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## Time-lapse videography reveals different morphokinetic profiles of human embryos displaying direct or reverse cleavage at different stages of development: A retrospective sibling embryo study

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### ABSTRACT

**Objective:** To investigate morphokinetic characteristics of embryos displaying either reverse cleavage or direct cleavage during the first, second or third cleavage cycle.

**Methods:** A total of 167 *in vitro* fertilization and/or intracytoplasmic sperm injection treatment cycles undertaken by 167 women [aged (35.0±4.6) years] were included for reverse cleavage analysis, and a total of 167 *in vitro* fertilization and/or intracytoplasmic sperm injection treatment cycles undertaken by 167 women [aged (33.8±4.3) years] were included for direct cleavage analysis in this study. Using a sibling-embryo design, morphokinetic profiles (both before and after the onset of abnormal event) of embryos displaying reverse cleavage ( $n=241$ ) or direct cleavage ( $n=244$ ) were compared with their unaffected siblings (the controls) in the first, second and third cell cycles ( $n=32$ ,  $n=142$ ,  $n=562$ ;  $n=195$ ,  $n=412$ ,  $n=205$ , respectively), at different developmental stages up to day 3.

**Results:** Direct cleavage embryos demonstrated significantly delayed cleavage rates prior to the event regardless of developmental stage of the occurrence, while reverse cleavage embryos showed similar cleavage rates to their unaffected siblings. Post event, direct cleavage embryos sped up cleavage rates while reverse cleavage embryos slowed down.

**Conclusions:** Altered morphokinetic profiles are displayed by direct cleavage embryos both before and after their occurrence and reverse cleavage embryos after the occurrence, which could potentially confound morphokinetic comparisons if not separated from their unaffected sibling embryos. Further study is warranted in order to fully understand the biological mechanisms of such events.

**KEYWORDS:** Time-lapse; Direct cleavage; Reverse cleavage; Morphokinetics; Cleavage abnormality

### 1. Introduction

The novel clinical application of time-lapse videography has enabled embryologists to observe in detail the preimplantation development of human embryos without disruption to culture conditions[1]. Whilst the reported embryo selection models employing time-lapse videography were mostly formulated by using quantitative morphokinetic parameters[2], time-lapse videography may also assist with qualitative embryo deselection *via* identification of cleavage abnormalities, which has recently been shown to have increased reproducibility between different laboratories[3]. Two of the most studied abnormal cleavage patterns are direct cleavage where one blastomere divides into three or more daughter cells[4] and reverse cleavage where cells fuse following division or blastomeres display failed cytokinesis (fail to separate post karyokinesis)[5]. This latter description of reverse cleavage did see the abnormal cleavage at the three cell cycles up to 8-cell, but the specific morphokinetic timings before or after reverse cleavage were not defined. Although clinical and biological causes of such abnormalities remain unclear, alterations in the embryo's subsequent morphokinetic profile can be expected[6]. Nevertheless,

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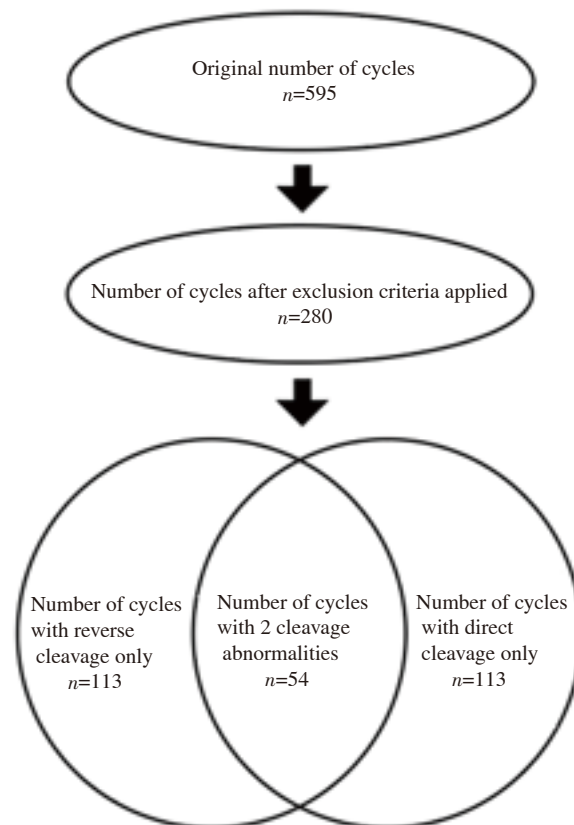
data demonstrating such morphokinetic differences in a well-controlled setting are lacking in the literature.

Whilst the reduced implantation potential of embryos displaying direct cleavage or reverse cleavage is well recognised, a systematic analysis of the morphokinetic dynamics of embryos with cleavage abnormalities at different stages is absent in the literature. The present study therefore aims to investigate the morphokinetic profile of embryos identified with direct cleavage or reverse cleavage during the first, second or third cleavage cycle as observed directly *via* time-lapse videography. Using a self-controlled sibling-embryo design, cleavage behaviours both before and after the onset of such events are evaluated up to three days post oocyte collection and compared with embryos showing normal cleavage patterns.

## 2. Materials and methods

### 2.1. Cycle inclusion criteria

Initial dataset included a total of 595 (Figure 1) consecutive *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycles using the women's own fresh oocytes undertaken at Fertility North between January 2017 and December 2018. Cycle records were downloaded and de-identified. Cycles resulting in only one zygote, or cycles with no embryos showing direct cleavage or reverse cleavage were excluded. Cycles with both direct cleavage and reverse cleavage embryos were included but the reverse cleavage embryos were removed when comparing direct cleavage embryos and unaffected sibling embryos, and *vice-versa* when direct cleavage embryos were excluded when comparing reverse cleavage embryos with unaffected sibling embryos. A total of 167 IVF and/or ICSI treatment cycles [167 women, aged (35.0±4.6) years at oocyte pick up] were included for reverse cleavage analysis (241 affected *vs* 657 control embryos), and a total of 167 IVF and/or ICSI treatment cycles [167 women, aged (33.8±4.3) years at oocyte pick up, using their own fresh oocytes] were included for direct cleavage analysis (244 affected *vs* 630 control embryos) in the current study (Figure 1, Table 1). This was a day three transfer program with Embryoscope™ (Vitrolife, Sweden) annotations being used to select and deselect embryos for transfer or subsequent cryopreservation[5]. All embryos contained two pronuclei, with one and multiple pronuclei embryos being excluded from analysis. Cycles were included if sibling embryos (within the same cycle) displayed both (i) direct cleavage or reverse cleavage and (ii) no cleavage abnormality embryos (control). Embryos demonstrating mixed or multiple direct cleavage or reverse cleavage events were excluded from the analysis, whereas cycles that contained both direct cleavage and reverse cleavage embryos were included. Embryos with poor conventional morphology as previously described[5], and those that were not cultured to day 3 in the time-lapse videography incubator were removed from the analysis.



**Figure 1.** Cell cycle number from the original data set and after the exclusion criteria is applied. The total cycle number for reverse cleavage and direct cleavage is shown, demonstrating the commonality of cycles. The conditions for exclusion: Cycles resulting in only one zygote, or cycles with no embryos showing direct cleavage or reverse cleavage are excluded.

### 2.2. Consent and ethical considerations

Identifiable patient details were removed prior to data analysis, and the patient's treatment was not impacted due to the retrospective nature of this study. Every patient, both male and female, had signed a Fertility North consent number 1 form for the use of stored data to be used for practice management and quality assurance. Since morphokinetic data were de-identified and transferred to a spreadsheet for subsequent analysis, the Australian National Statement on Ethical Conduct in Human Research (National Health and Medical Research Council, 2015) deemed such projects using de-identified data were classified as negligible risk and were exempted from ethical review[7].

### 2.3. Gamete preparation and embryo culture

Controlled ovarian hyperstimulation, gamete preparation and insemination *via* either conventional IVF or ICSI were performed as previously described[5]. Media from the G-series™ (Vitrolife, Sweden) were used to culture the gametes and embryos. G-IVF™ PLUS was used to co-incubate the gametes overnight in routine IVF,

or to perform the injection procedure for ICSI. Oocytes fertilised in IVF were moved to the Embryoscope™ the following day at the pronuclear stage for culture in the G-1™ PLUS media until three days post oocyte collection, whereas oocytes after the injection in ICSI were transferred directly into G-1™ PLUS until day 3. Embryo transfers were done on day 3 and supernumerary embryos not transferred underwent further culture to day 5 or 6 in G-2™ PLUS media prior to cryopreservation. Culture conditions in the Embryoscope™ were set at 6% CO<sub>2</sub>, 5% O<sub>2</sub> and balance N<sub>2</sub> at 37 °C, with images acquired across seven focal planes of each embryo every ten minutes.

#### 2.4. Time-lapse annotation of embryos

Annotation of embryos was performed by a number of experienced embryologists using the Embryosviewer® (Vitrolife, Sweden) software. Inter-operator consistency of time-lapse videography annotation was monitored *via* the External Quality Assurance Scheme for Reproductive Medicine (EQASRM, Northlands, Australia) as well as an internal quality control program. Developmental milestones of embryos were expressed in reference to pronuclear fading to remove timing variations arising from insemination method[8]. Milestone timing parameters analysed included two cell (t2), three cell (t3), four cell (t4), five cell (t5), six cell (t6), seven cell (t7) and eight cell (t8). Relative timing parameters considered included CC2 (duration of the two cell stage or t3-t2), CC3 (duration of the four cell stage or t5-t4) and synchrony of cell division at the two cell (S2=t4-t3) and four cell stages (S3=t8-t5).

Reverse cleavage was categorized as cell fusion following division (type I) or failed cytokinesis after karyokinesis (type II) as previously described[5]. Direct cleavage was categorized as multipolar cell division resulting in three or more daughter cells[4]. The nucleus inside the blastomeres were tracked and confirmed throughout all cleavage cycles in all embryos and used to differentiate blastomeres from large anucleated fragments, which was essential to accurately identify both direct cleavage and reverse cleavage. Developmental stages at which either direct cleavage or reverse cleavage occurred were classified into three categories; namely the first cleavage cycle where the event occurred at the one cell stage blastomere, the second cleavage cycle where the event occurred at the two to three cell stage blastomere, and the third cleavage cycle where the event occurred at the four to seven cell stage blastomere. Skipped cell stages in direct cleavage embryos were timed the same as the subsequent cell stage, *e.g.*, embryos with direct cleavage from one to three cell stages were annotated to have the same t2 and t3. Temporary cell stage in reverse cleavage embryos was not annotated with only the subsequent stable cell stage recorded, *e.g.*, embryos with a temporary cell stage of four cells were only annotated with the subsequently three cell stage.

#### 2.5. Statistical analysis

All timing parameters were tested for normal distribution, and if

normality was confirmed, expressed in the form of mean±standard deviation (mean±SD), with comparisons analyzed by using the Student *t*-test. If the distribution was found not to be normal, the comparisons were analyzed using the Mann Whitney *U* Test. The median, 1st and 3rd interquartile values were also calculated. The nonparametric values were expressed as median (interquartile range), namely, median (Q1-Q3). Statistical analysis was performed by using the IBM® SPSS® Statistics software platform, and a *P* value of <0.05 was considered statistically significant.

### 3. Results

#### 3.1. Cycle characteristics

Details on the cycles included in the study were shown in Table 1. There was no difference between cycles that had one or more embryos showing reverse or direct cleavage when considering the proportion of cycles using various ovarian stimulation protocols or insemination methods. The number of affected and control embryos per cycle was similar for both types of cleavage abnormalities.

#### 3.2. Reverse cleavage

Comparisons between reverse cleavage affected and unaffected (normal cleavage pattern) sibling embryos showed significantly delayed subsequent development (t2, t3, t4 and CC2; *P*<0.001, *P*<0.001, *P*<0.001 and *P*<0.001 respectively) when reverse cleavage occurred in the first cleavage cycle (Table 2). A similar delay was also detected in embryos post reverse cleavage in the second cleavage cycle (t4, t7, t8, CC3, S2, and S3; *P*<0.001, *P*<0.001, *P*=0.001, *P*<0.001, *P*<0.001 and *P*<0.001, respectively) (t5 and t6, *P*=0.050 and *P*=0.040, respectively), and in embryos when reverse cleavage occurred in the third cleavage cycle (t7, *P*=0.030; t8 and S3, *P*<0.001, *P*<0.001, respectively) (Table 2). No difference was observed in cleavage rates prior to embryos showing reverse cleavage as compared with their siblings (*P*>0.05), regardless of the developmental stages (the first cleavage cycle, the second cleavage cycle or the third cleavage cycle) at which reverse cleavage occurred (Table 2).

#### 3.3. Direct cleavage

Altered cleavage kinetics were also evident in embryos displaying direct cleavage when compared to their unaffected siblings. Firstly, direct cleavage embryos reached subsequent developmental milestones earlier than their unaffected siblings in the first cleavage cycle and the second cleavage cycle (Table 3); this can be attributed to the additional daughter cell(s) generated during the division. Direct cleavage occurring in the first cleavage cycle led to significantly reduced (faster) time to reach the three cell through to eight cell stages (t2, t3, t4, t5, t6, and t7; *P*<0.001,

$P < 0.001$ ,  $P = 0.001$ ,  $P < 0.001$ ,  $P < 0.001$ , and  $P = 0.005$ , respectively) ( $t_8$ ,  $P = 0.038$ ) in comparison to their unaffected siblings. Similarly, embryos reached the five cell through to seven cell stages ( $t_5$  and  $t_6$ ,  $P < 0.001$  respectively;  $t_7$ ,  $P = 0.026$ ) significantly earlier following direct cleavage in the second cleavage cycle. However, there was no detectable increase in development when direct cleavage occurred in the third cleavage cycle. Secondly, CC2 and CC3 were significantly shortened at either the first cleavage cycle (CC2 and CC3,  $P < 0.001$ , respectively) and the second cleavage cycle (CC3,  $P < 0.001$ ). Thirdly, S2 and S3 were prolonged in the first cleavage cycle (S2 and S3,

$P < 0.001$ , respectively) and the second cleavage cycle (S3,  $P < 0.001$ ).

Compared to reverse cleavage embryos, direct cleavage embryos seemed to slow down in their developmental progression prior to the onset of this abnormal cleavage event. This can be seen by the significantly delayed  $t_2$  (direct cleavage in the first cleavage cycle,  $P < 0.001$ ; and direct cleavage in the second cleavage cycle,  $P = 0.008$ ),  $t_3$  (direct cleavage in the second cleavage cycle,  $P < 0.001$ ; and direct cleavage in the third cleavage cycle,  $P = 0.022$ ), and  $t_4$  (direct cleavage in the second cleavage cycle,  $P = 0.002$ ).

**Table 1.** Characteristics of cycles including at least one reverse cleavage or direct cleavage embryo.

| Parameters                                      | Cycles with $\geq 1$ affected embryo |                  |
|---|--------------------------------------|------------------|
|   | Reverse cleavage                     | Direct cleavage  |
| No. cycles                                      | 167                                  | 167              |
| No. women                                       | 167                                  | 167              |
| Ovarian stimulation protocol [ $n(\%)$ ]        |                                      |                  |
| No. ultra-long down-regulation                  | 4(2.4%)                              | 2(1.2%)          |
| No. long down-regulation                        | 23(13.8%)                            | 32(19.2%)        |
| No. pill cross-over                             | 1(0.6%)                              | 0(0%)            |
| No. GnRH flare                                  | 6(3.6%)                              | 4(2.4%)          |
| No. GnRH antagonist                             | 133(79.6%)                           | 129(77.2%)       |
| Woman's age at OPU (years; mean $\pm$ SD)       | 35.0 $\pm$ 4.6                       | 33.8 $\pm$ 4.3   |
| Insemination method [ $n(\%)$ ]                 |                                      |                  |
| No. IVF   | 44(26.3%)                            | 38(22.7%)        |
| No. ICSI  | 107(64.1%)                           | 115(68.9%)       |
| No. split                                       | 16(9.6%)                             | 14(8.4%)         |
| Total No. embryos                               | 898(100%)                            | 874(100%)        |
| No. affected embryos                            | 241(26.84%)                          | 244(27.92%)      |
| Total No. of control embryos                    | 657(73.16%)                          | 630(72.08%)      |
| No. control embryos in:                         |                                      |                  |
| the first cleavage cycle                        | 32 <sup>a</sup>                      | 195 <sup>a</sup> |
| the second cleavage cycle                       | 142 <sup>a</sup>                     | 412 <sup>a</sup> |
| the third cleavage cycle                        | 562 <sup>a</sup>                     | 205 <sup>a</sup> |
| Total No. embryos per cycle [median (Q1-Q3)]    | 5(3.0-7.0)                           | 5(3.0-7.0)       |
| No. affected embryos per cycle [median (Q1-Q3)] | 1(1.0-2.0)                           | 1(1.0-2.0)       |
| No. control embryos per cycle [median (Q1-Q3)]  | 3(2.0-5.5)                           | 3(2.0-5.0)       |

Note: No. : Number; OPU: oocyte pick-up; IVF: *in vitro* fertilization; ICSI: intra-cytoplasmic sperm injection; Split: both IVF and ICSI used in same cycle. a: The control embryos in the first, second and third cell cycles do not equal the total number of control embryos, as some control embryos are utilized across more than one cell cycle.

**Table 2.** The morphokinetic and cleavage cycle parameters of embryos displaying reverse cleavage at different cleavage cycles.

| Parameters | 1st cleavage cycle        |                           |                     |           | 2nd cleavage cycle |                    |                     |           | 3rd cleavage cycle        |                           |                      |           |
|------------|---------------------------|---------------------------|---------------------|-----------|--------------------|--------------------|---------------------|-----------|---------------------------|---------------------------|----------------------|-----------|
|            | Affected<br>$n=6$         | Control<br>$n=32$         | $t/U$ value         | $P$ value | Affected<br>$n=41$ | Control<br>$n=142$ | $t/U$ value         | $P$ value | Affected<br>$n=194$       | Control<br>$n=562$        | $t/U$ value          | $P$ value |
| $t_2$      | 14.9 $\pm$ 3.5            | 2.3 $\pm$ 0.4             | 21.094 <sup>c</sup> | <0.001    | 2.7 $\pm$ 0.5      | 2.6 $\pm$ 0.6      | 1.159 <sup>c</sup>  | 0.274     | 2.7 $\pm$ 0.7             | 2.6 $\pm$ 0.7             | 0.666 <sup>c</sup>   | 0.554     |
| $t_3$      | 30.3 $\pm$ 6.7            | 13.7 $\pm$ 1.4            | 13.384 <sup>c</sup> | <0.001    | 14.0 $\pm$ 1.9     | 14.1 $\pm$ 1.2     | 0.540 <sup>c</sup>  | 0.597     | 13.8 $\pm$ 1.8            | 13.9 $\pm$ 1.3            | 0.745 <sup>c</sup>   | 0.447     |
| $t_4$      | 32.3 $\pm$ 7.5            | 14.7 $\pm$ 2.4            | 11.200 <sup>c</sup> | <0.001    | 27.1 $\pm$ 4.0     | 14.9 $\pm$ 1.6     | 29.301 <sup>c</sup> | 0.000     | 14.6 $\pm$ 1.7            | 14.7 $\pm$ 1.8            | 0.205 <sup>c</sup>   | 0.834     |
| $t_5$      | 35.5 (-)                  | 26.9 $\pm$ 2.5            | b                   | b         | 28.9 $\pm$ 4.8     | 27.7 $\pm$ 3.0     | 1.971 <sup>c</sup>  | 0.050     | 27.1 $\pm$ 3.4            | 27.4 $\pm$ 2.9            | 1.303 <sup>c</sup>   | 0.189     |
| $t_6$      | 36.8 (-)                  | 27.6 $\pm$ 2.5            | b                   | b         | 30.5 $\pm$ 5.2     | 29.0 $\pm$ 3.6     | 2.070 <sup>c</sup>  | 0.040     | 28.7 $\pm$ 3.5            | 28.4 $\pm$ 3.1            | 1.080 <sup>c</sup>   | 0.281     |
| $t_7$      | 37.9 (-)                  | 28.9 $\pm$ 2.7            | b                   | b         | 37.7 $\pm$ 7.1     | 29.9 $\pm$ 3.8     | 5.232 <sup>c</sup>  | <0.001    | 30.3 $\pm$ 4.4            | 29.6 $\pm$ 3.5            | 2.180 <sup>c</sup>   | 0.030     |
| $t_8$      | 39.0 (-)                  | 31.3 $\pm$ 4.6            | b                   | b         | 38.5 $\pm$ 7.0     | 31.4 $\pm$ 4.4     | 3.447 <sup>c</sup>  | 0.001     | 37.9 $\pm$ 5.9            | 31.0 $\pm$ 4.3            | 9.514 <sup>c</sup>   | 0.000     |
| CC2        | 15.4 $\pm$ 3.3            | 11.4 $\pm$ 1.2            | 5.426 <sup>c</sup>  | <0.001    | 11.2 $\pm$ 1.9     | 11.5 $\pm$ 1.0     | 1.044 <sup>c</sup>  | 0.294     | 11.1 $\pm$ 1.5            | 11.3 $\pm$ 1.3            | 1.076 <sup>c</sup>   | 0.254     |
| CC3        | 17.1 (-)                  | 12.5 $\pm$ 1.6            | b                   | b         | 0.9(0.3-1.7)*      | 12.9 $\pm$ 2.4     | 103 <sup>d</sup>    | <0.001    | 12.4 $\pm$ 2.9            | 12.8 $\pm$ 2.4            | 1.425 <sup>c</sup>   | 0.152     |
| S2         | 1.4(0.6-2.8) <sup>†</sup> | 0.5(0.2-1.2) <sup>‡</sup> | 65 <sup>d</sup>     | 0.222     | 13.2 $\pm$ 2.6     | 0.5(0.2-0.8)*      | 1 <sup>d</sup>      | <0.001    | 0.5(0.3-0.8) <sup>‡</sup> | 0.5(0.2-0.8) <sup>‡</sup> | 51711.5 <sup>d</sup> | 0.125     |
| S3         | 3.5(3.5-3.5) <sup>†</sup> | 3.3(2.1-4.9) <sup>‡</sup> | b                   | b         | 15.8 $\pm$ 2.9     | 2.8(2.0-5.5)*      | 9 <sup>d</sup>      | <0.001    | 14.0 $\pm$ 5.5            | 3.0(1.8-5.0) <sup>‡</sup> | 19215 <sup>d</sup>   | <0.001    |

Note: Times (hours) to reach the 2- to 8-cell stages ( $t_2$ - $t_8$ ) are expressed relative to pronuclear fading. CC2= $t_3$ - $t_2$ , CC3= $t_5$ - $t_4$ , S2= $t_4$ - $t_3$ , S3= $t_8$ - $t_5$ . The morphokinetic's and cleavage cycle parameters of embryos showing reverse cleavage are compared to those embryos showing no cleavage abnormality. b: Insufficient data available to calculate mean, standard deviation and/or significance for these parameters. \*: Values are expressed as median (interquartile range), namely, median (Q1-Q3). c:  $t$  value. d:  $U$  value.

**Table 3.** The morphokinetic and cleavage cycle parameters of embryos displaying direct cleavage at different cleavage cycles.

| Parameters | 1st cleavage cycle         |                           |                     |         | 2nd cleavage cycle        |                           |                     |         | 3rd cleavage cycle        |                           |                     |         |
|------------|----------------------------|---------------------------|---------------------|---------|---------------------------|---------------------------|---------------------|---------|---------------------------|---------------------------|---------------------|---------|
|            | Affected<br>n=62           | Control<br>n=195          | t/U value           | P value | Affected<br>n=129         | Control<br>n=412          | t/U value           | P value | Affected<br>n=153         | Control<br>n=205          | t/U value           | P value |
| t2         | 3.1±1.3                    | 2.5±0.4                   | 5.732 <sup>c</sup>  | <0.001  | 2.7±1.5                   | 2.5±0.4                   | 2.665 <sup>c</sup>  | 0.008   | 2.6±0.4                   | 2.6±0.9                   | 0.080 <sup>c</sup>  | 0.947   |
| t3         | 3.5±1.4                    | 13.8±1.2                  | 58.327 <sup>c</sup> | <0.001  | 14.8±2.2                  | 14.0±1.3                  | 5.326 <sup>c</sup>  | <0.001  | 14.3±1.4                  | 13.8±1.5                  | 2.305 <sup>c</sup>  | 0.022   |
| t4         | 13.4±3.6                   | 14.8±2.2                  | 3.547 <sup>c</sup>  | 0.001   | 15.5±3.2                  | 14.8±1.9                  | 3.188 <sup>c</sup>  | 0.002   | 15.0±1.5                  | 14.7±1.9                  | 1.174 <sup>c</sup>  | 0.245   |
| t5         | 16.8±4.7                   | 27.0±2.4                  | 22.661 <sup>c</sup> | <0.001  | 16.3±4.0                  | 27.5±2.7                  | 36.178 <sup>c</sup> | <0.001  | 27.7±3.0                  | 27.1±2.7                  | 1.400 <sup>c</sup>  | 0.160   |
| t6         | 19.9±7.2                   | 28.1±2.8                  | 12.947 <sup>c</sup> | <0.001  | 26.6±4.9                  | 28.5±3.0                  | 5.226 <sup>c</sup>  | <0.001  | 28.3±2.9                  | 28.1±2.9                  | 0.486 <sup>c</sup>  | 0.624   |
| t7         | 27.5±6.5                   | 29.4±3.3                  | 2.857 <sup>c</sup>  | 0.005   | 29.1±3.8                  | 30.0±3.6                  | 2.242 <sup>c</sup>  | 0.026   | 29.3±3.0                  | 29.5±3.6                  | 0.272 <sup>c</sup>  | 0.791   |
| t8         | 29.5±5.4                   | 31.0±3.9                  | 2.098 <sup>c</sup>  | 0.038   | 30.6±4.6                  | 31.3±4.1                  | 1.504 <sup>c</sup>  | 0.135   | 31.0±4.4                  | 31.4±4.5                  | 0.502 <sup>c</sup>  | 0.612   |
| CC2        | 0.0(0.0-0.05) <sup>*</sup> | 11.3±1.1                  | 0 <sup>d</sup>      | <0.001  | 12.1±2.1                  | 11.5±1.2                  | 3.983 <sup>c</sup>  | <0.001  | 11.7±1.3                  | 11.2±1.6                  | 2.073 <sup>c</sup>  | 0.038   |
| CC3        | 0.8(0.2-2.5) <sup>*</sup>  | 12.3±2.3                  | 1399 <sup>d</sup>   | <0.001  | 0.2(0.0-0.7) <sup>*</sup> | 12.7±2.5                  | 858 <sup>d</sup>    | <0.001  | 12.7±2.1                  | 12.4±2.3                  | 0.767 <sup>c</sup>  | 0.434   |
| S2         | 10.0±3.8                   | 0.5(0.2-1.0) <sup>*</sup> | 963 <sup>d</sup>    | <0.001  | 0.2(0.0-0.5) <sup>*</sup> | 0.5(0.2-1.0) <sup>*</sup> | 17652 <sup>d</sup>  | <0.001  | 0.5(0.2-1.0) <sup>*</sup> | 0.5(0.2-1.0) <sup>*</sup> | 5398 <sup>d</sup>   | 0.944   |
| S3         | 14.1±4.9                   | 3.2(2.0-5.1) <sup>*</sup> | 630.5 <sup>d</sup>  | <0.001  | 15.5±4.0                  | 3.1(2.0-5.2) <sup>*</sup> | 854.5 <sup>d</sup>  | <0.001  | 2.5(1.5-3.9) <sup>*</sup> | 3.2(2.2-5.3) <sup>*</sup> | 2949.5 <sup>d</sup> | 0.021   |

Note: Times (hours) to reach the 2- to 8-cell stages (t2-t8) are expressed relative to pronuclear fading. CC2=t3-t2, CC3=t5-t4, S2=t4-t3, S3=t8-t5. \*: Values are expressed as median (interquartile range), namely, median (Q1-Q3). c: t value. d: U value.

#### 4. Discussion

It is considered as a novel finding that direct cleavage embryos tend to slow down before its occurrence, which is previously unobserved in the literature. The cause of direct cleavage is largely unknown, however, a range of theories have been proposed. Previously, tripolar cell division in the first cleavage cycle was mostly observed in the trinucleated zygotes due to the additional pair of centrioles following polyspermic fertilization[4]. This however is unlikely in the current study as all zygotes were confirmed as binuclear at fertilization check. Another popular theory points to centrosomal dysfunction in direct cleavage embryos as an inherited defect from the sperm[4,9]. In addition, a further potential cause is the application of mitosis spindle toxins (such as paclitaxel, a cancer treatment) used to artificially induce tripolar mitosis[10]. Another group, Kalatova *et al*, illustrated that the immediate cellular mechanism of tripolar mitosis may originate from excessive duplication of centrosome before mitotic spindle formation[11]. This last theory may explain the observed delay prior to the onset of direct cleavage in the present study, where embryos with impaired centrosomal function may require extra time regulating associated replication activities before the occurrence of direct cleavage.

The present study also presents novel comparisons in embryo morphokinetic profile prior to the display of their reverse cleavage phenotype, showing no difference to sibling counterparts. By using sibling embryos as control, statistical analysis of the reverse cleavage embryo morphokinetic profile is considered reliable. At this stage, the cause of reverse cleavage also remains unknown, although previous reports have shown potential association with sperm motility and ovarian stimulation regime, but not female age[5]. Another group postulated that cell fusion could be linked with a defect in the cell membrane[12]. Results in the present study suggest that embryos displaying reverse cleavage may have an underlying intrinsic defect, which may not be evident in the cleavage kinetic prior to the event occurring. Studies at the ultrastructural and

molecular level may offer further insights.

The observed similar morphokinetic profiles between embryos showing direct cleavage in the third cleavage cycle and siblings are not expected since direct cleavage in one of the four cell stage blastomeres would have led to shorter t6 and onwards. Considering embryos in the present study were only cultured and observed for three days post oocyte collection, the opportunity to detect later stage (*e.g.*, nine cell or beyond) acceleration of embryo growth is lost. For example, direct cleavage in the last dividing four cell stage blastomere may not result in detectable differences in t8 and prior. Furthermore, the capability of “self-correction” is believed to arise in later stage human embryos, where “normal” cells tend to exclude genetically abnormal cells (as a result of direct cleavage in this case) which subsequently end up with developmental arrest[9].

The present study further expanded the annotation of direct cleavage events from the first mitotic division as was originally described in the first documented report[4] to the second and third round of mitotic divisions. Identification of later stage direct cleavage events is thought to require additional efforts by the annotating operator, which involves careful tracking of karyokinesis activities as well as cytokinesis with more cells in the field of view. A recent study made an attempt at identifying direct cleavage embryos and other irregularly dividing embryos using timing-parameter-based mathematical equations, however, it did not seem to outperform a qualitative-parameter-based model where abnormal biological events were manually annotated[13]. As such full automation in annotation for abnormal cleavage patterns such as direct cleavage and reverse cleavage seems to be a long way off, considering the commercially available time-lapse videography devices are currently incapable of automatically evaluating blastomere nuclei[2].

In previous studies the incidences of direct cleavage and reverse cleavage have been reported at various rates, probably due to different patient population. As such, the sibling-embryo design in the present study has avoided the risk of “comparing oranges to apples” by eliminating patient related confounding factors.



Furthermore, it is widely acknowledged that embryo morphokinetics alter between laboratories due to diverse laboratory[14] and patient[15] characteristics, even for those with known implantation outcomes[16]. It is therefore crucial, when comparing embryo cleavage timings, that not only laboratory/patient related confounding factors are taken into consideration but also that direct cleavage/ reverse cleavage embryos are removed from comparisons. For example, studies comparing morphokinetic features between different patient populations (such as smoker *versus* non-smokers) may consider removing direct cleavage and reverse cleavage embryos before performing statistical analysis on timing parameters[15]. Nevertheless, the full description of the behaviour and morphokinetic profiles of affected embryos in each of the three cell cycles is crucial as a first step in understanding these cleavage abnormalities. Further end-points will also reveal the impact of such cleavage abnormalities in the different cell cycles on the functional capacity of affected embryos, such as blastocyst formation rates, implantation rates and pregnancy viability, but these are outside the remit of the present study.

In conclusion, results in the present study indicate the significantly changed morphokinetic profiles by direct cleavage embryos both before and after their occurrence and reverse cleavage embryos after the occurrence. It is therefore proposed that such embryos are removed from morphokinetic comparisons to avoid biased and erroneous interpretation of data.

### Conflict of interest statement

The authors declare that they have no conflict of interest.

### Authors' contributions

All authors have contributed positively to this manuscript. Phillip L. Matson and Yan-he Liu conceived and designed this study. Chloé Brits carried out data collection, statistical analysis and manuscript writing. Katie Feenan and Vince Chapple facilitated data collection and patient management. All authors contributed significantly to the proofreading, revision and final approval of the manuscript.

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