 10hr period of expansion, including the trophectoderm cells. In order to acquire qSEA measurements, no blastocysts were removed from the Embryoscope for biopsy or vitrification until at least 10 hours had elapsed from their times of initial blastocyst formation (tB). This was operationally defined as the beginning of progressive expansion of the trophectoderm enclosed cavity against either its intact zona pellucida or through the laser ablation slit. This area was recorded in µ2 either manually or automatically by computer using a customized AI platform [20]. These measurements served, exclusively, to rank order individual blastocysts within cohorts from the highest (ranked #1) to the least expanded at the end of the assay. The #1 ranked blastocyst from a given cohort was used for each patient's first single blastocyst transfer in Groups A, B, and C, irrespective of its tB time after ICSI. Double blastocyst transfers in patients >37yrs (Group D) utilized the two highest qSEA ranked, unbiopsied blastocysts. In some biopsy cases, euploid blastocyst ranking was performed within one gender subset. Morphology assessment was always used after ranking and prior to transfer to confirm that the cells of the ICM and trophectoderm from selected blastocysts had grades of A or B or that no other cohort blastocyst had an unequivocally higher SG score; however, no blastocysts were deselected on this basis in this study. Other than confirmation of two pronuclear formation, other cleavage stage observations (e.g. fragmentation or direct unequal cleavage during the first two cell cycles) did not alter any selection of the qSEA selected blastocysts for transfer. Following the embryo transfer, a positive HCG was defined by HCG doubling (undoubled HCG rises were considered negative). Clinical pregnancy required confirmation of a heartbeat by 8-10 weeks, and live birth was defined as confirmed delivery after 22 weeks.

DATA ANALYSIS

Characteristics of patients were summarized by mean and range for continuous variables and frequency and percentage for categorical variables. Normality of the continuous measure was assessed by quantile-quantile plot. Two sample t tests were used to compare age and numbers of blastocysts vitrified between biopsy and non-biopsy groups and chi-square or Fisher's exact tests were used to compare frequencies between the two groups for categorical variables described in [Table 1,2]. All the statistical analyses were conducted by using SAS 9.4 and the significance level was set at 0.05.

RESULTS /FINDINGS

For outcome analysis, patients were stratified into four groups based upon both age and biopsy status (Tables 1,2):

Group A: <37yr, single euploid blastocysts (n=72); Group B: <37yr, single unbiopsied blastocysts (n= 55); Group C: ≥37yr single euploid blastocysts (n=29); Group D: ≥37yr, double unbiopsied blastocysts (n= 23).

1. Pregnancy results using the highest qSEA ranked single euploid or single unbiopsied blastocysts in patients <37ys

Comparison of results in treatment Groups A and B that represent patients <37yr are shown in Table 1 and Figure 2. There were a total of 80 biopsies in Group A with intention to treat after biopsy. 72/80 (90.0%) had at least one normal euploid available that was transferred, with such calls comprising 49.4% of the total blastocysts biopsied, resulting in an average of 3.9 normal euploids per cohort. Group A maternal ages were significantly older by 1.9 years compared to Group B (32.4yrs vs 30.5yrs, p= 0.006). Biopsied Group A also had significantly more averaged blastocysts cryopreserved (8.0 vs 6.3, p= 0.011).

Both initial doubling of HCG and sustaining pregnancy rates/transfer were both of high quality and statistically comparable between both Groups. These rates per transfer ranged from 86.1% and 89.1% for HCG doubling to 73.6% and 74.5% for live births for Groups A and B, respectively. These difference were not statistically significant (p= 0.62 and 0.41, respectively). On an ITBx basis for Group A, the pregnancy rate revisions did not alter the statistical evaluation of live birth (p= 0.30). Notably, there was one monozygotic twin from the biopsy Group A (1/72= 1.4%), but no twinning was observed in unbiopsied Group B. Although at lower levels, the same parity was also seen at points during pregnancy between single euploids (Group C) and double unbiopsied blastocysts (Group D).

Figure 3 correlates different blastocyst cohort size bin groups with both the number of patients receiving a transfer from those bin groups (A) and their HB+ pregnancy outcomes (B). Biopsied Group A cases that had no euploid calls (10% of the total, Table 1) are further graphically concatenated as light grey bars above the black bars representing the cases having euploids. These no euploid call cases were largely distributed across smaller bin groups, particularly bin groups with 1-4 blastocysts (Figure 3A). Assuming that a similar distribution of no normal euploid call cases also existed in unbiopsied Group B, these should have acted to reduce the pregnancy rate/transfer in these smaller bin group regions; however, no evidence for such an effect was seen for any bin group other than the single smallest bin group 1-2 that had relatively fewer patients (Figure 3B). Quite to the contrary, bin group 3-4 represented the bin group having both the highest number of unbiopsied blastocyst transfers and the highest pregnancy rate/transfer of all bin groups. Moreover, pregnancies from qSEA selected unbiopsied blastocysts appeared robust across the entire spectrum of cohort size bin groups. Although these results suggest the possibility that there may be a pregnancy rate risk from biopsy from smaller cohort sizes, these differences did not reach statistical in this younger patient group (Table 1).

2. Pregnancy results from the highest qSEA ranked single euploid and double unbiopsied blastocysts in patients ≥37ys .Comparison of treatment Groups C and D, representing patients ≥37yr, is shown in Table 2 and Figure 2. Patients in biopsied Group C were significantly younger by 1.3 years (38.9yrs vs 40.2yrs; p= 0.02), although each group had similar numbers of blastocysts vitrified (5.0 vs 5.2, p= 0.58). A total of 62 patients had biopsies with intention to treat after biopsy. Notably, while 29/62 (46.8%) had at least one euploid available for transfer, 33/62 (53.2%) of these biopsy cases had no normal euploid calls. Cohorts with normal euploids averaged 1.8 euploid/cohort, which represented 36% of these cohort's average size. In unbiopsied Group D, there were 23 cases that received a transfer of the two highest qSEA ranked unbiopsied blastocysts.

Both initial HCG doubling and sustaining pregnancies in patients receiving a transfer were both of high quality and statistically comparable in both Groups C and D (Table 2). These rates ranged from 79.3% and 73.9% for HCG doubling and 62.1% and 60.1% live births for biopsied Group C and unbiopsied Group D, respectively. These difference were not statistically significant (p= 0.65 and 0.93, respectively). While there was one dizygotic

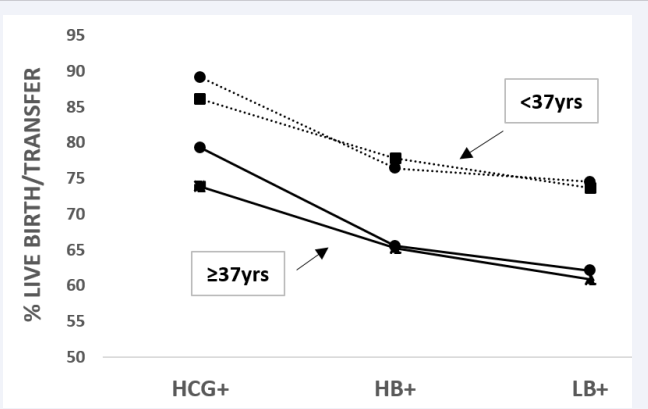

Figure 2 PREGNANCY PROGRESSION BY GROUP: From initial HCG doubling to live birth there was parity between biopsied Group A (box, dotted line) with unbiopsied Group B (circles, dotted line) in patients <37yrs utilizing single blastocyst transfers. There was also similar parity for patients ≥37yrs at all time points between single euploid blastocysts in Group C (box, solid line) and unbiopsied double blastocysts in Group D (circles, solid line).

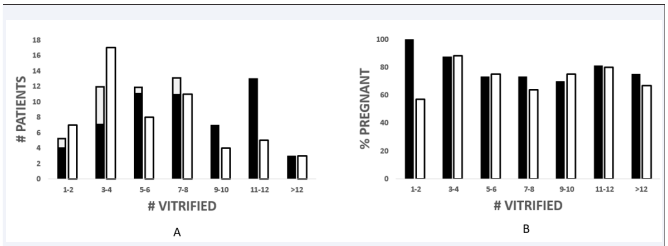

Figure 3 Comparison of Outcomes in Patients <37yrs based upon blastocyst cohort size. A: Bin groups are compared on the basis of the number of patients in each bin group. Solid white bars represent patient transfers using unbiopsied single blastocysts. Upwardly concatenated bars represent additional patients without a transfer who had no normal euploid calls. B: Bin groups are compared on the basis of the sustained live birth pregnancy rate in each bin group. Solid white bars represent single blastocyst transfers of unbiopsied blastocysts. Solid dark bars represent single euploid blastocyst transfers.

twin from unbiopsied Group D (1/23 = 4.3%), no monozygotic twinning was observed in biopsied Group C. Figure 2 also graphically compares the kinetics of pregnancy losses on a per transfer basis in Groups C and D from initial HCG doubling to live birth. As seen in the younger patient groups, the loss rates were identical in both groups, with proportionally greater losses seen before clinical heartbeats than afterwards.

Figure 4 correlates blastocyst cohort size bin groups in Groups C and D with both the number of patients receiving a transfer from those bin groups (A) and each bin group's HB+ pregnancy outcome (B). Biopsied Group C cases that had no euploid calls (53.2% of the total) were widely distributed among cohort sizes and are graphically concatenated as light grey bars above the black euploid bars in panel A. In contrast to the younger patients in Groups A and B (Figure 3), most bin groups showed a significant proportion of biopsies with no euploids calls. These occurred mostly from the smaller bin group sizes 1-6 (Figure 4A), where they typically represented more than half of each group's biopsy cases. The presence of a similarly distributed set of no normal euploid calls in unbiopsied Group D cases would have predicted a significant and proportional reduction in pregnancy rates/transfer within comparable bin groups. Quite to the contrary, the pregnancy rates were actually equal to or higher for unbiopsied blastocysts in the two smallest bin groups (1-4, Figure 4B), which actually represented the majority of Group D transfers (Figure 4A). Although bin groups having >4 blastocysts showed higher pregnancy rates using biopsied euploids, the number of patients in these larger bin groups was relatively small (Figure 4A). In contrast to the results for younger Groups A and B patients (Figure 2), the presence of so many of these cases with no euploid calls resulted in a significant reduction in pregnancy rate/transfer on an ITBx basis (29.0%) which was significantly lower than for the unbiopsied Group D (p = 0.007).

As shown in Figure 5, a significant proportion of these no euploid call cases had at least one call that did not involve uniformly whole chromosome aneuploidy (e.g. mosaics, segmental aneuploids). In those with <4 blastocysts biopsied, (7/16) 44% had at least one such calls. In those with ≥4 blastocysts biopsied, 15/17 (88%) contained at least one such call, despite the absence of any normal euploids.

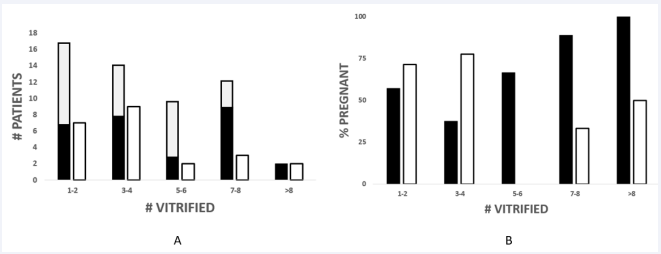

Figure 4 Comparison of Outcomes in Patients ≥37yrs based upon blastocyst cohort size. A: Bin groups are compared on the basis of the number of patients in each bin group. Solid white bars represent patient transfers using unbiopsied single blastocysts. Upwardly concatenated bars represent additional patients without a transfer who had no normal euploid calls. B: Bin groups are compared on the basis of the sustained live birth pregnancy rate in each bin group. Solid white bars represent double blastocyst transfers of unbiopsied blastocysts. Solid dark bars represent single euploid blastocyst transfers.

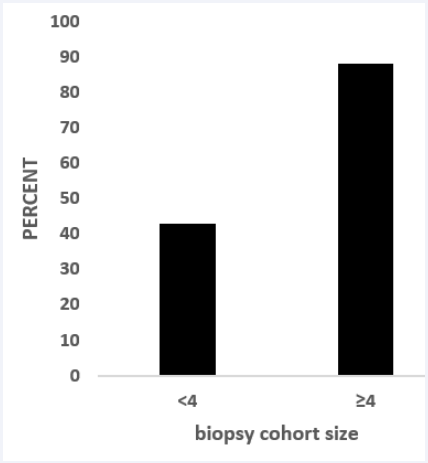

Figure 5 Percent of biopsy cases with that had no normal euploid calls after biopsy from patients ≥37yrs that also had at least one call of unknown clinical significance (mosaics, segmental abnormalities).

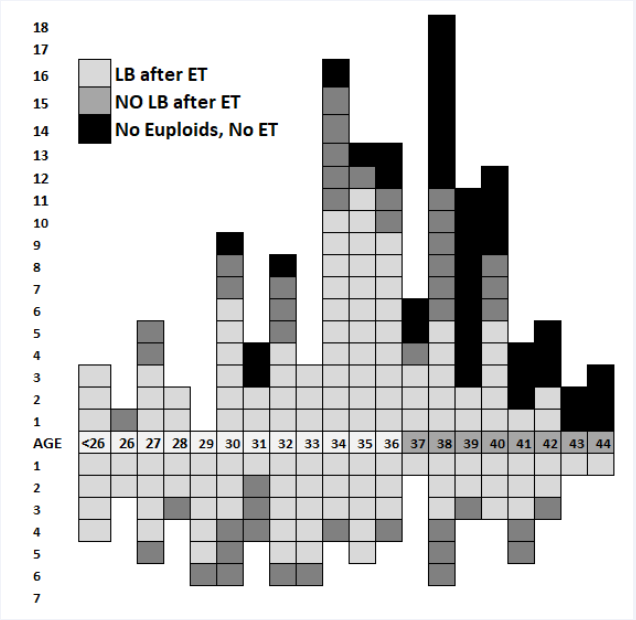

Figure 6 Live birth outcome histogram plot by individual maternal age in years (central X-axis) and biopsy status. Individual biopsy case outcomes are reflected above this axis while unbiopsied cases are reflected below this axis. Each patient outcome is represented by a single box as either 1) successful live birth (light grey) or 2) live birth failures (dark grey). The distribution of additional cases having at least two blastocysts biopsied but with no normal euploid calls (and no embryo transfer) are further concatenated (black) above the biopsied blastocyst transfer cases.

An outcome histogram plot of pregnancy results for each individual patient from all Groups by age on an ITBx basis is shown in Figure 6. When considering only cases that had an embryo transfer, the pregnancy outcomes both above and below the age axis closely mirror one another, reflecting the parity in pregnancy outcomes (Figure 2). Although there were more total cases in the biopsied Groups A and C, in only 4/20 (20%) of the chronological year groups (28, 30, and 31, and 33yr) was the ratio of live birth successes to failures higher from the biopsy

pathway than from the unbiopsied pathway on a per transfer basis; however, after adding in the cases with no normal euploid calls and no transfer from with at least two blastocysts biopsied for patients ≥37yrs, these additional cases acted to significantly reduce the pregnancy rates on an ITBx (Table 2).

DISCUSSION

Results presented here describe pregnancy outcomes of first from cohort transfers using blastocysts selected exclusively using a quantitative physiological bioassay (qSEA) rather than standard morphology grading. The rationale for this approach is based upon a previous retrospective study [19], showing that averaged euploid expansion was significantly greater than averaged aneuploids in this assay. By treating expansion as a continuous variable, these expansion distributions suggested that rank ordering unbiopsied blastocysts within cohorts simply based upon the values of this variable should identify the single blastocyst having the highest odds or likelihood of being euploid [28,29]. The results described here support this prediction, particularly for patients <37ys, where there is both a higher averaged normal euploid prevalence within cohorts (49.4%) and relatively few cases having no normal euploid calls (10% of biopsies). In these younger patients, such cases mostly occurred in blastocysts biopsied from smaller sized blastocyst cohorts (Figure 3). While this lowered the pregnancy rates on an ITBx basis, this suppression did not reach statistically significance (Table 1).

On a per transfer basis, there was also statistical parity in pregnancy rates between single euploids with the transfer of two qSEA selected unbiopsied blastocysts in the ≥37yr patient group. This reflects a likely limitation of qSEA ranking for selection of single blastocysts from a population with a lower prevalence of normal euploid calls (36% compared to 49.4% in the younger cohort). This was also noted in an earlier study [19]. In addition, the biopsy pathway appears to have resulted in a significant live birth rate suppression on an ITBx basis in this age group due to a large number of biopsy cases having no normal euploid calls (p= 0.007); (Tables 2). The presence of a comparable group of such cases should have also resulted in a pregnancy rate reduction in Group C cases, particularly from smaller, unbiopsied blastocyst cohorts (Table 2; Figure 4); to the contrary, this was not observed. This supports observations of others that there may be inaccuracies between some abnormal biopsy calls with a blastocyst's true totipotentiality [30-33]. This may be due to mosaic or segmental calls rather than other meiotically derived whole chromosomal aneuploidies [34,35]. While only speculative, a totipotent subgroup of such blastocysts might be prioritized for transferred after ranking using qSEA analysis, while remaining untransfered after biopsy analysis. Thus, while biopsy and PGT-A for all IVF cases might appear to be rational to optimize pregnancy rates per patient, the data presented here suggest a pregnancy rate risk for some of the poorer responding patients having smaller cohort sizes that attends the intention for greater reproductive certainty.

The pregnancy results described here can also be discussed in relation to larger prospective and randomized studies reported by Munne et al. [36], and Ozgur et al. [37], with the caveat that their blastocyst selection was performed using standard grading. These studies also suggested that pregnancy may be compromised using the biopsy pathway, either from damage to blastocysts or from genetic screening inaccuracies with developmental potential [9, 38-40]. The STAR trial [36], initially observed a pregnancy benefit from biopsy on a per transfer basis only in patients >35ys; however, even this benefit was statistically lost on an ITBx basis as defined in their study arms. The possible superiority of qSEA selection on an ITBx basis (Tables 1, 2) also argues that the interpretation of biopsy results, perhaps more than biopsy damage to the embryo, may be a more critical issue. This point was also addressed in the nonselection study of Tiegs et al. [6], who found no clear evidence for biopsy damage to developmental potential based upon comparative pregnancy outcomes/ET with aged matched controls of unbiopsied embryos outside of their study arms. On the other hand, they also identified classes of abnormal biopsy calls consistent with normal totipotentiality other than whole chromosome aneuploidies. This supports the hypothesis that such abnormals (e.g. mosaics and segmentals) can explain some of the pregnancy rate gap between Groups C and D on an ITBx basis (Table 2). Taken together, these results support the interpretation that deselection of blastocysts for transfers solely on the basis that they are not "normal euploid" may significantly limit and/or delay some patient's pregnancy chances. While whole chromosome meiotic aneuploidies, unquestionably, should be avoided, many abnormal calls clearly do not fall into this category.

The current study also helps to address a longstanding problem that high pregnancy rates achieved in younger, good prognosis patients using double blastocyst transfers is associated with unacceptably high twinning rates [1, 41,42]. With the advent of extended culture, considerable evidence has subsequently accrued that the qualitative degree of blastocyst expansion is one of the key performance indices that have value for blastocyst selection [2,43-48]. Nevertheless, the inability for the field to more consistently achieve similarly high pregnancy rates with single blastocysts using SG has likely contributed to the adoption of biopsy with comprehensive chromosome screening across a wide age range [5,6,49]. Given these historical considerations, the data presented here suggest that qSEA selection algorithm represent a pathway capable of closing this outcome gap between traditional standard grading and invasive genetic screening for single blastocyst transfers, particularly for younger patients and, possibly, for better responding older patients with more limited blastocyst cohort sizes.

This study also has several recognized limitations. First, these results derived from a retrospective case control study design with some groups having a relatively smaller sample size; thus, the results described justify the need for a prospective randomized control study design powered with more appropriate group sample sizes. Despite differences in mean ages and some differences in biopsy group sizes, the age paired groups had reasonable numbers of blastocysts within cohorts available

for transfer. Secondly, relatively smaller group sample sizes, particularly in the more highly selected Group D patients ≥37ys, limits the strength of the interpretation of outcome comparisons between these biopsied and unbiopsied groups. In addition, Groups C and D patients represented only the subset of better responders to gonadotropin stimulation in this older age group given the significant number of cases (32%) that had all cleavage stage embryos vitrified on Day 3 (Figure 1) due to their more limited ovarian response.

This study also has several strengths. First, the blastocysts transferred were all prospectively selected identically and quantitatively using qSEA analysis ranking, and clinical outcomes included consecutive patient first from cohort transfers across all ages over a four year period, with relatively few exclusions. These results extend an earlier pilot study using this same methodology [20], that showed high quality sustained pregnancy rates using qSEA selection as analyzed using an artificial intelligence based image segmentation protocol. Perhaps more significantly for IVF laboratories that use time lapse imaging, qSEA selection represents a simple and transparent algorithm for the selection of first single blastocysts to transfer from cohorts by all personnel, regardless of experience. Such first transfers are particularly important given that failure can have a profound psychosocial impact on patients and their return for subsequent transfers [49,50]. Good responder patients typically have several comparable highly grading blastocysts, and selection of the single "best" one (as is often described in the literature) has actually remains quite problematic for reproducibility both within and between laboratories; in contrast, qSEA selection suggests redefining "best" as the "winner" of a physiologically based time trial that begins at blastocyst formation rather from fertilization [34]. Thus, once tB is determined, all personnel can uniformly choose the same blastocyst, with standard grading used as confirmation for that selection. As a practical matter, qSEA selection provides the laboratory with an objective, quality assurance tool to choose quantitatively between cohort blastocysts that have comparable cellular morphology grades.

There also remain both known and unknown limitations of qSEA selection of blastocysts for transfer. First, this report only describes first transfers from cohorts, and it is not yet clear how efficacious this approach will be for subsequent transfers. Recent observations suggest that >90% live birth rates can be achieved after sequential transfer of 3 euploid blastocysts [51]. However, this benchmark not commonly achieved for most patients >37ys, who averaged only 1.8 euploids/cohort (Table 2). This low averaged number further supports the use of double blastocyst transfers in Group D and explains its low incidence of twinning. qSEA selection may, hypothetically, act to include for transfer the most totipotent of these blastocysts that would have had calls of unclear reproductive potential (Figure 5). Second, qSEA selection will likely be more useful in comparing blastocysts with relatively little cell loss from fragmentation or blastomere loss [52,53]. The loss of cell mass undoubtedly reduces the qSEA expansion measurements in some blastocysts that may be both euploid and developmentally totipotent [19]. Third, a limitation of qSEA selection is that it cannot identify cohorts that either truly have no genetically normal totipotent blastocysts or those at risk for the more clinically significant aneuploidies such as trisomies. Recent work suggests that these as well as segmental aneuploidies and mosaics are likely to be among some of the more higher ranked embryos using qSEA analysis [35,54]. Fourth, some degree of inter-operator variability to identify tB remains problematic for some embryos. This can critically impact ranking designations and work continues to further standardize this critical time point which would be needed for cross-validation studies by other laboratories.

Taken together, such considerations certainly support continued work to further evolve the qSEA platform as well as the search for other corroborating and complementary markers of euploidy and totipotentiality [19, 55, 56]. A number of other non-invasive analytic platforms have also been developed using time lapse imaging [11, 57,58]. Some have utilized a similar hierarchical modelling approach based upon multiple cell cycle timing events of cleavage stage embryos [59]. More recently, artificial intelligence platforms have also emerged in conjunction with both single time point imaging and more expansive time-lapse imaging periods [18, 59-65]. Several of these appear to be quite statistically robust. These may even be more predictive than the qSEA approach; however, an advantage of the described qSEA approach is that it is simple, transparent, and can easily be adopted using any time lapse imaging system without proprietary software. It only seems reasonable to suggest that including quantitative metrics of blastocyst expansion will be beneficial to any of these other emerging algorithms.

CONCLUSIONS, LIMITATIONS, AND RECOMMENDATIONS

qSEA selection of single blastocysts can result in sustained pregnancy rate parity between biopsied unbiopsied blastocysts in patients <37ys. Such parity in patient's ≥37yrs required both double blastocysts in patients with a better clinical response. This approach offers a quality assurance pathway that standardizes blastocyst selection within a laboratory. Although pregnancy rates from additional transfers from cohorts requires further study, these results support the continued use of double unbiopsied blastocyst transfers in patients ≥37yrs.

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Appendix

qSEA: quantitative standardized expansion assay; PGT-A: preimplantation genetic testing for aneuploidy; ICSI: intracytoplasmic sperm injection; HCG: human chorionic gonadotrophin; HB: heartbeat; ITBx: intention to treat after trophectoderm biopsy; AI: artificial intelligence.

Statements and Declarations

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