Human Reproduction, Vol.32, No.12 pp. 2549–2560, 2017
Advanced Access publication on November 8, 2017 doi:10.1093/humrep/dex324

human reproduction

ORIGINAL ARTICLE ***Reproductive genetics***

The incidence and origin of segmental aneuploidy in human oocytes and preimplantation embryos

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Submitted on August 1, 2017; resubmitted on September 10, 2017; accepted on October 3, 2017

STUDY QUESTION: What is the incidence, origin and clinical significance of segmental aneuploidy in human oocytes and preimplantation embryos?

SUMMARY ANSWER: Segmental aneuploidy occurs at a considerable frequency in preimplantation embryos with a majority being mitotic in origin.

WHAT IS KNOWN ALREADY: In recent years, accurate techniques for the detection of aneuploidy in single cells have been developed. Research using such methods has confirmed that aneuploidy is a common feature of human oocytes and preimplantation embryos. However, thus far research has mainly focused on loss or gain of whole chromosomes. We utilized sensitive molecular methods to study another important form of cytogenetic abnormality at the earliest stages of human development, namely segmental aneuploidy.

STUDY DESIGN, SIZE, DURATION: Chromosomal copy number data was obtained from oocytes and embryos of 635 IVF patients, who requested chromosome screening for various reasons, most commonly for advanced maternal age or previously unsuccessful IVF treatments. A total of 3541 samples comprising of 452 human oocytes, 1762 cleavage stage and 1327 blastocyst stage embryos were investigated in the present study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Whole genome amplification (Sureplex, Illumina) was performed on cells biopsied from oocytes and embryos of IVF patients who requested chromosome screening. The samples were subsequently processed and analyzed for their chromosome complement using microarray comparative genomic hybridization (aCGH), (Illumina, Cambridge, UK).

MAIN RESULTS AND THE ROLE OF CHANCE: Segmental abnormalities, involving loss or gain of chromosomal fragments in excess of 15 Mb, were found to occur at a high frequency. The incidence of such abnormalities was 10.4% in oocytes, but this increased dramatically during the first 3 days of embryonic development (24.3%), before starting to decline as embryos reached the final (blastocyst) stage of preimplantation development (15.6%). While some segmental errors were clearly of meiotic origin, most appear to arise during the first few mitoses following fertilization. The reduction in frequency at the blastocyst stage suggests that many cells/embryos affected by segmental abnormalities are eliminated (e.g. via arrest of the affected embryos or apoptosis of abnormal cells). Interestingly, sites of chromosome breakage associated with segmental aneuploidy were not entirely random but tended to occur within distinct chromosomal regions. Some of the identified hotspots correspond to known fragile sites while others may be considered novel and may be specific to gametogenesis and/or embryogenesis.

LIMITATIONS REASONS FOR CAUTION: The cytogenetic analysis was performed on biopsies of embryos, which might not be representative of the true incidence of mosaic segmental aneuploidy of the entire embryo.

WIDER IMPLICATIONS OF THE FINDINGS: The findings of this study are valuable for understanding the origin of subchromosomal duplications and deletions, a clinically important class of abnormalities that are a common cause of congenital abnormalities and miscarriage. Furthermore, the results provide additional evidence that control of the cell cycle is more relaxed during the first few mitotic divisions following fertilization, permitting DNA double-strand breaks to occur and persist through cell division. The data are also of great relevance for

preimplantation genetic testing, where the detection of segmental aneuploidy is currently considered problematic for embryo diagnosis and patient counseling.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by institutional funding (Reprogenetics UK). Additionally, DW is supported by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre Programme. DB was supported by the University of Oxford's Clarendon funding. No conflict of interests to declare.

Key words: preimplantation genetic testing for aneuploidy (PGT-A) / segmental aneuploidy / chromosomal abnormalities / embryo selection / IVF

Introduction

Chromosomal abnormalities in human oocytes and preimplantation embryos are common, their prevalence increasing with advancing maternal age from ~25% of blastocysts affected when women are in their mid-twenties to over 65% above the age of 40 (Munné et al., 2007; Franasiak *et al.*, 2014). The high frequency of aneuploidy is thought to be one of the main explanations why less than a third of embryos generated using IVF technology succeed in producing a child. It is thought that a similar situation also exists for naturally conceived embryos. In most cases, aneuploidy causes developmental arrest of embryos or failure of implantation in the uterus (Scott et al., 2012). Of the small proportion of aneuploid embryos that do succeed in establishing a pregnancy, the great majority eventually miscarry (Nagaoka et al., 2012).

In recent years, cytogenetic methods capable of examining the entire chromosome complement of single cells, such as array comparative genomic hybridization (aCGH), have been widely applied for the purpose of preimplantation genetic testing for aneuploidy (PGT-A), a strategy that aims to improve IVF treatment by identifying euploid embryos, which can then be prioritized for transfer to the uterus. Studies reporting cytogenetic data from oocytes and embryos, obtained using comprehensive chromosomal screening methodologies, such as aCGH, have thus far primarily focused on the incidence of abnormalities affecting whole chromosomes. However, it is clear that losses or gains of chromosomal fragments, resulting in segmental aneuploidy, also occur at appreciable frequencies (Voullaire *et al.*, 2000; Wells and Delhanty, 2000; Vanneste *et al.*, 2009; Rabinowitz *et al.*, 2012). While the impact of segmental aneuploidies in terms of preimplantation embryo viability and IVF outcome are poorly understood at this time, the clinical effects at later stages are well-known—segmental aneuploidy has been detected in ~6% of established pregnancies that miscarry, while the abnormalities that are compatible with live birth are usually associated with a range of congenital abnormalities in the affected children (Martínez *et al.*, 2010; Wellesley *et al.*, 2012).

Segmental imbalances in the cells of preimplantation embryos were first observed half a century ago in murine models using classical cytogenetic methods (Vickers, 1969). However, it was only with the development of advanced molecular cytogenetic techniques that the occurrence of segmental aneuploidy in individual human embryonic cells could be reliably evaluated (Wells *et al.*, 1999). Two early studies on human cleavage stage embryos using such methods revealed multiple segmental imbalances and also demonstrated the occurrence of *de novo* chromosomal breakage during the first few mitoses following fertilization, giving rise to reciprocal losses/gains of the same chromosome fragment in different cells of the embryo (Voullaire *et al.*, 2000; Wells and Delhanty, 2000).

Although the occurrence of chromosomal breakage and segmental aneuploidy in embryos has been previously reported, little work has been done to determine their incidence and origin. The most significant study in this regard, published by Vanneste and colleagues several years ago, investigated cleavage stage embryos using microarray technology and found that ~70% contained at least one cell affected by segmental imbalance (Vanneste *et al.*, 2009). This high prevalence could have important implications for natural and assisted reproduction, potentially contributing to the low fecundity and relative inefficiency of IVF in humans. However, the sample size assessed was very limited (23 embryos tested) and all samples were derived from a single IVF clinic. Confirmation in a greater number of samples, preferably generated by multiple laboratories is urgently required to verify these results. More recently, a study investigating the incidence and origin of segmental aneuploidy in blastocyst stage embryos showed that majority of segmental aneuploidies were of mitotic origin (Vera-Rodríguez *et al.*, 2016).

The current study examines chromosome breakage in a large population of human oocytes and preimplantation embryos. A total of 3541 samples were assessed: fertilized oocytes were tested following completion of both meiotic divisions; cleavage stage embryos were examined 3 days post-fertilization; embryos at the blastocyst stage were evaluated 5 or 6 days after fertilization. The results obtained provide information on the frequency and variety of segmental imbalances existing prior to implantation and give an insight into the origin and likely fate of this form of chromosome anomaly.

Materials and Methods

Patient details

The PGT-A data obtained from oocytes and embryos was collected during the course of routine clinical analysis. Patients received counseling and a signed consent for embryo screening was obtained. Embryological techniques, including polar body, cleavage stage and blastocyst biopsy were carried out according to standard protocols in each clinic. In all cases of oocyte analysis, the first and the second polar body were biopsied after completion of meiosis I and meiosis II, respectively, and were thereafter tested. Cleavage stage biopsy involved the removal of a single blastomere three days post-fertilization, while for blastocyst stage analysis a trophectoderm biopsy comprising ~5 cells was obtained 5 or 6 days after fertilization. We have previously validated the 24Sure™ aCGH platform for

aneuploidy detection and have confirmed accurate cytogenetic evaluation in >98% in single blastomeres, polar bodies and trophectoderm samples (Fragouli *et al.*, 2011; Gutiérrez-Mateo *et al.*, 2011). The sensitivity and resolution of the aCGH (24Sure™ platform) for the detection of segmental aneuploidies was established using cell lines with previously defined segmental aneuploidies (Fragouli *et al.*, 2017). Moreover, an additional 32 embryo samples from 11 patients with different reciprocal translocations were used to validate the resolution of segmental aneuploidy detection on 24Sure™aCGH platform (data not presented). The embryo samples from these patients were initially analyzed utilizing 24Sure + aCGH platform (Illumina), which has been validated previously for the detection of reciprocal translocations (Fiorentino *et al.*, 2011; Alfarawati *et al.*, 2011; Colls *et al.*, 2012).

Sample preparation and analysis

The polar body and embryo biopsies were carefully washed to remove any DNA contaminants and were collected in ~2 µl PBS/0.1% (v/v) polyvinyl alcohol. The SurePlex amplification system (Illumina, Cambridge, UK) was utilized to perform cell lysis and subsequent whole genome amplification according to the manufacturer's instructions. The amplified DNA, along with male and female reference samples, was subsequently labeled with fluorescent probes utilizing the Fluorescence Labeling System (Illumina, Cambridge, UK) as previously described (Fragouli *et al.*, 2010b, 2011). Briefly, the fluorescently labeled samples were placed on 24Sure Cytochip microarrays (Illumina, UK) and incubated overnight. Post-incubation, the microarrays were washed and scanned using an InnoScan 710 (Innopsys, France). The resulting images were analyzed using BlueFuse software for their corresponding chromosomal copy number analysis (Illumina, UK). The criteria for categorizing a segmental aneuploidy was that losses or gains should be at least 15 Mb in size with the results of all of the probes within the affected region exceeding the threshold used by the analysis software for calling aneuploidy. A chromosomal fragment size of 15 Mb is well within the limit of resolution as determined by the validation experiments described above. For statistical analysis of differences in the data between different sample groups, Fisher's exact test with a 95% confidence interval was applied. A *P*-value of <0.05 was considered to be statistically significant.

Assessment of the number of cells affected by segmental imbalances

Fifteen embryos with segmental imbalances were subjected to further analysis in order to determine the proportion of cells affected and to confirm whether or not the abnormality was present in a mosaic form. Eight embryos were at the cleavage stage and had been identified as carriers of segmental anomalies following array-CGH analysis of a single cell. Seven embryos were at the blastocyst stage and had been detected to carry segmental imbalance after biopsy and analysis of ~5 trophectoderm cells. The fluorescence *in situ* hybridization (FISH) method was essentially as described by Fragouli *et al.* (2011) and utilized different combinations of telomere and centromere probes mapping to regions affected by the segmental imbalance in each embryo (Fragouli *et al.*, 2011). Signals from FISH probes were visualized using an Olympus BX61 fluorescent microscope with a cooled charge-coupled device (CCD) system, and appropriate filters for the fluorochromes used. These embryos were surplus from clinical requirements and donated with patient consent (Institutional Review Board ethics approval WIRB 20060680).

Results

Cytogenetic data were obtained from a total of 3541 samples comprising of 452 human oocytes, 1762 cleavage stage and 1327 blastocyst stage embryos (Table I). For patients having oocytes analyzed, 45/120 (37.5%) had one or more oocytes affected by segmental imbalance(s), while for the cleavage and blastocyst stages the percentage of patients having a least one affected sample was 167/251 (66.5%) and 79/264 (29.9%) respectively (Table II). An example of an embryo with a segmental chromosome imbalance revealed by array-CGH is shown in Figure 1.

The average age of female patients producing one or more oocytes/embryos with a segmental aneuploidy was not significantly different from those who had no affected samples (Table II). Additionally, advancing maternal age, a key factor affecting the risk of whole chromosome losses/gains, has no significant correlation with the rate of segmental aneuploidy (Fig. 2).

Table I Patient and sample characteristics.

Stage analysis performed	Number of patients	Number of embryos/oocytes	Mean maternal age (y)	Age range (y)
Oocyte	120	452	40.4	36–46
Cleavage stage	251	1762	38.36	26–47
Blastocyst	264	1327	38.21	28–45

Table II Patients having oocytes or embryos affected by segmental abnormalities.

Stage analysis performed	Patients with ≥1 affected* embryos/oocytes	Mean age (y) of patients with an affected* embryo/oocyte	Age range (y) of patients with an affected* embryo/oocyte	Patients without any affected* embryos/oocytes
Oocyte	45 (37.5%)[a,b]	39.4	36–44	75
Cleavage stage	167 (66.5%)[a,c]	39.7	26–46	84
Blastocyst	79 (29.9%)[b,c]	38.4	31–43	185

*Affected by segmental aneuploidy. Statistical comparisons, using Fisher's exact test: (a) $P < 0.0001$; (b) p = not significant; (c) $P < 0.0001$.

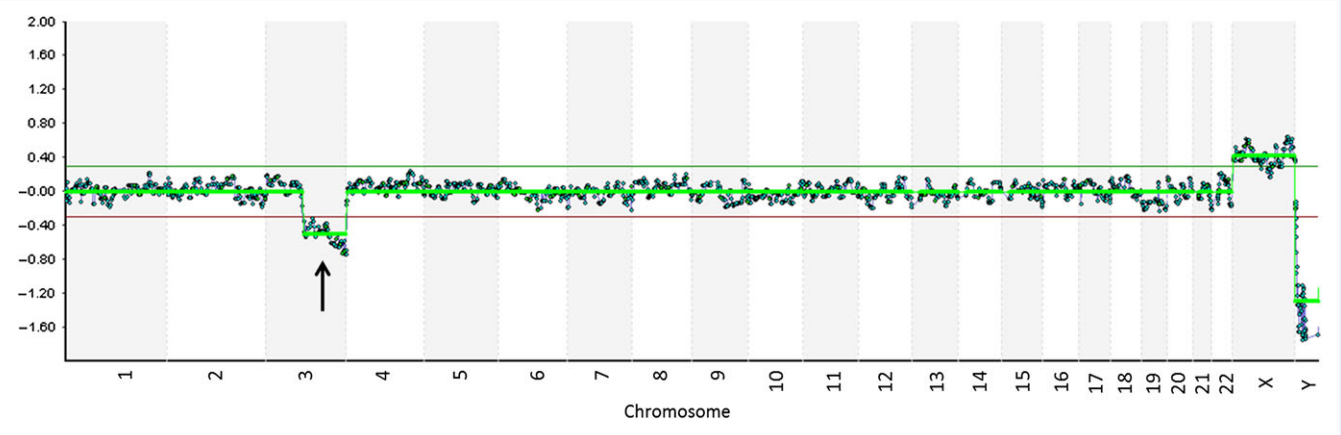


Figure 1 Molecular karyotype of an embryo generated after array-CGH showing a segmental loss (indicated by an arrow) affecting the long arm of chromosome 3.

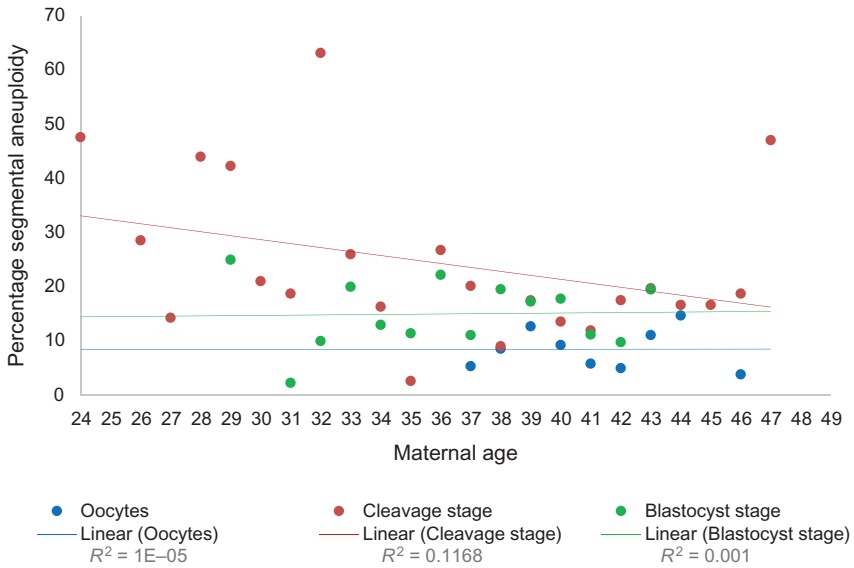


Figure 2 No significant correlation was observed between the rate of segmental aneuploidy and advancing maternal age at three different stages of preimplantation development; fertilized oocytes ($R = 0.003$), cleavage stage embryos ($R = 0.341$) and blastocyst stage embryos ($R = 0.032$).

Forty-seven of four hundred fifty-two oocytes (10.39%) were seen to carry segmental chromosomal errors (Table III and Fig. 3). There were over three times more segmental abnormalities detected in second polar bodies, extruded from oocytes at the completion of the second meiotic division compared to first polar bodies produced at the end of meiosis I (MI) ($P < 0.0001$) (Table IV). Three days after fertilization, at the cleavage stage, a significant increase in segmental aneuploidy was observed ($P < 0.0001$), affecting 24. 3% of embryos (428/1762). A further 2 days later, at the blastocyst stage, the rate of segmental abnormalities showed a decline relative to the cleavage stage, falling to 15.6% (207/1327, $P = 0.001$) (Table III). However, the incidence of segmental errors at this stage of development still remained slightly higher than observed in mature oocytes ($P = 0.021$). Of all the oocytes affected by segmental aneuploidy, about a quarter (12/47; 25.53%) were affected by multiple segmental aneuploidy (2 or more), the rate of which remained similar at cleavage stage (112/428; 26.12%) and at blastocyst stage (42/207; 20.29%) of development (P = not significant). The distribution of segmental aneuploidy across all the chromosomes at various stages of preimplantation development is depicted in Fig. 4.

Segmental errors appeared to occur independently of aneuploidy affecting whole chromosomes. However, given the high frequency of aneuploidy, it was not surprising that the two types of abnormality often coincided in the same sample. Indeed, most segmental abnormalities occurred alongside whole chromosome aneuploidies. Of all the oocytes assessed, only 2.0% (9/452) were affected exclusively by segmental aneuploidy, increasing significantly to 5.8% (103/1762; $P = 0.0013$) of embryos at the cleavage stage. At the blastocyst stage the

Table III Prevalence of oocytes/embryos affected by segmental abnormality.

Stage analysis performed	Number of oocytes/embryos with a segmental aneuploidy	Ratio of loss:gain of material
Oocyte	47 (10.39%)[a,b]	0.5 (30/60)
Cleavage stage	428 (24.29%)[a,c]	1.38 (324/234)
Blastocyst	207 (15.59%)[b,c]	1.57 (168/107)

Statistical comparisons, using Fisher's exact test: (a) $P < 0.0001$; (b) $P = 0.0207$; (c) $P < 0.0001$.

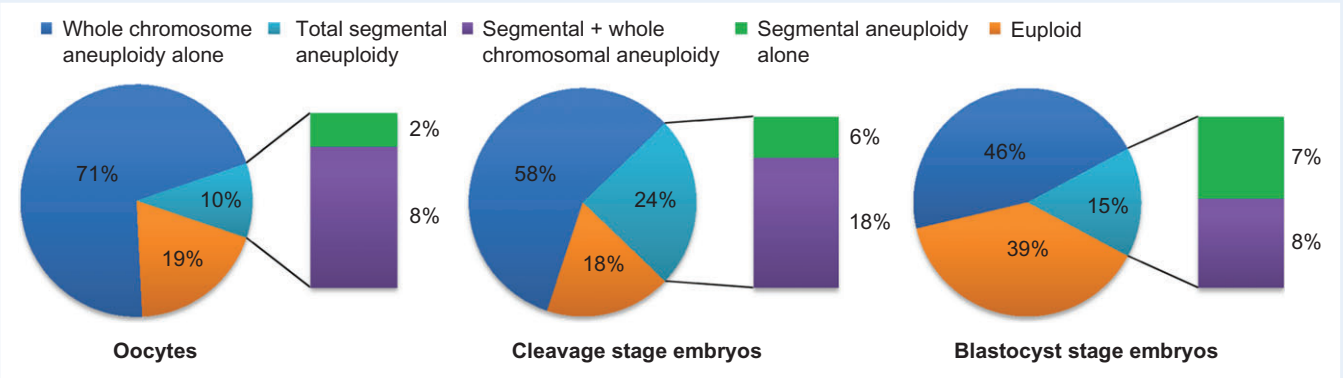


Figure 3 The frequency of segmental aneuploidy increased significantly from oocytes to the cleavage stage embryos but decreased from cleavage to blastocyst stages. Most of the segmental imbalances occurred along with other whole chromosomal aneuploidies with 8.4% (38/452) in oocytes, 18.4% (352/1762) in cleavage stage embryos and 8.1% (108/1327) at blastocyst stage. It was rare to see segmental aneuploidies occurring alone in oocytes (9/452; 1.99%) however, this was seen significantly more often at the cleavage (103/1762; 5.85%; $P = 0013$) and blastocyst stages (99/1327; 7.46%; $P < 0.0001$). No significant difference was observed in the occurrence of segmental aneuploidy alone between cleavage and blastocyst stage embryos.

occurrence of embryos with a segmental aneuploidy as the sole abnormality was not significantly different from that observed at the cleavage stage (7.5%; 99/1327; $P = 0.085$) (Fig. 3).

Dividing segmental aneuploidy according to whether the abnormality involved duplication or deletion of chromosomal material, it was apparent that oocytes have ~50% more gains of chromosomal material in comparison to losses (60/90 versus 30/90 respectively). Losses of chromosome fragments were more common at the cleavage and blastocyst stages, accounting for 234/558 (41.9%) and 107/275 (38.9%), respectively ($P < 0.0001$), compared to oocytes (30/90; 33.3%). There was no significant difference ($P = 0.4110$) in the relative frequency of segmental losses versus gains when comparing cleavage and blastocyst stages, both displayed a similar preponderance of segmental deletions (Fig. 5). The segmental imbalances were also assessed in terms of the site at which chromosome breakage had occurred and divided into two broad categories: 'distal' (where the break occurred along the chromosome arm, meaning that one of the resulting fragments would not have a centromere); 'centromeric' (where an entire chromosome arm was involved, giving the possibility that both of the resulting fragments might have a functional centromere). The frequency of centromeric segmental imbalances, including segmental losses and gains, was essentially the same between the three developmental stages assessed (Fig. 6). However, the frequency of distal chromosomal breakages leading to segmental losses was significantly higher in cleavage and blastocyst stage embryos ($P < 0.0001$) as compared to oocytes (Fig. 6). Additionally, the analysis of chromosomal breakpoints revealed a similar pattern across oocytes, cleavage and blastocyst stage embryos. Almost 25% of chromosomal breakages occurred at/near centromeres whereas ~75% of breakages were situated at more distal positions along chromosome arms (Table V).

The distribution of chromosomal breakpoints across all the chromosomes is shown in Fig. 7. Additionally, it was clear that the location of breakages was not entirely random but had a tendency to occur at distinct hotspots. Some of the identified hotspots corresponded to known fragile sites in the human genome while others are likely to be novel fragile sites, which might be specific to gametogenesis or embryogenesis (Fig. 7). Some chromosomes appeared to be more susceptible to breakages as compared to the others, especially chromosomes 16 and 19 (Fig. 7).

Since both polar bodies were analyzed from every oocyte tested, it was possible to build up an accurate picture of segmental abnormalities in oocytes following completion of both meiotic divisions. However, it is likely that the incidence of such abnormalities was underestimated for embryos. It is clear that some segmental aneuploidies exist in a mosaic form and these might not always be detected when sampling small numbers of cells from the embryo. To allow an estimation of the true incidence of segmental imbalance in preimplantation embryos, eight embryos at the cleavage stage and seven blastocysts were subjected to FISH analysis, specifically targeting chromosomal regions previously shown to be affected by segmental abnormality by array-CGH. Data was available for 40 cells derived from the cleavage stage embryos and 115 cells from the blastocysts. In most cases the segmental abnormality

was found to be mosaic, affecting some of the cells but not all, ranging from 5.3 to 100%. However, some uniformly abnormal embryos were detected at both developmental stages (Table VI). Cells sharing the original abnormality identified by array-CGH, or with a reciprocal form of the anomaly, were detected by FISH in all cases, confirming the accuracy of the aCGH method. However, in several instances the affected cells represented a small minority of the total (Table VI).

Discussion

Segmental aneuploidy arises as a consequence of DNA double stranded breaks (DSBs), which can be induced by various endogenous and exogenous factors (reviewed by Mehta and Haber, 2014). Oxidative stress or the action of certain mutagens can cause strand breakage, while another common mechanism involves stalling of replication forks. This latter problem may occur due to DNA damage, insufficient raw materials for efficient DNA synthesis, or challenges related to replication of regions of the genome that have extensive secondary structure (e.g. repetitive sequences). If DNA synthesis cannot be reinitiated a double-strand break may occur at the site of the collapsed replication fork. Additionally, in gametes, programed strand breaks facilitate meiotic recombination and can result in chromosome breakage if not resolved appropriately (reviewed by Richardson *et al.*, 2004). In most cell types, problems that predispose DNA to DSBs or the breaks themselves, induce checkpoints which subsequently pause the cell cycle. If the problem cannot be corrected, cell cycle arrest is maintained and apoptotic pathways are activated, eliminating the affected cell (Aguilera and García-Muse, 2013).

Table IV Incidence of segmental aneuploidy in MI and MII stage in oocytes.

Stage analysis performed	Number of segmental aneuploidies
MI (polar body 1)	21 (23.3%)[P]
MII (polar body 2)	69 (76.7%)[P]

Statistical comparison, using Fisher's exact test: $P < 0.0001$.

To the best of our knowledge, this is the first study to present a detailed investigation of incidence of segmental aneuploidy in large numbers of human oocytes and embryos at different stages of preimplantation development. The results confirm that such abnormalities, which are very rare in most cell types, are common during the earliest stages of human development. This poses important questions concerning the mechanisms by which these cytogenetic anomalies occur, their eventual fate and clinical impact. Unlike meiotic aneuploidy, which typically affects whole chromosomes and shows a strong association with advancing female age, no correlation was observed between age and the incidence of segmental abnormalities in oocytes or embryos (Munné *et al.*, 1995, 2007). This suggests that the molecular processes leading to segmental aneuploidy are likely to be distinct from those responsible for loss or duplication of intact chromosomes.

After completion of both meiotic divisions ~10% of oocytes were predicted to contain a segmental abnormality. It is possible that some oocytes suffer unusually high levels of DNA damage, which overwhelm intracellular repair mechanisms. It is certainly the case that not all oocytes collected from the ovary during IVF treatment are of equal competence. The follicles from which oocytes are retrieved can vary in terms of size, levels of damaging oxygen radicals and extent of blood supply (Agarwal *et al.*, 2005; Tatone *et al.*, 2008). Disruption of the blood supply has been shown to affect replication fork progression in

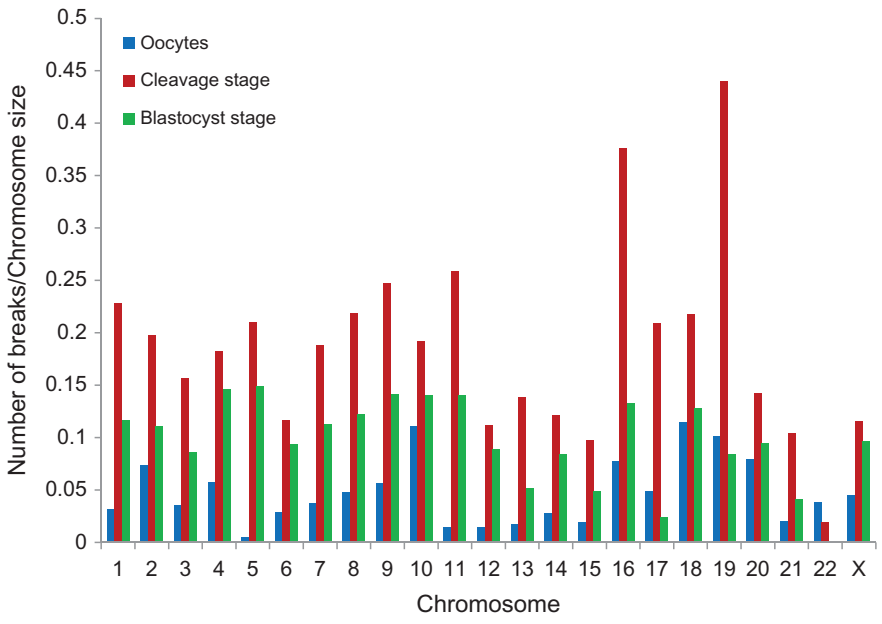
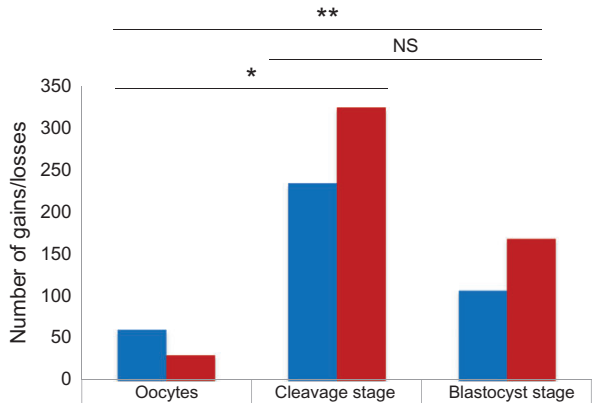


Figure 4 Graph depicting the total number of segmental errors per megabase of chromosome length in oocytes (blue), cleavage stage embryos (brown) and blastocyst stage embryos (green) across all chromosomes.

certain cell types and may directly lead to chromosomal breakages (Hammond *et al.*, 2002). Individual oocytes/embryos with multiple segmental abnormalities were detected more often than would be expected by random chance, suggesting that some gametes are predisposed to chromosome breakage, either due to intrinsic cellular defects or deficiencies of the follicular microenvironment in which they matured. Some of these problems could conceivably be influenced by factors that are patient-specific or IVF treatment related.

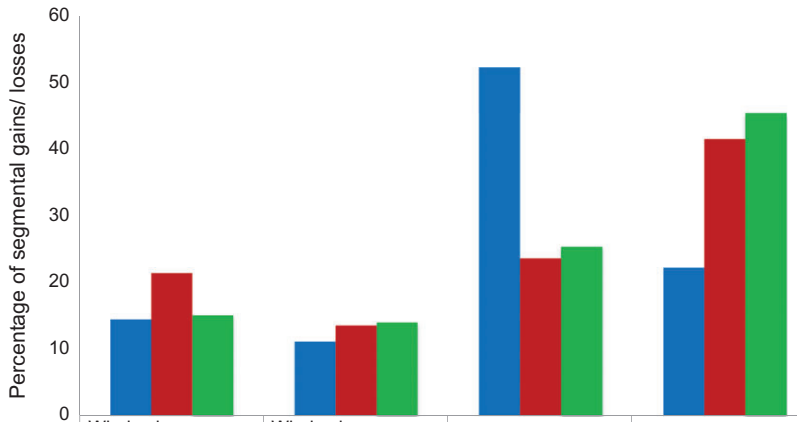


	Oocytes	Cleavage stage	Blastocyst stage
Gains	60	234	107
Losses	30	324	168

Figure 5 A significant increase in the segmental chromosomal losses was observed from oocytes to cleavage stage (*$P < 0.0001$) and blastocyst stage (**$P < 0.0001$). However, there was no significant difference in segmental gains or losses between cleavage and blastocyst stage embryos. NS, not significant.

Interestingly, three times more segmental imbalances were detected after the second meiotic division compared with the first. This may be explained by pieces of a fragmented chromosome being held in place during MI, tethered to their sister chromatid. The defect would then only become apparent after separation of chromatids at anaphase of MII and subsequent extrusion of the second polar body. It is also noteworthy that in the context of IVF (and thus relevant to the samples analyzed during the current study) the second meiotic division is completed *in vitro*, opening up the possibility of an influence of suboptimal oocyte/embryo culture systems on chromosome integrity. The majority of the predicted segmental aneuploidies in oocytes involved a gain of chromosomal material. This is in line with expectations as fragments of chromosome lacking a centromere are unable to attach to the meiotic spindle and would be more likely to remain in the oocyte than end up in the polar body. These acentric chromosomal fragments are predicted to be lost in subsequent mitotic divisions, which is consistent with the excess of segmental losses that were observed at later embryonic stages.

The current study clearly demonstrated a marked increase in the frequency of segmental aneuploidy 3 days after fertilization, at the cleavage stage of embryo development. One possible source of the additional chromosomal breakages is the DNA contributed by the fertilizing sperm. Previous studies have demonstrated that sperm samples with high levels of DNA damage, including chromosome breakage, are capable of fertilization and are associated with poor IVF outcome (Spanò *et al.*, 2000). In previous work, the origin of aneuploidies, including those of a segmental nature, was determined by using SNP microarrays to trace the inheritance of alleles on affected chromosomes from parents to embryos (Konstantinidis *et al.*, 2016). The research demonstrated that segmental aneuploidies of meiotic origin were twice as likely to affect paternal chromosomes compared with those derived from the mother, a finding that implicates meiotic processes specific to males and/or sperm DNA damage in the origin of segmental



	Whole chromosome arm gain	Whole chromosome arm loss	Acentric gain	Acentric loss
Oocytes	14.44	11.11	52.22$^{(a,b)}$	22.22$^{(a,b)}$
Cleavage stage embryos	21.39	13.53	23.58$^{(b,c)}$	41.48$^{(b,c)}$
Blastocyst stage ambryos	15.13	14.05	25.4$^{(a,c)}$	45.4$^{(a,c)}$

Figure 6 The frequency of centromeric segmental gains and losses was similar between oocytes, cleavage and blastocyst stage embryos (P = not significant). However, the oocytes had a higher incidence of acentric gains while the cleavage and blastocyst stage embryos had a significantly higher incidence of acentric losses. Statistical comparison, using Fisher's exact test: (a) $P < 0.0001$, (b)$P < 0.0001$, (c)$P = 1.000$; not significant.

aneuploidy. However, the same study demonstrated that segmental errors arising during the mitotic divisions of the embryo were ~2.5 times more common than those of a meiotic origin. This agrees with the findings of the current research and other published work, in which testing of multiple cells from the same embryos has demonstrated that most segmental abnormalities are mosaic and must therefore arise during the post-zygotic cell divisions (Voullaire et al., 2000; Wells and Delhanty, 2000; Vanneste et al., 2009; Vera-Rodríguez et al., 2016).

The observation that the peak incidence of segmental aneuploidies occurs at the cleavage stage of development echoes results of studies looking at malsegregation of whole chromosomes. These have shown that errors in chromosome segregation are common during the first few mitotic divisions, leading to a high frequency of chromosomal mosaicism in the early embryo (Bean et al., 2002; Wells et al., 2005; Fragouli et al., 2010b). The degree of genetic instability seen at the cleavage stage may be related to the fact that the human embryonic genome is not activated until the 4–8 cell stage (Braude et al., 1988) and consequently most processes occurring during the first 2–3 days after fertilization remain dependent on a pool of mRNAs and proteins provided by the oocyte. The lack of dynamic gene expression during the early mitotic divisions is likely to reduce the capacity of cells to adjust to challenging situations and may increase the rigidity of cell cycle control. The relatively rapid cell cycles that characterize the first few mitotic divisions may also increase the risk of errors occurring and suggest that checkpoints, that usually serve to monitor factors such as DNA damage and chromosome alignment on the spindle, may be relaxed or absent.

Since the oocyte governs the first few mitoses, factors related to oocyte competence, including the adequate resourcing of the female gamete with the raw materials needed for nucleotide metabolism and/or components of critical DNA repair pathways, may be relevant to genetic abnormalities seen at the cleavage stage (Fragouli et al., 2010a). It is also possible that suboptimal embryo culture systems may induce replicative and/or other stresses, ultimately causing double stranded DNA breaks and segmental aneuploidy. We have noted that the rates of segmental abnormalities detected in preimplantation embryos vary between different IVF clinics, potentially implicating

Table V Sites of chromosome breakage.

Stage analysis performed	Chromosome breakage detected near the centromere	Acentric breakage detected
Oocyte	23	67
Cleavage stage	152	412
Blastocyst	92	231

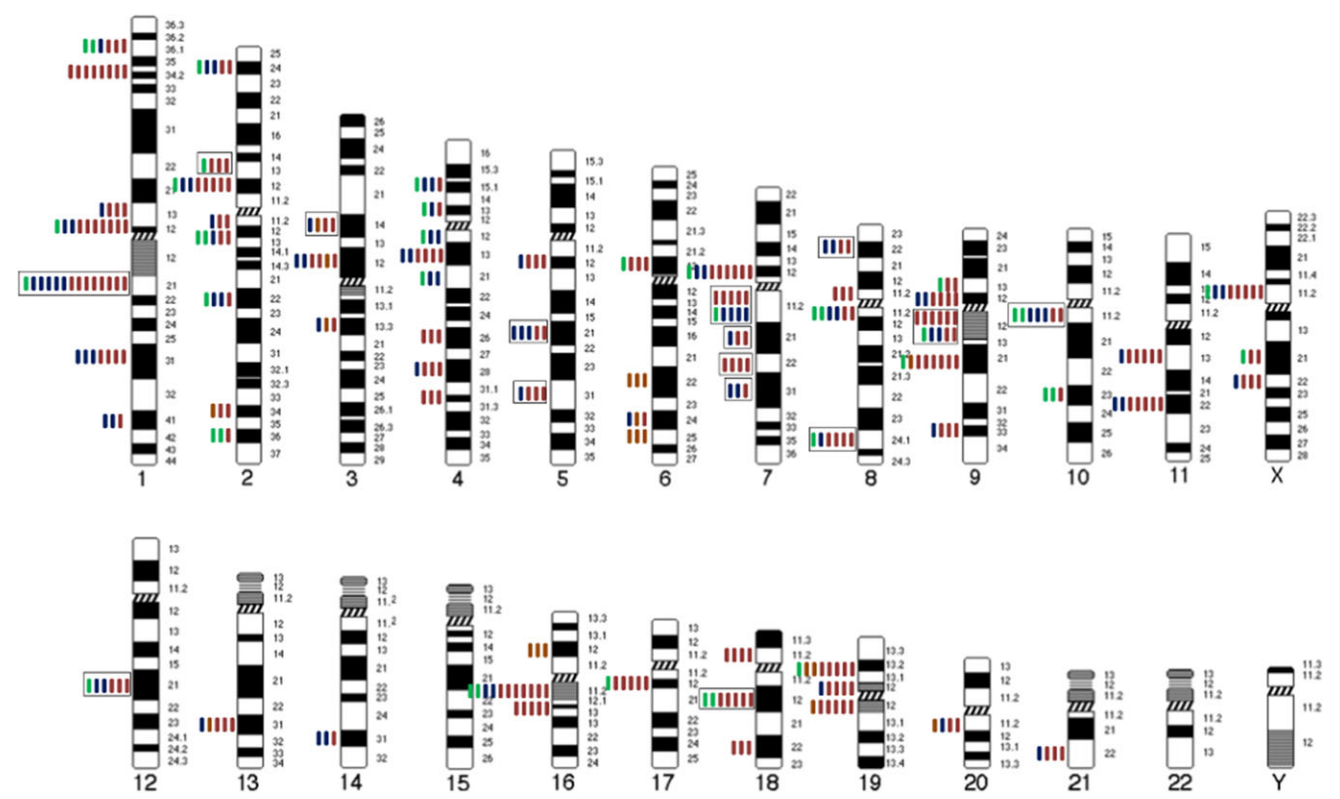


Figure 7 This ideogram shows the locations of chromosomal breakage sites observed in oocytes (green lines), cleavage stage embryos (brown lines) and blastocyst stage embryos (blue lines). Numerous breakpoints formed hotspots at distinct locations on chromosomes. The loci affected by more than three segmental aneuploidies are shown on this ideogram. The black rectangular boxes indicate the breakpoints corresponding to known fragile sites in the human genome (Durkin and Glover, 2007).

Table VI Proportion of cells affected by segmental abnormalities in embryos at the cleavage or blastocyst stages.

Embryo	Developmental stage	Cells available for analysis	Cells affected by the segmental imbalance	Percentage segmental aneuploidy (%)
1	Cleavage stage	3	3	100
2	Cleavage stage	4	1	25
3	Cleavage stage	5	2	40
4	Cleavage stage	7	1	14.3
5	Cleavage stage	4	4	100
6	Cleavage stage	8	2	25
7	Cleavage stage	7	1	14.3
8	Cleavage stage	2	2	100
Total proportion of abnormal cells in cleavage stage embryos with a segmental abnormality		16/40 (40%)		
9	Blastocyst stage	19	5	26.3
10	Blastocyst stage	12	10	83.4
11	Blastocyst stage	19	1	5.3
12	Blastocyst stage	8	3	37.5
13	Blastocyst stage	22	2	9.1
14	Blastocyst stage	28	3	10.7
15	Blastocyst stage	7	7	100
Total proportion of abnormal cells in blastocyst stage embryos with a segmental abnormality		31/115 (27%)		

Note: Analysis at the cleavage stage involves testing of a single cell from each embryo, whereas analysis of blastocyst stage embryos is accomplished by testing a clump of ~5 cells. The sensitivity of array-CGH is such that chromosomal imbalance can be detected in the clump of cells if at least 30–50% of them carry the anomaly.

aspects of the treatment, such as ovarian stimulation protocol and embryo culture method, in the origin of this class of abnormalities (Wells *et al.*, 2017).

The blastocyst represents an important developmental phase, during which the first cellular differentiation becomes apparent and apoptotic pathways can potentially be activated. Studying the ratio of embryos with only whole chromosomal aneuploidies to euploid embryos, we observed a 2.7 times decline from cleavage stage to blastocyst stage (58:18 versus 46:39). However, embryos with segmental abnormality in addition to whole chromosomal aneuploidy seem to be even more compromised. Relative to euploid embryos, they decline five-fold from the cleavage to blastocyst stage (18:18 versus 8:39). Embryos carrying only segmental chromosomal abnormalities decline 1.9 times from cleavage to blastocyst stage (6:18 versus 7:39). This suggests that having a combination of segmental plus whole chromosomal aneuploidy is associated with the greatest impact on viability, followed by whole chromosomal aneuploidy alone and lastly the presence of isolated segmental abnormality. All of these forms of abnormality display reduced survival from the cleavage to the blastocyst stage in comparison to euploid embryos. The decline in whole chromosomal and segmental aneuploidy during the transition to the blastocyst stage is suggestive of selection against affected cells and/or embryos. It is possible that embryos with these types of abnormalities undergo developmental arrest. Alternatively, affected cells may cease dividing or be eliminated through the initiation of apoptotic pathways.

The sites of chromosome breakage revealed that the prominent heterchromatic blocks on chromosomes 1, 9 and 16 were associated with numerous instances of chromosome breakage, leading to a high incidence of segmental imbalances affecting these chromosomes. The constitutive heterochromatin in these blocks is comprised of highly repetitive DNA sequences, which may hinder replication fork progression, thereby causing it to stall, collapse, and produce DNA breaks (reviewed by Saksouk *et al.*, 2015). Chromosome 19 was also affected by a disproportionately large number of segmental abnormalities given its relatively small size. This chromosome has the highest GC content amongst all the chromosomes, potentially leading to challenges for DNA replication. It also has a comparatively high recombination rate and consequently more strand breaks are induced during meiosis and need to be resolved correctly.

Many of the chromosomal breaks that resulted in segmental imbalance in this study did not appear to be random but rather concentrated at various loci on different chromosomes. This finding is similar to the occurrence of *de novo* chromosome rearrangements found in prenatal samples, wherein the breakpoints are often associated with defined fragile sites. A high incidence of chromosome rearrangements at fragile sites is also observed in tumor cells (Dillon *et al.*, 2010), which have compromised cell cycle checkpoints reminiscent of the deficiencies of cell cycle control seen in human preimplantation embryos (Wells *et al.*, 2005). Fragile sites are chromosomal regions, which are susceptible to breakage induced by perturbations in the DNA replication/repair process, often associated with regions of DNA sequence that are challenging for replication due to the presence of secondary structures (Hewett et al. 1998). To date, over 100 different fragile sites have been identified, distributed across the human genome (Durkin

and Glover, 2007). Some of the identified hotspots of chromosomal breakpoints in this study lie in the vicinity of these fragile sites, indicating a potential relationship between the two. Additional sites of recurrent breakage identified in the oocytes and embryos during this study may correspond to novel fragile sites unique to gametogenesis and/or embryogenesis or induced by specific deficiencies of embryo culture media formulations (Fig. 7).

It is not certain whether the segmental aneuploidies investigated in the present study are representative of what might be observed following natural conception, or whether they are in some way associated with the underlying infertility of the patient or the treatments they received (ovarian stimulation and embryo culture). It is known that *de novo* chromosomal rearrangements, similar to those described here, occur in natural pregnancies at a low frequency as shown by a study based on amniocentesis (Warburton, 1991). However, unbalanced chromosome rearrangements are frequently lethal and embryos carrying such errors are most likely lost early during development. The data from the current study may be the closest we can come to an estimate of the true rate of segmental aneuploidy around the time of conception, before any selection against abnormalities has occurred.

The clinical significance of segmental abnormalities in oocytes and embryos remains unclear. Those detected in oocytes are of the most concern, since they are predicted to produce embryos with a non-mosaic segmental abnormality. Segmental aneuploidy affecting entire chromosome arms, following a breakage event at the centromere, seemed to typically have a meiotic origin, as there was no significant change in the frequency of such errors at subsequent embryonic stages. Considering that this type of aneuploidy is likely to be present in all the cells of embryo, and the fact that a functional centromere will increase the chances of stable transmission of the abnormal chromosome, the risk of an affected embryo producing an abnormal pregnancy or birth is predicted to be highest for this class of segmental abnormality. Based upon the size of the chromosomal fragments involved, we estimate that at least 80% of those seen in oocytes would be incompatible with a viable pregnancy and would either fail to implant of miscarry. This means only ~2% of all oocytes are likely to be affected by a breakage that would be detected during gestation or at birth, although it is possible that this figure may be higher if smaller chromosomal fragments, below the threshold of detection of the array-CGH technology used in this research, also experience loss/duplication in oocytes. Hence, the origin of segmental aneuploidy (meiotic vs mitotic) as well as the chromosomal breakage site and size of the affected fragment should all be taken into account for clinical management of embryos carrying segmental aneuploidy. This is of particular importance in a scenario where there are no euploid embryos present for transfer. It is known that the transfer of embryos with segmental abnormalities can sometimes result in healthy euploid births, but the numbers of cases in which this has occurred with appropriate follow up is too small for any firm conclusions to be reached at this time. Given the current paucity of data, if any embryos with a segmental aneuploidy are chosen for transfer, prenatal testing (ideally amniocentesis) is strongly recommended to confirm the genetic status of any resulting fetus.

It is important to define the true rate of segmental chromosome imbalances in oocytes and embryos, since a high frequency would be expected to have a negative impact on IVF success rates and lead to miscarriages and congenital abnormalities. A study by Vanneste and colleagues reported that 70% of cleavage stage embryos (Day-3/-4 post-fertilization) contained segmental abnormalities, but provided no data concerning incidence in oocytes or at later preimplantation stages (e.g. blastocyst). Additionally, the number of embryos tested was small and all of the data was derived from a single clinic. The results of the current study suggest that the incidence of chromosome breakage at the cleavage stage is actually considerably lower than 70%, which is more in keeping with other investigations using a range of cytogenetic techniques (Clouston *et al.*, 1997; Voullaire *et al.*, 2000; Wells and Delhanty, 2000; Vera-Rodríguez *et al.*, 2016).

A limitation of the present investigation is that it did not involve analysis of every cell from each of the embryos tested. However, extrapolating from the data, we calculate that the maximum proportion of human cleavage stage embryos containing segmental imbalance in at least one of their cells is unlikely to exceed 40%. This assumes that embryos contain 6–8 cells, which is typical for this stage of development, and that each cell is independent in terms of chromosome imbalance occurring. In reality, this likely overestimates the frequency as some embryos have the same segmental error in every cell, due to a meiotic error. Others have the same abnormality in several cells, resulting from an error in the first or second mitotic division. Finally, some embryos have cells with reciprocal abnormalities due to segregation of derivative chromosome fragments into different cells. Another limitation, relevant to any study that assesses chromosome copy number based on quantification of DNA, including the current research, is the possibility of artifacts in copy number assignment for individual chromosomal regions related to asynchrony of DNA replication across the genome (early replicating areas of the genome transiently having higher copy number than late replicating during S-phase of the cell cycle) (Van der Aa *et al.*, 2013). However, while this remains a theoretical source of false positive segmental aneuploidy, we are yet to see any convincing evidence of this artifact having occurred.

It can be concluded from the data analyzed that segmental imbalances, while common in human oocytes, can be detected at a much high frequency in preimplantation embryos. There appears to be a high risk of chromosomal breakage during the first few mitotic divisions following fertilization of the oocyte with terminal regions being affected more frequently than centromeres. It is probable that this reflects the natural situation, although it is possible that an underlying susceptibility might be exacerbated by the application of assisted reproductive techniques. As embryonic development progresses from the cleavage to the blastocyst stage, the rate of segmental aneuploidy decreases. This is likely to be explained by selection against affected embryos or the abnormal cells they contain. Selection mechanisms could involve apoptosis, which might eventually lead to the demise of the embryo, if the proportion of abnormal cells is high, or normalization of the karyotype by elimination of abnormal subpopulations of cells in mosaic embryos.

It is noteworthy that there is no evidence for an increased rate of segmental aneuploidy in children born following fertility treatments (Bonduelle *et al.*, 1995; Woldringh *et al.*, 2010). Importantly, the frequency of segmental aneuploidies in the neonatal population following natural or assisted conception is extremely low, suggesting that affected cells/embryos are eliminated with high efficiency at early developmental stages.

Authors' roles

Conception and design of the study: D.W. and E.F. Data acquisition: E.F., D.B., A.F. and K.S. Data analysis and interpretation: D.B., D.W.,

E.F., A.F. and K.S. Drafting of the manuscript: D.W. and D.B. Supervision and critical revision of the manuscript for important intellectual content: D.W., E.F. and D.B. Final approval of the version to be published: D.B., E.F., A.F., K.S. and D.W. All authors have read, and confirm that they meet, the authorship criteria.

Funding

This study was supported by institutional funding (Reprogenetics UK). Additionally, DW is supported by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre Programme. DB was supported by the University of Oxford's Clarendon funding.

Conflict of interest

None declared. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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