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Embryos with morphokinetic abnormalities may develop into euploid blastocysts

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KEY MESSAGE
Irregularly cleaved embryos should be cultured to blastocyst stage as they have the potential to become euploid. They are observed to exclude cells from compaction. These cells should be analyzed to investigate a possible aneuploidy rescue mechanism. It is also recommended that their collection during biopsy procedures is avoided to prevent misdiagnosis.

A B S T R A C T

Irregular cleavage divisions are expected to produce chromosomally deviant embryos. We investigated whether embryos from irregular cleavages could develop into euploid blastocysts, and, if so, whether any evidence existed of a self-correction mechanism of the embryo. We also investigated the role of different dynamic aspects of morula compaction in this process. A total of 791 embryos from 141 patients undergoing pre-implantation genetic screening were retrospectively analysed using a time-lapse imaging system, and multiple cell divisions were evaluated. A total of 276 embryos developed into blastocysts suitable for biopsy and chromosome screening through array-comparative genomic hybridization. As well as testing trophectoderm biopsy specimens for aneuploidy, excluded cells of 18 blastocysts, which developed from partially compacted morulas, were also analysed. Unique data on the developmental fate of embryos with cleavage abnormalities are presented, and a potential mechanism of 'aneuploidy rescue' is postulated through which mosaic embryos may form partially compacted morulas to exclude aneuploid cells. In addition, this process seems to be less efficient in older women. The data obtained also provide further evidence that excluded cells should not be used to infer the cytogenetic status of the embryo.
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Introduction

With the introduction of time-lapse imaging technology into clinical practice, it has become possible to continuously track embryo development up to the day of transfer. Time-lapse technology potentially allows embryo to be observed in a more accurate and objective manner, and permits precise detection of abnormal morphological or cell kinetic events occurring during in-vitro development. Anomalies of this type cannot be readily detected by conventional static

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observation owing to their dynamic nature and the transience of nuclear appearance and fading (Kirkegaard et al., 2012; Stecher et al., 2014). Associations between morphokinetic variables and embryo viability have been proposed, but only a few with ploidy status (Basile et al., 2014; Campbell et al., 2013; Chavez et al., 2012; Yang et al., 2014). Aneuploidy was observed either in the first cell cycle parameters up to day 3 of development (Basile et al., 2014; Chavez et al., 2012) or in late embryo development (Campbell et al., 2013; Yang et al., 2014), where delayed 'start blastulation' and 'full blastulation' timings were associated with aneuploidy with the building of a predictive model of risk classification (Campbell et al., 2013). The significance of morphokinetic variables in predicting aneuploidy is yet to be determined, and future well-designed prospective trials are needed.

Many other aspects of embryo development can be investigated: time-lapse technology, for example, makes it possible to distinguish between a blastomere and a large anucleate fragment, by observing the phase of the cell cycle during which the fragment forms and enabling the positioning of the nucleus before division to be examined. Other abnormal cleavage events, such as direct cleavage from a one-cell zygote to a three-cell embryo, most likely associated with a multipolar spindle, are also clearly revealed, but typically go unnoticed when using traditional assessments because of their dynamism and because their formation occurs at a time of day when observations are not generally undertaken.

Until now, different irregular cleavage events, correlated with embryo implantation potential and considered as de-selection parameters, have been observed, i.e. indicators of reduced viability (Chavez et al., 2012; Cruz et al., 2012; Hlinka and Coombes, 2012; Meseguer et al., 2011; Rubio et al., 2012; Wong et al., 2010; Zaninovic et al., 2013), but how embryos from these irregular divisions might be able to develop into euploid blastocysts has not yet been discussed.

Irregular cleavages, defined by timing (very short or very long), of the cell cycle and their pattern of division in the early stages (Berrisford and Cater, 2015), may be a consequence of various different mechanisms, such as abnormalities in spindle formation, diploid gametes, fertilization with more than one sperm and failure to extrude the second polar body. Also, the centrioles that control the first mitotic divisions of the oocyte and are introduced by the spermatozoa may have a role in this process, with defective centrosomes causing abnormal cleavage and compromising embryo development (Sathananthan, 1998).

With the evolution of cytogenetic techniques for preimplantation genetic diagnosis, an important insight into the chromosomal status of human gametes and preimplantation embryos has been made, revealing that errors leading to aneuploidy can arise during meiosis and also after fertilization, during preimplantation embryonic development (Delhanty, 1997; Fragouli et al., 2010; Kuliev et al., 2003; Vanneste et al., 2009). Some evidence shows that the timing of cell division is affected by the cytogenetic status of the embryo and that an abnormal chromosomal complement is more frequent in embryos cleaving in a time frame that is different from what is expected (Magli et al., 2007). In the present study, we sought to investigate whether embryos displaying irregular cleavage, presumably chromosomally abnormal, were able to develop into euploid blastocysts and, if so, whether there was any evidence of a self-correction mechanism, rescuing the embryos from aneuploidy. We hypothesized that incomplete morula compaction in embryos with abnormal cleavage might provide a means of excluding the anomalous cells. We analysed embryos developing from irregular cleavage events using time-lapse imaging technology and conducted cytogenetic evaluation of the embryo and any excluded cells using array-comparative genome hybridization (array-CGH).

Materials and methods

In this retrospective study, 791 embryos obtained from 141 patients (39.9 + 4.4 years) undergoing 145 pre-implantation genetic screening (PGS) cycles in our centre (9.baby, Bologna, Italy) were analysed between May 2013 and January 2015. The couples included in the analysis were indicated for PGS because of severe male factor, advanced maternal age, recurrent unexplained pregnancy loss and repeated implantation failure; only a few patients had no particular indication for PGS. The ages of the women ranged between 26 and 48 years (mean 39.9 + 4.4 years). All the embryos were retrospectively morphokinetically analysed. A total of 276 were suitable for trophectoderm biopsy and underwent chromosomal investigation. Cleavage aberrations affected 111 of the 791 embryos (14%) and only 24 of them developed into blastocyst stage. As well as PGS to detect aneuploidy affecting embryos, excluded cells of 18 blastocysts, developed from partially compacted morulas, were analysed.

Approval for the study was obtained from the local Institutional Review Board on 5 October 2014, and patients provided written informed consent to embryo biopsy, chromosome analysis and the anonymous use of clinical data for statistical evaluation and research purposes.

Ovarian stimulation protocol and blastocyst grading

Ovarian stimulation protocol and blastocyst grading procedures were carried out as previously described by Borini et al. (2006) and Lagalla et al. (2015).

Semen preparation and ICSI procedures

Semen preparation and intracytoplasmic sperm injection procedures were carried out as previously described by Borini et al. (1996).

Embryo culture and transfer

Embryo culture was carried out in EmbryoScope (UnisenseFertiliTech, Denmark), an integrated embryo-culture time-lapse microscopy system with N2/CO2/O2 (89:6:5, v/v) at 37°C without control of humidity. Embryos were placed inside pre-equilibrated slides (EmbryoSlide; Unisense Fertilitech) containing 12 droplets each of 25 µl fresh cleavage medium (Cook IVF) covered by 1.2 ml of mineral oil (SAGE, Biocare Europe, Rome, Italy). On day 3, cleavage medium was replaced with blastocyst medium (Cook IVF) for culture until day 5 or 6.

Embryo transfers were carried out in fresh PGS cycles or after freeze-all cycles. Blastocysts were cryopreserved using a vitrification protocol with a closed-system device (HSV straw, Cryo Bio System, France) and (Kitazato BioPharma Co., Japan) as previously described by Cobo *et al.* (2008).

Time-lapse analysis and recording of kinetic parameters

The EmbryoScope was set to acquire images every 15 min at seven different focal planes for each embryo. Time-lapse sequences were

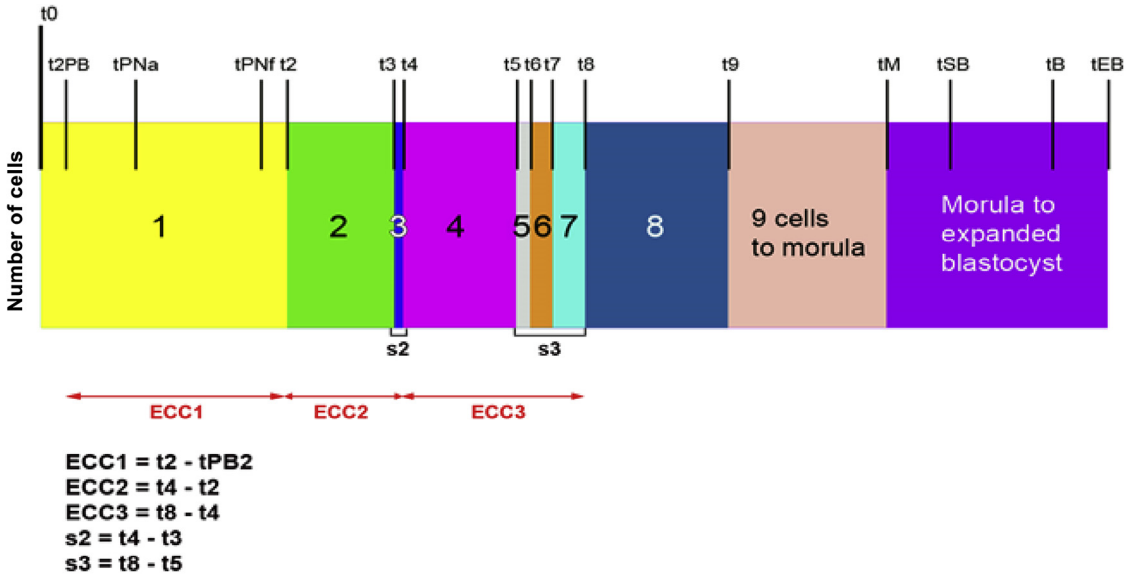


Figure 1 – Definitions of the morphokinetic variables analysed in this study.

replayed by the EmbryoViewer workstation and the timing of each developmental event was annotated manually by an embryologist according to Meseguer et al. (2011) and Dal Canto et al. (2012). Time of insemination was defined as t0, corresponding to the time of microinjection for intracytoplasmic sperm injeciton. In the EmbryoScope, the time of insemination was programmed by the operator when the slide was inserted. All time points are reported as hours. This study recorded the timing of sundry developmental parameters from t0 to tEB (expanded blastocyst formation: blastocoele filled the embryo, blastocyst had increased in by over 50% in diameter and the zona pellucida started to thin). The timings recorded in our Embryoscope database, based on the model by Ciray *et al.* (2014), are shown in **Figure 1**. Some of the main variables related to cell-cycle duration were also recorded: t3–t2, the second cell cycle; t4–t3 (S2), the second synchrony; t4–t2 and t8–t4, respectively the length of the second and the third cell cycle. Each anomalous cell division was evaluated, paying particular attention not to confound fragments with blastomeres by the presence of a visible nucleus. Irregular divisions that were considered for data analysis are described below. Direct cleavage 1–3 indicates an irregular cleavage whereby one blastomere divides directly into three or more daughter cells and was first used by Rubio et al. (2012) to describe a fast division from two to three cells within the arbitrary value of within 5 h. In the present study, the more accurate term 'rapid cleavage', as suggested by Ciray *et al.* (2014) and clearly described in the Atlas by Campbell and Fishel (2015) is used. In contrast 1–3 cleavage was named 'direct cleavage' when t3–t2 = 0: this different nomenclature allows 'direct cleavage' to be distinguished as an aberrant cleavage from a single cell directly to three daughter cells and is defined as trichotomous mitosis (Kola et al., 1987; Ciray *et al*, 2014) whereas 1–3 rapid cleavage, where t3–t2 <5 h, is when a short step at two cells is present (Rubio et al., 2012; Ciray *et al*, 2014; Berrisford and Cater, 2015). The other variables that were taken into consideration in this study were also all the 2–5 cleavages (Zaninovic et al., 2013), reverse cleavages or blastomere fusion (Hickman et al., 2012; Liu et al., 2014) and prolonged S2 phase, where S2 >4 h (VerMilyea et al., 2014).

Morphokinetic parameters related to blastocyst formation were also recorded, in particular morula compaction pathway, with particular attention paid to cells excluded from this event. Those cells were only considered when they clearly displayed the presence of nuclei (**Supplementary Video S1**).

Morphological and kinetic events of the embryos were recorded by a single, certified, senior embryologist. All morphokinetic readings were blinded to embryo ploidy status.

Blastocyst grading

As previously described by Lagalla et al. (2015), blastocysts were evaluated according to the degree of expansion and quality of the inner cell mass and trophectoderm cells. The inner cell mass was evaluated depending on the number of cells and the degree of compaction, whereas the trophectoderm cells were evaluated according to the number, dimension of the cells and the appearance of the epithelium (cohesive or loose), as described in the modified Gardner and Schoolcraft grading system by Cornell's group (Veeck and Zaninović, 2003).

Statistical analysis

Chi-square test and Student's t-test were used for data analysis as appropriate. Differences were considered significant when $P \leq 0.05$.

Biopsy procedures

At 113–142 h after insemination, all fully expanded blastocysts having a visible blastocoele, where an inner cell mass could be identified and with at least a few cells forming the trophectoderm epithelium, underwent trophectoderm biopsy.

All biopsy procedures were conducted on a heated stage in a dish prepared with two 20 µl droplets of Sydney IVF Blastocyst Medium (Cook IVF, Brisbane, Australia) overlaid with pre-equilibrated mineral oil. Each blastocyst had its own dish where it was left until transfer or cryopreservation, so that the identification procedures for each biopsied embryo were safer and the time used to carry out the biopsy was shorter (pre-equilibrated medium has been used during biopsy). A diode laser (Saturn 3, Research Instruments, Cornwall TR11 4TA,

Table 1 – Frequency of abnormally cleaved embryos in all pre-implantation genetic screening cycles.

Number of pre-implantation genetic screening cycles	145
Inseminated oocytes	1164
Fertilized oocytes	804
Embryos	791
Irregularly cleaved embryos, n (%)	111 (14)
1–3 direct cleavage	24 (22)
1–3 rapid cleavage	12 (11)
2–5 cleavage	26 (23)
Reverse cleavages	20 (18)
Prolonged S2	29 (26)

UK) was used to assist the opening of a 10–20 µm hole in the zona pellucida on day-3. Four to eight trophectoderm cells were then aspirated into the biopsy aspiration pipette (Cook, Brisbane, Australia), followed by laser-assisted or mechanical removal of the trophectoderm cells from the epithelium. During trophoectoderm biopsy procedures, cells remained in the perivitelline space were collected in a separate polymerase chain reaction tube.

Microarray CGH: chromosome analysis and interpretation

24Sure Cytochip V3 microarrays (Illumina Ltd, Cambridge, UK) was used for chromosome analysis. The procedures were carried out as previously described by Fragouli et al. (2013; 2014).

For image analysis and interpretation, ClearScan (Blugnome, Cambridge, UK) and Blue-Fuse multi software (Illumina, Cambridge, UK) were used.

Results

This investigation includes the analysis of 791 embryos, obtained in 145 PGS cycles, in 141 couples. Average female age of this patients group was 39.9 years. All the 791 embryos were retrospectively evaluated by time-lapse morphokinetics analysis. A total of 111 embryos (14%) displayed cell division aberrations: 24 embryos (22%) showed 1–3 direct cleavage (t3–t2 = 0), 12 embryos (11%) 1–3 rapid cleavage (t2–t3 <5 h), 26 embryos (23%) cleavage from two to five cells, 20 embryos (18%) reverse cleavages (in which two cells fuse together) and 29 embryos displayed an abnormally prolonged S2 phase (> 4 h) (**Tables 1 and 2**).

Irregularly cleaved embryos and female age

Statistical analysis showed that anomalous divisions are not age-related (**Figure 2**). Out of 111 abnormally cleaved embryos, 22 out of 147 embryos came from women who were younger than 35 years (15%), 31 out of 238 embryos were from women aged between 35 and 39 years (13%) and 58 out of 406 embryos were from women aged over 39 years (14.3%).

Irregularly versus normally cleaved embryos: incidence of arrested embryos

Out of the 111 embryos with cell division aberrations (group A), 87 arrested or were discarded (78.4%): 54 arrested at less than the 8/9 cell stage (48.6%), 22 at morula stage (19.8%) and 11 formed poor-quality blastocysts (9.9%). Twenty four of the 111 embryos (21.6%) became developing/transferrable blastocysts and underwent trophectoderm biopsy and microarray CGH analysis.

Out of the 680 normally cleaved embryos (group B), 428 arrested or were discarded (62.9%): 157 arrested at less than eight to nine cell stage (23.1%), 135 at morula stage (19.9%) and 136 developed into not developing blastocysts (20%). A total of 252 out of the 680 embryos (37.1%) became developing/transferrable blastocysts and underwent trophectoderm biopsy and microarray CGH analysis.

Data analysis revealed that irregularly cleaved embryos arrested more than normally cleaved embryos ($P < 0.01$). This different incidence of arrested embryos is particularly clear in the first stages of development, at less than eight to nine cell stage ($P < 0.01$) (**Table 2**).

Irregularly versus normally cleaved embryos: ploidy

Chromosomal analysis was carried out on trophectoderm cells from all developing blastocysts. In group A (irregularly cleaved embryos)

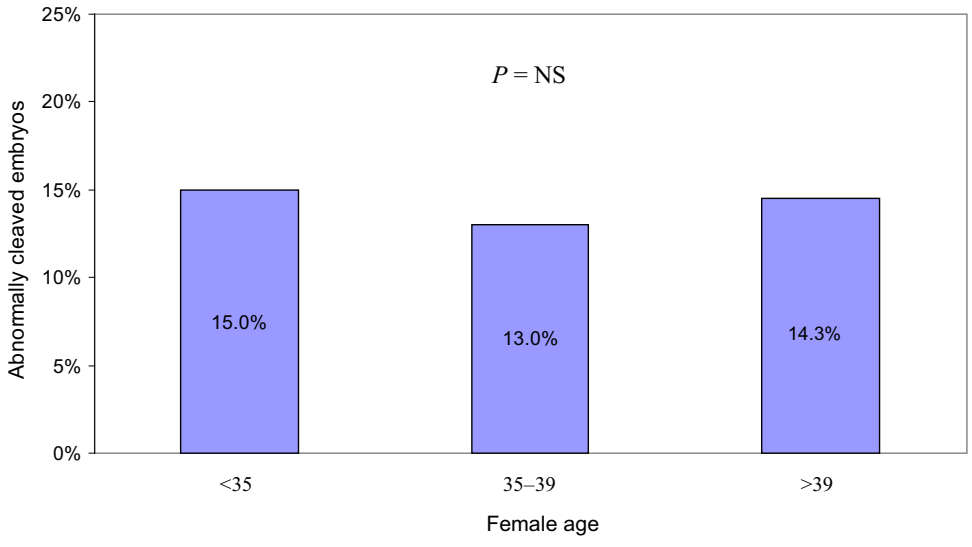


Figure 2 – Irregular divisions distribution on the basis of female age. NS = non-significant.

Table 2 – Incidence of arrested embryos, ploidy and morphokinetic analysis in irregularly versus normally cleaved embryos.

	Group A: irregularly cleaved embryos n (%)	Group B: normally cleaved embryos n (%)	*P*-value
Number	111	680	
Arrested or discarded embryos	87/111 (78.4)	428/680 (62.9)	<0.01
Embryos arrested at <8/9 cell stage	54/111 (48.6)	157/680 (23.1)	<0.01
Embryos arrested at morula stage	22/111 (19.8)	135/680 (19.9)	NS
Discarded blastocysts	11/111 (9.9)	136/680 (20)	<0.05
Biopsied blastocysts	24/111 (21.6)	252/680 (37.1)	<0.01
Euploid blastocysts (proportion of all embryos)	18/111 (16.2)	124/680 (18.2)	NS
Euploid blastocysts (proportion of biopsied blastocysts)	18/24 (75)	124/252 (49.2)	<0.05
Biopsied blastocysts derived from partially compacted morulas	19/24 (79.2)	114/252 (45.2)	<0.01
Euploid blastocysts derived from partially compacted morulas	18/18 (100)	57/124 (46.0)	<0.01

NS = non-significant.

18 out of 24 developing blastocysts (75%) were found to be euploid: the distribution of ploidy in the different groups of irregularly cleaved embryos is shown in **Table 3**. In group B (normally cleaved embryos) only 124 out of 252 developing blastocysts (49.2%) were found to be euploid. A statistical difference was found in the number of chromosomally normal blastocysts between groups A and B ($P < 0.05$), with a significantly higher proportion of euploid blastocysts in group A (**Table 2**). Considering the percentage of euploid blastocysts out of the whole embryo cohort (also those which did not reach blastocyst stage), no difference was found between group A (16.2%) and group B (18.2%) (**Table 2**).

Irregularly versus normally cleaved embryos: morphokinetics analysis

The morphokinetic analysis of embryos revealed that all the euploid blastocysts in group A (irregularly cleaved embryos) were derived from partially compacted morulas (**Figure 3**). It is hypothesized that all irregularly cleaved embryos that developed into euploid blastocysts excluded some cells during the compaction process, and that this could represent a potential mechanism of 'aneuploidy rescue'. In group B (normally cleaved embryos), only 46% of euploid blastocysts were derived from partially compacted morulas (A versus B = 100% versus 46%; $P < 0.01$) (**Table 2**).

Cytogenetic analysis of cells excluded during compaction process, at morula stage

To explore the possibility of a potential mechanism of 'aneuploidy rescue' of the embryo, array-CGH was carried out on both trophodectoderm cells, and those excluded during morula compaction derived from 18 blastocysts which cleaved irregularly in the first stages of development (from Group A) (**Table 4**).

Table 3 – Distribution of ploidy in the different groups of irregularly cleaved embryos.

	Overall	1–3 direct cleavage	1–3 rapid cleavage	2–5 cleavage	Reverse cleavages	Prolonged S2
Irregularly cleaved embryos	111	24	12	26	20	29
Arrested or discarded embryos n (%)	87/111 (78.4)	23/24 (95.8)	6/12 (50)	21/26 (80.8)	15/20 (75)	22/29 (75.9)
Euploid blastocysts n (%)	18/111 (16.2)	0	5/12 (41.7)	4/26 (15.4)	3/20 (15)	6/29 (20.7)
Aneuploid blastocysts n (%)	6/111 (5.4)	1/24 (4.2)	1/12 (8.3)	1/26 (3.8)	2/20 (10)	1/29 (3.4)

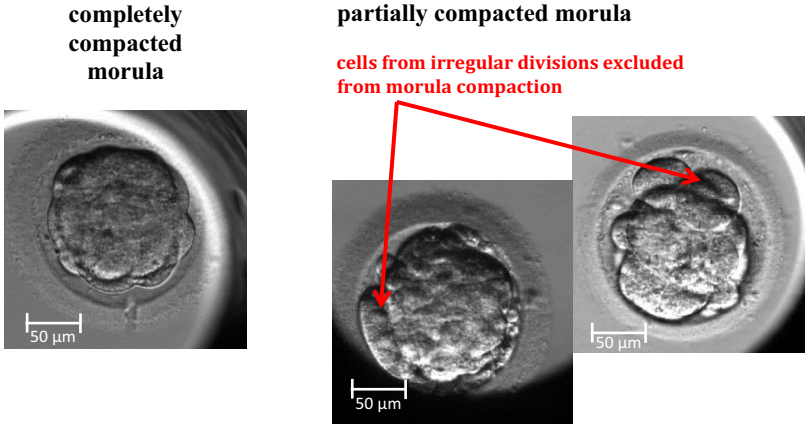


Figure 3 – Images showing the two different ways of compacting during embryo development: complete morula and partially compacted morulas with excluded cells. Bar = 50 µm.

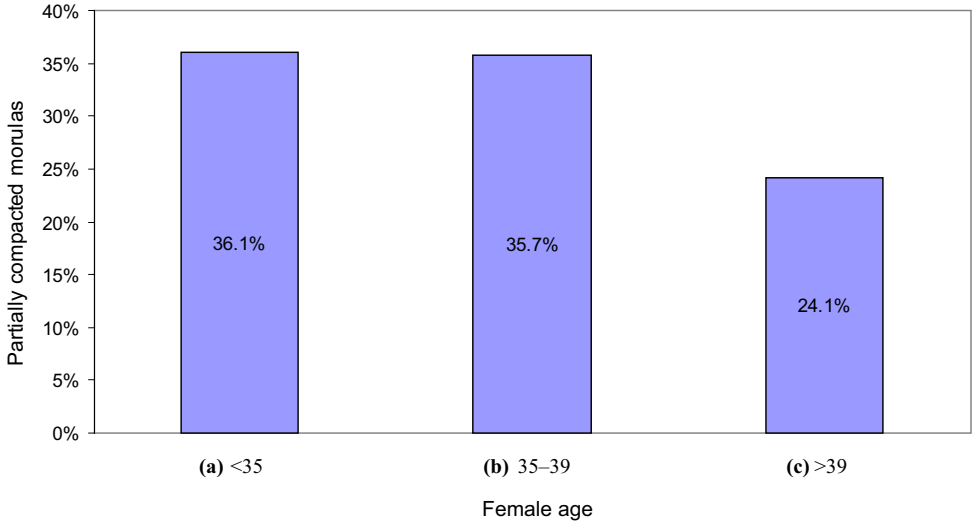
Table 4 – Array-comparative genomic hybridization results: simultaneous analysis of trophoectoderm and excluded cells of 18 biopsied blastocysts.

	Trophectoderm chromosomal assessment	Excluded cells chromosomal assessment
Blastocyst 01	46XX	46XX
Blastocyst 02	46XX	46XX
Blastocyst 03	46XX	45XX (+11; –14; +19; –20; –21; many segmental aberrations)
Blastocyst 04	46XX	38XX (–1; –2; –3; –7; –13; –18; –20; –22)
Blastocyst 05	46XY	48XXY (+19; many segmental aberrations)
Blastocyst 06	46XX	Anomalies of almost all chromosomes
Blastocyst 07	46XY	45XO (+3; +4; –9; –16; –20)
Blastocyst 08	46XY	No amplification
Blastocyst 09	46XX	No amplification
Blastocyst 10	46XY	No amplification
Blastocyst 11	46XX	No amplification
Blastocyst 12	46XY	No amplification
Blastocyst 13	46XY	No amplification
Blastocyst 14	45XO	48XX (+19; +21; many segmental aberrations)
Blastocyst 15	48XX (+12; +13)	48XX (+12; +13; mosaic9; segmental aberrations)
Blastocyst 16	46XX (+15; –21)	51XX (-1; –10; –20; –21; +3; +4; +9; +11; +12; +15; +16; +18; +19)
Blastocyst 17	45XY (–21)	47XX (+21; segmental aberration 19)
Blastocyst 18	47XX (+15)	45XX (–15)

The trophectoderm biopsy specimen revealed that 13 of the 18 blastocysts derived from partially compacted morulas were euploid and five were aneuploid. The cytogenetic analysis of the excluded cells within the 13 euploid blastocycts revealed aneuploidy in five (38.5%), no amplification owing to the presence of fragmented/degraded DNA in six cases (46.2%) and euploidy in only two cases (15.4%). The array-CGH analysis of the excluded cells from the five aneuploid blastocysts revealed aneuploidy in all the five samples (100%), with an increased complexity of aneuploidy with respect to the respective trophectoderm cells in four out of the five cases (80%) (**Table 4**). These data revealed that the excluded cells have a higher incidence of aneuploidies in relation to corresponding trophectoderm cells, providing evidence for a potential self-correction mechanism for mosaic embryos.

Partial compaction and female age

Statistical analysis shows that partial compaction is age-related. Out of 791 embryos, 236 developed into partially compacted morulas (29.8%): 53 embryos out of 147 in women aged younger than 35 years (36.1%), 85 embryos out of 238 in women aged between 35 and 39 years (35.7%) and 98 embryos out of 406 in women age older than 39 years (24.1%) (**Figure 4**). A statistical difference was found in the



- **(a)** *versus* **(b)**: $P = \text{N.S.}$
- **(b)** *versus* **(c)**: $P < 0.01$
- **(c)** *versus* **(c)**: $P < 0.01$

Figure 4 – Partial compaction distribution on the basis of female age.

number of embryos that underwent partial compaction at the morula stage between women who were older than 39 years and both the other two groups of younger patients (35–39 years and womn aged less than 35 years), with a significantly lower proportion of partial compaction in the group of older patients ($P < 0.01$). No statistically significant difference was found between women younger than 35 years and women aged between 35 and 39 years.

Discussion

In this study, research into embryos that displayed irregular cleavage and other abnormalities of mitosis during their early development are reported. The study combined the use of time-lapse analysis and comprehensive aneuploidy detection methods, allowing the progress of embryos to be carefully monitored and their cytogenetic status confirmed. Although embryos that underwent irregular cleavage were at increased risk of developmental arrest, a subset succeeded in reaching the blastocyst stage, and in many cases produced chromosomally normal embryos. While transitioning through the morula stage, it was observed that such embryos often displayed incomplete compaction, with exclusion of some cells, in a less efficient way in embryos from older women. These findings led us to hypothesize that this could represent a 'correction' mechanism, rescuing the embryo from the mosaic aneuploidy that typically occurs after abnormal cleavage by preferentially eliminating anomalous cells.

Morphokinetic anomalies affecting cell division, reported in this study, focused upon timing of mitosis (very short or very long cell cycles) and the pattern of cleavage. Anomalies of the cell cycle or abnormal forms of divisions are associated with different biological events (Berrisford and Cater, 2015). Prolonged cell cycles in the human preimplantation embryo are likely to be associated with activated DNA repair processes, incorrect attachment of chromosomes to the spindle, or failure to complete previous phases of the cell cycle appropriately. Conversely, unusually rapid cell cycles may be related to inadequate cell cycle checkpoints (Ramos and de Boer, 2011). All of these possibilities are considered to be associated with an increased risk of mitotic chromosome malsegregation, potentially leading to mosaic aneuploidy in the embryo.

Analysis of irregular cleavage events in relation to female patient age allowed confirmation that the frequency of abnormal cell divisions are not age-related (women under 35 years [15%]; women aged 35–39 years [13%] and women over 39 years [14.5%]). The total proportion of embryos displaying cleavage abnormalities was in accordance with previous studies of these phenomena (14% of the whole cohort) (Cruz et al., 2012; Meseguer et al., 2011; Rubio et al., 2012; Zaninovic et al., 2013). Irregularly cleaved embryos were found to arrest more often than those displaying normal cell divisions, which is also in concordance published research (group A: 78.4% versus group B: 62.9%; $P < 0.01$. Embryonic arrest was especially common in the early cell divisions up until eight to nine cell stage (group A: 54/111, 48.6% versus group B: 157/680, 23.1%; $P < 0.01$).

As recently reported by Stecher et al. (2014), if embryos with deviant morphokinetic parameters succeed in developing to the blastocyst stage, some can go on to produce healthy babies. The possibility that mosaic embryos, arising after aberrant cell divisions, may become more chromosomally normal as development proceeds, owing to selection against aneuploid cells, has long been suspected. It is well established that the frequencies of aneuploidy and mosaicism are lower for blastocysts than at earlier stages of development (Coonen et al., 2004; Fragouli and Wells, 2011; Fragouli et al., 2008, 2014; Magli et al., 2000; Santos et al., 2010; van Echten-Arends et al., 2011). Studies looking at the chromosomal content of the same embryo on day 3 and day 5 have presented data suggesting that a progressive normalization of chromosomal status is possible in some embryos (Barbash-Hazan et al., 2009). The data from the present study confirm that embryos that begin development with irregular divisions can sometimes form blastocysts and that when they do they are frequently euploid. Indeed, those embryos that suffered irregular cleavage, but succeeded in producing blastocysts, had the same euploidy rate as blastocysts from embryos with a history of normal cleavage (group A: 16.2% versus group B: 18.2%). This finding suggests that any chromosomal abnormalities induced by mitotic anomalies are typically resolved by the blastocyst stage.

The loss of many embryos with irregular cleavage before morula formation was expected, but a more surprising finding was that embryos with abnormal cleavage had higher rates of euploidy at the blastocyst stage compared with embryos that had a history of normal cell divisions (Group B) (75% versus 49.2%; $P = 0.015$). The results suggest that embryos that display abnormal divisions, but subsequently form blastocysts, have lower aneuploidy rates than those developing from normally cleaving embryos. This finding seems counterintuitive and a definitive explanation is not currently available. One possibility is that embryos that are already aneuploid due to a meiotic error may be less able to tolerate additional abnormalities resulting from mitotic anomalies and are more likely to undergo developmental arrest, thus removing them from the blastocyst cohort.

When considering different classes of cleavage aberrations individually, it was clear that 1–3 cleavage, whereby the zygote divides directly into three or more daughter blastomeres, produced no euploid embryos. This distinguished 1–3 cleavage from all other types of mitotic aberration, where chromosomally normal cells can still potentially be found within the embryo. This finding is in keeping with the theory that if a zygote immediately divides to three cells, the most likely explanation is a tri-polar spindle (Kola et al., 1987), which will cause the chromosomes to be pulled in three directions instead of the usual two, leading to their random dispersion. It is highly likely that the resulting embryos would be mosaic, containing a mixture of aneuploid cells in the absence of any that were chromosomally normal. Rapid 1-2-3 division is also considered to be an abnormal feature (too fast and asynchronous) and may be associated with an increased risk of chromosome malsegregation and mosaicism. This type of division, however, does not guarantee an error in chromosome segregation, so it is not surprising to find some euploid blastocysts developing from affected embryos. Embryos that divide 1–2–5 seem to have completed the first division correctly, so the first two cells produced are likely to be identical. The next division is normal for one cell, but abnormal for the other, potentially producing two cells with correct chromosome segregation and three cells with a very high probability of chromosome abnormality.

Another form of abnormal mitosis examined during this study was reverse cleavage. This phenomenon involves the fusion of two blastomeres, resulting in a hybrid cell containing two nuclei, and is currently not well documented. Hickman et al. (2012), however, found an association between cell fusion and multinucleation, and postulated that susceptibility to this form of abnormality may be influenced

by the follicular environment of the maturing oocyte. In their study, reverse cleavage did not seem to impair embryo development to the blastocyst stage and was not linked to ploidy status. A more recent study by Liu et al. (2014), however, suggested otherwise. In another study, Balakier et al. (2000) studied cell fusion in thawed embryos and reported that, depending upon the number of fused cells per embryo, reverse cleavage caused embryos to be either entirely polyploid or mosaic. It was postulated that abnormal cells resulting from fusion may be sequestered to the trophoblast and later the placenta.

Regarding the anomaly of prolonged S2 phase (time from an embryo to cleave from three to four cells), researchers found this parameter reflective of euploidy at the cleavage stage (Chavez et al., 2012) and gene expression (Wong et al., 2010). In the EEVA score model (VerMilyea et al., 2014), S2 phase was considered anomalous when greater than 4 h.

All the morphokinetic abnormalities taken into consideration in this study are associated with a high probability of generating chromosome anomalies, but, with the exception of direct cleavage 1–3, each of them also has a possibility that the embryo will contain some normal cells, which may help to promote blastocyst formation. Although most abnormalities of the early cleavage divisions are likely to produce mosaicism, the small number of cells sampled during trophectoderm biopsy might not permit this to be detected. Mosaic embryos may give a normal, aneuploid or mosaic result depending on which population(s) of cells is sampled during biopsy.

Retrospective analysis of the evolution of irregularly cleaved embryos throughout preimplantation development, using time-lapse methods, revealed a fascinating phenomenon: all the blastocysts that provided a euploid result following trophectoderm biopsy and comprehensive chromosome screening exhibited partial compaction at the morula stage, with some cells excluded from the embryo. The phenomenon was also seen in embryos with normal cleavage, but at a much lower frequency (group A: 100% versus group B: 46%; $P < 0.01$). Given the high likelihood that an embryo experiencing anomalous cleavage will be mosaic, it was interesting to consider the possibility that the elimination of specific (abnormal) cells during compaction could represent a corrective mechanism, allowing the formation of euploid blastocysts. The assumption that this hypothetical phenomenon is less effective in embryos deriving from older women was also explored. In fact, partial morulas from women older than 39 years were 24.1% compared with 36.1% in women younger than 35 years ($P < 0.01$) and with 35.7% in women aged between 35 and 39 years ($P < 0.01$); however, no statistically significant difference was found between embryos from the two younger groups of patients. These findings suggest that embryos derived from older women are less able to undergo this hypothetical self-correction mechanism.

To examine further the elimination of specific (abnormal) cells during compaction, excluded cells were carefully removed from the embryo and subjected to cytogenetic analysis. Excluded cells were collected from 18 blastocysts that developed from partially compacted morulas. Biopsy of the trophectoderm was also undertaken, providing information concerning the cytogenetic status of cells from the actual embryo. Twelve embryos gave results from both types of sample, whereas, for six, a result was obtained from the trophectoderm, but the DNA of the excluded cells was too degraded for analysis. Degraded DNA is a hallmark of apoptosis, which represents the most likely fate for highly abnormal or genetically unstable cells. Of the 12 samples of excluded cells that yielded cytogenetic results, only two were found to be euploid. In both of these cases the corresponding trophectoderm sample was also normal. The remaining 10 samples were all aneuploid. In five cases, abnormalities were detected in the excluded cells, whereas the corresponding trophectoderm sample was euploid. The other five embryos displayed aneuploidy in both samples, although interestingly the excluded cells were almost always more abnormal than the corresponding trophectoderm specimen, having multiple aneuploidies. No instances of chromosomally normal excluded cells associated with an aneuploid trophectoderm sample were occurred. Clear evidence of chromosomal mosaicism was detected in all the aneuploid embryos, emphasizing the link between this phenomenon and cleavage abnormalities. For example, reciprocal loss and gain affecting the same chromosome, a consequence of mitotic non-disjunction, was detected in trophectoderm and excluded cell samples from some embryos.

To date, the chromosomal status of the embryo on day 4 has received little attention, despite the fact that during this period important morphological and developmental changes such as compaction and cavitation occur (Mertzanidou et al., 2013). Compaction occurs as a result of increased cell-to-cell adhesion and the formation of gap and tight junctions between blastomeres, usually commencing after the third mitotic division. This marks the beginning of key processes that culminate in the differentiation of the inner cell mass and the trophectoderm (Montgomery, 2015). Clinical embryo evaluations have focused heavily on the cleavage and blastocyst stages, with the morula stage relatively neglected.

In the 1990s, from the observation of day 3 embryos fragmentation, it was hypothesised that elimination of selected blastomeres may reflect embryo effort in restoring or maintaining viability when anomalies affect particular blastomeres as, for instance, limited diploid mosaicism, preventing their contribution to the inner cell mass or the trophectoderm of the blastocyst (Alikani et al., 1999; Munné et al., 1995). Nowadays, the technology implementation allows an in-depth analysis of these issues. Recently, an interesting and innovative study (Mertzanidou et al., 2013) investigated potential mechanisms of correcting aneuploidy by analysing 13 morulas. Rates of aneuploidy and mosaicism were found to be similar to those observed in day 3 embryos, suggesting that any processes of correction have not been completed by the morula stage. This study, however, did not consider morulas displaying excluded cells.

As discussed above, incomplete compaction occurs when not all cellular material has been incorporated into the morula, leaving some cells excluded. Few studies have examined this feature, although what little information exists suggests that embryos displaying incomplete compaction are of reduced implantation potential (Ivec et al., 2011). The exclusion of cells from the formation of a compact embryo may have many different reasons; for instance, it has been seen to be caused by abnormal tight junctions (Watson, 1992), or the failure of the blastomeres excluded to express or to relocate the necessary proteins for adhesion and compaction (Alikani, 2005; Alikani et al., 1999). Currently, however, little is known about this condition.

In the **Supplementary Video S1**, it is evident that a cell coming from a rapid cleavage 1–3 is excluded by the morula compaction.

This study presents unique data concerning the developmental fate of embryos with common forms of cleavage abnormality and presents evidence for a potential mechanism allowing correction of aneuploidy in mosaic embryos. We suggest that our results be interpreted with caution because of the numerical asymmetric comparison made between the study groups and emphasize that similar investigations should be replicated in larger cohorts of embryos before firm conclusions are drawn. Nonetheless, we believe that this

is an observational and qualitative study, in which the data obtained may represent an important starting point for the investigation of the consequences of abnormal cleavage. The results suggest that human embryos might have an active capacity, that decreases with age, to recover from chromosome abnormalities that have originated during the early mitotic divisions, an extremely common form of error.

The study also highlights the value of time-lapse imaging for the study of embryos and suggests that, if a laboratory does not have access to this technology, it is advisable to culture embryos until blastocyst stage in order to avoid discarding the subset of potentially viable embryos that recover from earlier cleavage abnormalities. The data obtained also have relevance to PGS, providing further evidence that excluded cells should not be used to infer the cytogenetic status of the embryo (Ottolini et al., 2015).

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2016.11.008.

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