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Conception to ongoing pregnancy: the 'black box' of early pregnancy loss

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Even when conditions are optimal, the maximum chance of a clinically recognized pregnancy occurring in a given menstrual cycle is 30–40%. Increasing evidence points to preclinical pregnancy loss rather than failure of conception as the principal cause for the relatively low fecundity observed in humans. While sensitive assays for hCG have provided a glimpse of the events occurring between implantation and the missed menstrual period, new cytogenetic techniques have further opened this 'black box', providing novel insights into the causes of early pregnancy wastage. In this article, the evidence and causes of preclinical or 'occult' pregnancy are reviewed, and the implications for the infertile patient are addressed.

Key words: aneuploidy/early pregnancy loss/human chorionic gonadotrophin/infertility/IVF

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Introduction

The continuing growth in the world population would appear to stand as testament to the fecundity of the human race. However, when human reproduction is examined in terms of efficacy, it becomes clear that the growth in population has occurred in spite, rather than as a result, of our reproductive performance. The relative inefficiency of humans in achieving live births was graphically highlighted when the number of births registered in England in 1970 was compared with the number of births that might have been expected, given the estimated number of fertile ovulatory cycles exposed to coitus in the same year and population (Roberts and Lowe, 1975). Since only 22% of cycles at risk of pregnancy resulted in live birth (Table I) the authors posed the question ''Where have all the conceptions gone?'' More recent studies have indicated that even when circumstances for conception are deemed optimal, the maximal chance of conceiving a clinically recognized pregnancy in one cycle is ~30% (Zinamen *et al.*, 1996; Slama *et al.*, 2002), rising to 40% in young women undergoing timed insemination with sperm from donors of proven fertility (van Noord-Zaadstra *et al.*, 1991).

Where are the pregnancies going? Pregnancies may be lost at any time between fertilization and implantation, or up to term. A proportion of these losses are clinically revealed as miscarriages. However, since the first report of a pregnancy loss detected by the measurement of hCG (Morris and Udry, 1967), it has become clear that a large number of conceptions fail before the woman

Table I. The estimated fetal loss for married women aged between 20 and 29 years in England and Wales, 1971 (Modified from Roberts and Lowe, 1975).

	Number in millions
Married women aged 20–29 years	2.4
Annual acts of coitus (twice a week)	253.4
Annual acts of unprotected coitus (1:4)	63.4
Unprotected acts within 48 h of ovulation (1:14)	4.5
Assume one in two of these results in fertilization	2.3
Number of infants born to these women	0.5
Estimated loss (2.3–0.5)	1.8
Percentage loss [(1.8/2.3)×100]	78

becomes aware that she might have been pregnant. These early pregnancy losses, sometimes termed 'occult' pregnancies (Walker *et al.*, 1988), have been defined as pregnancies, ''That terminate so soon after implantation that no clinical suspicion exists as to its having existed'' (Bloch, 1976). The introduction of sensitive assays for hCG and the possibility provided by IVF to observe the events from ovulation to on-going pregnancy, has enabled the previously elusive 'black box' of early pregnancy to be explored. With new techniques in cytogenetics, our understanding of the natural limits of human fecundity has grown, with clear implications for where the limits of success may lie for IVF.

Preclinical pregnancy loss

The 'black box' of early pregnancy was first opened in a literal sense in the classic studies of Hertig *et al.* in which the uterine contents of hysterectomy specimens removed from ovulatory, fertile women were meticulously examined (Hertig *et al.*, 1959; Hertig and Rock, 1973). Studying specimens from 107 women deemed to have been at optimal 'risk' of conceiving prior to undergoing hysterectomy, they identified evidence of early pregnancy in the form of implantation sites or embryos in just 34 cases. Ten of the 34 'fertilized ova' recovered during the first 17 days of development were found to be abnormal. They concluded that the failure to identify evidence of early pregnancy in the other specimens was due to failure of fertilization or ''The disintegration of an ovum once fertilized''. The greatest losses appeared to occur at the preimplantation stage or during the first week of implantation. Although the high rate of early pregnancy loss before the time of the first missed period was thus clearly demonstrated, analysis of the stage of development at which early pregnancy failure occurred was hampered by a lack of information as to the precise time at which ovulation occurred. The age of the specimen was determined indirectly by morphology of the conceptus, the clinical history of the patient and histological dating of the endometrium (Noyes *et al.*, 1950) and the corpus luteum (White *et al.*, 1951).

Later, studies of oocyte donors undergoing periovulatory insemination and uterine lavage confirmed the large variability in the viability of the recovered zygotes (Buster *et al.*, 1985; Formigli *et al.*, 1987). However, further knowledge as to the precise stage at which early pregnancies fail required the availability of biochemical markers for conception and implantation.

hCG as a marker for pregnancy loss

The early conceptus produces a wide range of metabolites which may be measured *in vitro* (Gardner *et al.*, 2001). Only a limited number of these embryonic factors are secreted in concentrations sufficient to allow detection in maternal serum or urine. The most commonly employed marker of pregnancy, and that on which studies of occult pregnancy have been based, is hCG. In-vitro studies have shown that hCG is produced by trophoblastic cells of the unhatched blastocyst and may be detected from 7 days onwards after fertilization (Shutt and Lopata, 1981; Fishel *et al.*, 1984; Lachlan and Lopata, 1988; Lopata and Hay, 1989). Messenger RNA for the β-subunit of hCG may be detected as early as the 8-cell stage, ~4 days post-fertilization (Blonduelle *et al.*, 1988). However, hCG produced by a conceptus is generally considered to become readily detectable in maternal serum or urine only from the time of implantation onwards. In practical terms this means that it becomes detectable 6.5–9.5 days after the LH surge (Hay *et al.*, 1986; Lenton and Woodward, 1988). The stability of hCG makes it suitable for study since, particularly in early pregnancy, measurements in urine are accurate and consistently represent measurements in the serum (Wehmann *et al.*, 1981; Norman *et al.*, 1987). Furthermore, hCG is very stable even in urine which has been repeatedly thawed and frozen (McCready *et al.*, 1978; Wilcox *et al.*, 1985). The development of antibodies towards the β-subunit of hCG was an improvement on earlier assays (Vaitukaitis *et al.*, 1972), and the development of immunoradiometric assays finally provided very sensitive and specific means of measuring serum and urinary hCG (Wilcox *et al.*, 1985; Canfield *et al.*, 1987).

Preliminary investigations using hCG to study early pregnancy demonstrated great variations in the rate of unrecognized pregnancy loss (Table II). These discrepancies reflect the limitations of the hCG assays used, the different patient populations involved and methodological problems relating to the timing of ovulation. The detection of 'background hCG' in non-pregnant women may have further confused the issue (Landesman *et al.*, 1976; Seppala *et al.*, 1978; Tamsen and Eneroth, 1986). Many of these problems were addressed by a landmark study in which daily urine samples from 221 women, collected during 6 months of attempted conception, were analysed for hCG (Wilcox *et al.*, 1988). The degree of sensitivity of the hCG assay employed meant that background hCG produced by

Table II. Early studies using hCG as marker for early human pregnancy after unprotected intercourse in an unselected group of women. The non-pregnant control groups and means of timing ovulation employed by each study in order to interpret hCG results are given

Authors	Control group	Ovulation timing	Cycles	Pregnancies (%)
Chartier *et al*., 1979	12 lab workers	BBT curve	321	71 clinical (22) 49 occult (15)
Miller *et al*., 1980	None	Cycle length	623	102 clinical (16) 50 occult (8)
Edmonds *et al*., 1982	50 sterilized	Mean cycle length	198	51 clinical (26) 67 occult (34)
Whittiker *et al*., 1983	None	Cycle length	226	85 clinical (38) 7 occult (3)

BBT = body basal temperature.

the endometrium of non-pregnant women (Wolkersdorfer *et al.*, 1998) could be detected. Therefore, a control group of women who had undergone sterilization by tubal ligation were also studied, and cut-off values for identifying pregnancy were determined. Of the 198 pregnancies thus detected, 31% were subsequently lost. Twenty-two percent of all pregnancy losses were 'occult'; occurring before the woman could have been aware of the pregnancy. In a similar study in which 200 women collected daily urine specimens for hCG analysis over three menstrual cycles, an overall pregnancy loss rate of 31% was reported (Zinamen *et al.*, 1996). While these observations were consistent with those of Wilcox (Wilcox, 1988), the percentage of occult pregnancies observed was lower (13% compared with 22%). This discrepancy may be partly explained by the lower sensitivity of the assay used in the study of Zinamen *et al*. and the lack of a control group to aid in determining the threshold level for hCG indicative of pregnancy. However, when taken together, data from the published studies point to a rate of pregnancy loss prior to implantation of 30%, a further 30% following implantation but prior to the missed period, and 10% as clinical miscarriages (Figure 1).

The embryo or the endometrium?

In pregnancies destined for term, an exponential rise in serum hCG to a peak at 8–10 weeks gestation is observed. In patients with extrauterine or intrauterine pregnancies destined for failure, lower serum hCG concentrations are produced (Lenton *et al.*, 1982; Sinosich *et al.*, 1985; Hay *et al.*, 1986; Yovich *et al.*, 1986; Liu *et al.*, 1988a; Liu and Rosenwaks 1991; Bjercke *et al.*, 1999).

Two types of occult pregnancy loss have been identified, based on the pattern of hCG expression (Lenton *et al.*, 1988). When the process of implantation, initiated at the normal time, is terminated abruptly, hCG becomes detectable from around day 9 post-LH surge but fails to rise exponentially, declining over the next 2 days. This pattern of hCG secretion may suggest an endometrial factor preventing completion of implantation. However, if implantation is delayed, the rate of rise of hCG is reduced and detection occurs later in the cycle. This pattern of hCG rise may indicate abnormal embryonic development after implantation has occurred. Both of these patterns of hCG secretion have been observed in IVF pregnancies destined to miscarry (Liu and Rosenwaks, 1991).

Data from IVF studies on the impact of timing of implantation on pregnancy outcome cannot be directly extrapolated to the situation following spontaneous conception because the ovarian stimulation treatment and the underlying cause of infertility may themselves distort the timing of implantation and hence the pattern of hCG secretion (Tur-Kaspa *et al*., 1990; Macklon and Fauser, 2000). Data are therefore required from spontaneous conceptions. This has been addressed in a study of 221 women attempting to conceive from whom daily urine samples were collected for up to 6 months (Wilcox *et al.*, 1999). The first appearance of hCG, taken to indicate implantation, varied between 6–12 days after ovulation, with 84% implanting on day 8, 9 or 10. Delayed implantation was clearly associated with occult pregnancy loss, such that no clinical pregnancy was recorded if implantation occurred >12 days post-ovulation (Figure 2). Early loss was least likely when implantation occurred by day 9. These findings were consistent with an earlier study in women with no known fertility problems (Stewart *et al.*, 1993) and confirmed earlier reports of increased risk of early pregnancy loss with late implantation (Lenton *et al.*, 1991; Liu *et al.*, 1991; Stewart *et al.*, 1993).

The late implantation of the conceptus may be either the cause or result of abnormal circumstances in early pregnancy. Abnormal embryos may develop slowly or implant abnormally, leading to delayed and reduced production of hCG. The corpus luteum may thus be 'rescued' from involution too late to allow pregnancy to continue (Liu and Rosenwaks 1991). Should a normal embryo begin implanting later in the luteal phase, endometrial receptivity may be impaired (Hearn *et al.*, 1991; Rogers *et al.*, 1995) as the window of receptivity is closing (Lessey *et al.*, 2001). The question as to whether the embryo or endometrium is the primary determinant of the timing of implantation has also been addressed (Bergh and Navot, 1992). Embryos of identical age were transferred into endometria of different ages in women who had

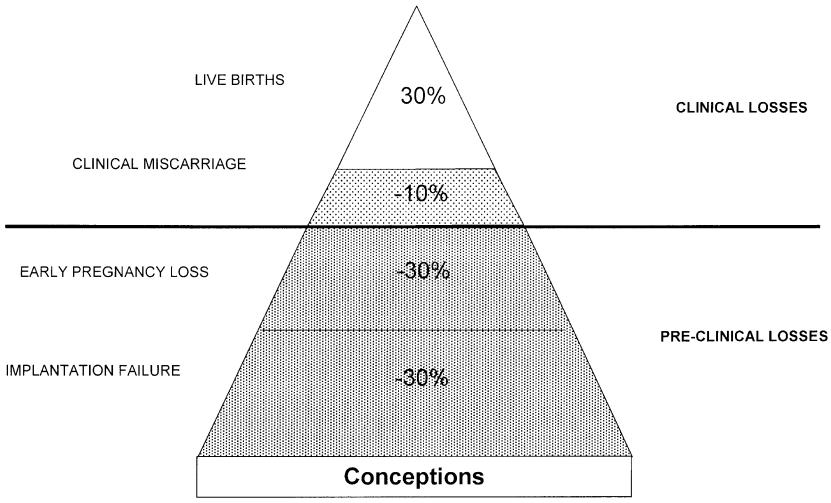

Figure 1. The Pregnancy Loss Iceberg: an overview of the outcome of spontaneous human pregnancy. A total of 70% of conceptions are lost prior to live birth. The majority of these losses occur prior to the time of the missed menstrual period, and are not revealed. (Adapted from Chard, 1991.)

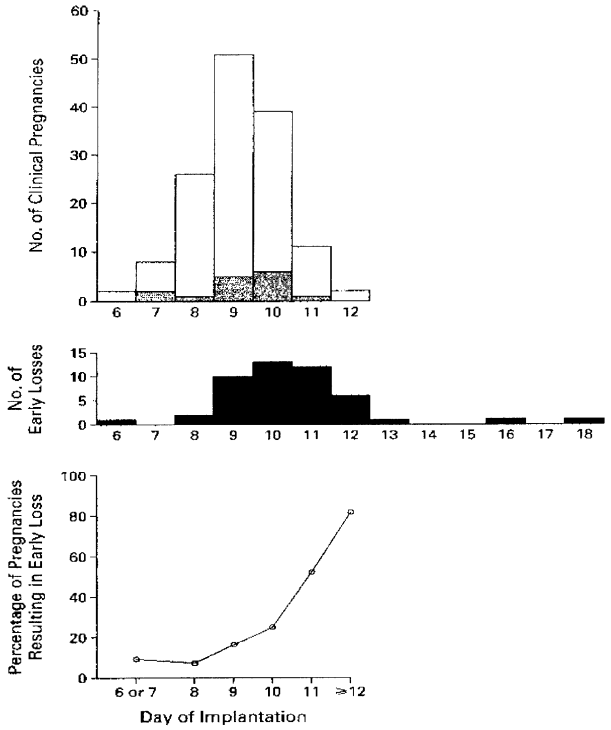

Figure 2. Timing of implantation in 189 naturally-occurring pregnancies and the risk of early pregnancy loss. The day of ovulation represents day 0. The upper panel demonstrates the day of implantation in 141 clinically recognized pregnancies (continuing beyond 6 weeks after the previous menstrual period), 15 of which ended in miscarriage (shaded area). The mid-panel shows the day of implantation in 48 pregnancies which ended in early pregnancy loss (within 6 weeks of the last menstrual period). A clear delayed implantation is seen. The increasing proportion of early loss with later implantation is demonstrated in the lower panel (P for trend, < 0.001). (Reproduced with permission from Wilcox *et al.*, 1999. Copyright © 1999 Massachusetts Medical Society. All rights reserved.)

not received exogenous hCG. The day of the first embryonic hCG signal indicated that the window of implantation extends between cycle day 20 and 24. Within this window, the timing of implantation appeared to be dependent on the embryonic age and not that of the endometrium.

While studies such as these have helped us in understanding where the missing pregnancies 'have gone', the fate of the fertilized oocyte prior to implantation remains unclear. Implantation begins around day 8 after the LH surge (Lenton *et al.*, 1982; Enders and Lopata, 1999). Although sensitive assays may be capable of detecting rises in hCG produced by the embryo before it implants (Chang *et al.*, 1998), in-vivo studies of the preimplantation conceptus require a marker which becomes detectable from the moment of fertilization onwards. A promising candidate is a substance which is secreted into the maternal serum 12–16 h after fertilization. This 'early pregnancy factor' was first detected in 1977 (Morton *et al.*, 1977) and was subsequently found to be secreted by preimplantation embryos (Bose, 1989, 1991; Bose *et al.*, 1989). The presence of early pregnancy factor in the culture media of cleaved zygotes and its absence in media from unfertilized oocytes indicate that it is a product of the developing embryo (Daya and Clark, 1986, 1988). More recent studies have focused on the sensitivity and specificity of early pregnancy factor for detecting conception *in vivo* (Fan and Zheng, 1997). Although it has been characterized (Cavanagh, 1996; Haq *et al.*, 2001), current assays remain cumbersome and the utility of this marker is not yet established.

It is clear that studies using markers for conception, implantation and ongoing pregnancy can only provide limited information as to the fate of the oocyte following fertilization. Much of the progress made in improving our understanding of the events of very early pregnancy has been made in the field of cytogenetics.

Understanding early pregnancy loss: lessons from cytogenetics

The likely importance of genetic abnormalities as a cause of early pregnancy loss was made clear by studies of spontaneous abortions in which chromosome abnormalities were encountered in >50% of pregnancies (Boué *et al.*, 1975; Eiben *et al.*, 1987). Since the early eighties, the frequency of chromosome abnormalities in oocytes, zygotes and early stages of embryo development have also been studied. Due to legal restrictions in most countries, human embryos for study originate within the context of infertility treatment and therefore do not reflect the normal situation. However, it has become possible to study normal oocytes from couples with male factor infertility and *vice versa*, and the introduction of new cytogenetic methods has allowed more unselected studies to be carried out. The various types of fluorescence in-situ hybridization (FISH) now available have led to reliable estimates of aneuploidy rates in individual blastomeres, and the contribution of chromosomal mosaicism to early embryonic death to be determined.

Cytogenetic techniques

Cytogenetic studies can be carried out on metaphase chromosomes or on interphase cells. In the former case, classical karyotypes are made following mitotic arrest, while in the latter, non-dividing cells are studied by in-situ hybridization. In-situ hybridization can be used to visualize specific DNA sequences in a microscopic preparation. This method is ideal for the study of chromosomes in interphase nuclei. FISH allows the detection of numerical as well as structural chromosome abnormalities in single cells.

The visualization of chromosomes at the single cell level with great accuracy and optimal efficiency is a formidable task. In this respect some new approaches have been proposed for the study of oocytes and first polar bodies (Durban *et al.*, 1998; Marquez *et al.*, 1998), second polar bodies (Verlinsky and Evsikov, 1998) and single blastomeres (Evsikov and Verlinsky, 1999). If chromosomes have been obtained, newer staining methods such as multicolour FISH and spectral karyotyping simultaneously visualize each human chromosome in a specific colour. Both methods only differ in the way these colours are produced (Lichter, 1997). These methods have already been applied for the single cell diagnosis of aneuploidy (Fung *et al*., 1998; Marquez *et al*., 1998).

Another promising approach is comparative genomic hybridization (CGH). This technique was originally described for cytogenetic analysis of solid tumours (Kallioniemi *et al*., 1992). Recently this approach has been applied in preimplantation embryos (Wells and Delhanty, 2000; Wilton *et al.*, 2001) and in early spontaneous abortion (Fritz *et al*., 2001). The principle of this method is that control and study DNA are simultaneously

hybridized onto a normal metaphase. On the basis of a shift in the ratio of two colours, quantitative fluorescence microscopy reveals chromosomes (in case of aneuploidy) and chromosomal regions (in case of unbalanced structural rearrangements) which are lost or amplified.

In the future, molecular genetic techniques, such as fluorescent PCR of small and short tandem repeats (Adinolfi *et al*., 1997; Findlay *et al*., 1998) and microarrays for the measurement of DNA copy number (Snijders *et al*., 2001) may replace cytogenetic methods.

Aneuploidy in oocytes

Since the first report on the study of meiosis in human oocytes in 1968, a large number of publications have described the cytogenetic analysis of inseminated oocytes that failed to fertilize in an IVF programme. Data on 2434 oocytes, reported by 12 groups, have shown that the total incidence of chromosome anomalies ranges from 8 to 54% with an average of 27%. (13% hypohaploidy, 8% hyperhaploidy, 2% structural abnormalities and 4% diploidy) (Plachot, 2001). In another study of 400 oocytes showing no sign of fertilization or cleavage, 124 were unanalysable and 79 were normal haploid (Angell, 1994). The remaining oocytes were abnormal, but no instance of hyperhaploidy was noted. An increase in maternal age results in an increased embryonic aneuploidy rate as well as an increased frequency of spontaneous abortion. Both increases result from non-disjunction giving rise to autosomal trisomy (Hook, 1981). For all chromosomes, except the largest, the non-disjunction rate increases with age. On the other hand, monosomy X shows an inverse maternal age effect. The maternal age effect on aneuploidy which is known to be present in spontaneous abortions and liveborns has also been demonstrated in morphologically and developmentally normal preimplantation embryos (Munné *et al*., 1995). This corroborates the hypothesis that oocytes of older women are more prone to non-disjunction caused by meiotic errors at the gamete level. However, the effect of maternal age on fetal aneuploidy, well documented at birth, has not yet unambiguously been found to be a consequence of an increased rate of aneuploid oocytes. Intra- and extrafollicular influences (perifollicular microvasculature, oxygenation, the presence of residues from cigarette smoke) are able to disturb maturation leading to immaturity and aneuploidy. Oocyte meiosis is very sensitive to endogenous or exogenous factors, which could lead to chromosomally abnormal oocytes and as a consequence, to abnormal zygotes (Plachot, 1997).

The difference in age-related aneuploidy between oocytes and miscarried embryos might be explained on the basis of preimplantation or early post-implantation loss. For chromosomes 13, 18 and 21, aneuploidy in clinically recognized pregnancies increases from 1.3% in the 35–39 years of age group to 4.3% in the 40–45 years group. However, in cleavage stage embryos it has been observed that the aneuploidy rates for chromosomes X,Y, 13, 18 and 21 increased from 5 and 10% in the 20–34 and 35–39 years of age groups respectively to 28% in the 40–45 years group (Munné *et al*., 1995). If only normally developing embryos are considered the differences are even greater, increasing from 4% in the 20–34 years group to 37.2% in the 40–45 years group.

There seems to be no effect of maternal age on triploidy and tetraploidy, which originate at fertilization and during pre-implantation development respectively. This confirms that maternal age is a factor that influences only meiosis. The cause of non-disjunction in oocytes of older women is largely unknown. Although an increase in maternal age is accompanied by a reduction in chiasma frequency of all chromosomes, the distribution of the meiotic errors is not identical for every chromosome. Trisomy 16, for example, always show a maternal meiosis I (MI) error. Trisomy 18, on the other hand, results predominantly from MII errors.

Premature centromere division at MI has been suggested as an alternative mechanism for trisomy formation (Angell, 1991, 1997). Unfertilized oocytes obtained following ovarian stimulation for IVF were studied for MI and MII errors. None of the chromosome complements studied had an extra whole chromosome. This finding was not in line with the theory of non-disjunction that proposes that both chromosomes of the bivalent fail to disjoin at MI so that both move to one pole and result in an additional whole chromosome at MII metaphase. The only class of abnormality found in the MII oocytes had single chromatids (half chromosomes) replacing whole chromosomes. Analysis of the chromosomally abnormal oocytes revealed an extremely close correlation with data on trisomies in spontaneous abortions, with respect to chromosome distribution, frequency and maternal age, and indicated the likelihood of the chromatid abnormalities being the MI non-disjunction products that lead to trisomy formation after fertilization. In the light of data showing that altered recombination patterns of the affected chromosomes are a key feature of most MI origin trisomies, the oocyte data imply that the vulnerable meiotic configurations arising from altered recombination patterns are processed as functional univalents in older women.

The association between non-disjunction and maternal age in MII oocytes using FISH has been analysed in a study of oocytes and their corresponding polar bodies (Dailey *et al.*, 1996). The chromosomes of both division products complemented each other and provided an internal control to differentiate between aneuploidy and technical errors. Two mechanisms of non-disjunction were determined. First, non-disjunction of bivalent chromosomes going to the same pole and second, non-disjunction by premature chromatid separation (predivision) of univalent chromosomes producing either a balanced (2 + 2) or unbalanced (3 + 1) distribution of chromatids into the first polar body and MII oocytes. Balanced predivision of oocytes, proposed by Angell as a major mechanism of aneuploidy (Angell, 1997), was found to increase significantly with time in culture, which suggests that this phenomenon should be interpreted carefully. Unbalanced predivision and classical non-disjunction were unaffected by ageing of the oocyte. In comparing oocytes from women <35 years of age with oocytes from women >40 years, a significant increase in non-disjunction of full dyads was found in the oocytes with analysable polar bodies and no FISH errors. Premature predivision of chromatids was also found to cause non-disjunction, but it did not increase with maternal age.

When discussing these results it should be kept in mind that the study material in these investigations was far from ideal. Women undergoing IVF are not a random sample from the population and the behaviour of surplus oocytes might not be representative of the whole cohort. However, a clear age-related effect is seen on the genetic constitution of oocytes and embryos. These data are

consistent with clinical data which show a great impact of maternal age on the chance of spontaneous conception (van Noord-Zaadstra *et al.*, 1991) and success from IVF treatment (Templeton *et al.*, 1996; Hunault *et al.*, 2002).

Aneuploidy in sperm

Using FISH, aneuploidy rates have been shown to vary from 0.03% for the X chromosome to ~1% for chromosome 1 (Pieters and Geraedts, 1994). A wide range was observed in the percentages of disomy for the different chromosomes. The median value for all chromosomes reported is in the range of ~0.3%. The large variations observed between studies can be attributed to technical factors such as decondensation and denaturation of the DNA in the specimen and distortion of the morphology after pretreatment.

If the observed 0.3% is truly representative of the disomy rate among all the 23 chromosomes in sperm, an overall rate of 7% would be found using the FISH technique. This figure is in the range of the findings using the zona-free hamster technique (Martin and Rademaker, 1990; Pellestor, 1991). Besides technical factors explaining a proportion of the variation observed, it should not be forgotten that differences might be observed between patients. In more recent studies it has been shown that the percentage of numerical abnormalities is significantly increased in a group of patients with abnormal semen characteristics (Pfeffer *et al.*, 1999; Vegetti *et al.*, 2000). A higher incidence of numerical chromosome abnormalities in sperm has also been detected in ICSI zygotes (Macas *et al*., 2001). Finally, it has been observed that sperm of non-obstructive azoospermic men had a higher incidence of chromosome abnormalities, of which sex chromosome abnormality was the most predominant (Palermo *et al*., 2002).

Abnormalities in zygotes and preimplantation embryos

The reported rate of abnormal fertilization observed during IVF varies from 2 to ~9%. About 20 h after insemination, the zygotes are studied for the presence of pronuclei. The two main categories of abnormalities are parthenogenetic activation (1 pronucleus) and triploidy (3 pronuclei). Tripronuclear zygotes, which result in most cases from dispermy (Plachot *et al*., 1989), are never transferred to the uterus.

The first cytogenetic studies of early embryos were carried out using classical techniques. To obtain sufficient metaphases for study, cleaving embryos were required. Only a minority of the embryos studied this way could be analysed. Furthermore, the proportion of cases in which results were obtained in all cells was even less (Jamieson *et al.*, 1994; Pellestor *et al.*, 1994a). The rate of reported abnormalities using this technique varied from 23 to 90%.

From the study of preimplantation embryos arising from tripronuclear zygotes it has become clear that they display a variety of chromosomal abnormalities (Pieters *et al*., 1992). They include (i) complete triploidy in all cells after regular division, (ii) gross abnormalities in all cells due to chaotic chromosome movement after multipolar spindle division, (iii) cell subpopulations with either a haploid or a diploid chromosomal content because of extrusion of a haploid nucleus during the first cleavage division, and (iv) cell subpopulations with a diploid or triploid chromosomal content as a result of extrusion of a haploid nucleus during the first cleavage division and subsequent incorporation in one of the two nuclei.

The development of FISH technology enabled arrested human embryos to be studied. Since interphase cells could be studied with high efficiency it was possible to draw conclusions about the presence of normal and abnormal chromosome copy numbers. FISH studies performed on nuclei of embryos resulting from abnormal fertilization revealed mostly mosaic chromosome complements (Coonen *et al.*, 1994). In human preimplantation embryos, especially those with abnormal morphology, chromosomal mosaicism has been shown to be a normal feature (Bongso *et al.*, 1991; Pellestor *et al.*, 1994a,b). Reported proportions of chromosomal abnormal preimplantation embryos ranged between 30 and 70% in embryos at day 2/3 of development (Munné *et al.*, 1993, 1997; Delhanty *et al.*, 1997; Laverge *et al.*, 1997). The data reported appear to depend on the number of probes applied simultaneously, the type of probes used, embryo morphology, embryo development and the presence of multinucleated blastomeres.

In total, at least 29% of morphologically normal embryos are chromosomally abnormal (Munné *et al.*, 1995) at the cleavage stages. In human blastocysts, chromosomal mosaicism was reported in 29% (Benkhalifa *et al.*, 1993). Others (Bielanska *et al.*, 2000; Ruangvutilert *et al.*, 2000) reported that during preimplantation development, the percentage of embryos showing chromosomal mosaicism increases to almost 100% at the blastocyst stage. The percentage of abnormal cells per embryo was 16%. Studies applying the comparative genome hybridization technique reported an identical result of three out of 12 preimplantation embryos consisting of only normal cells. (Voullaire *et al.*, 2000; Wells and Delhanty, 2000). Even though there is a partial selection against chromosomally abnormal embryos, extended culture to day 5 or 6 cannot be used as a reliable tool to select against clinically relevant chromosome abnormalities such as trisomies (Sandalinas *et al.*, 2001).

Abnormalities in clinical miscarriages and in liveborn children

Chromosomal studies of spontaneous abortion specimens are difficult to carry out. Frequently the material is not suitable for cytogenetic preparation. Occasionally the abortion product has been fixed in preservative for histological examination, making culture and karyotyping impossible. A proportion of the specimens that are properly sent to the cytogenetic laboratory appear to be contaminated after the culture has been started. A few specimens do not show any growth because intrauterine death occurred long before the tissue was expelled and removed. Furthermore, the yield from some cultures suffers because of a lack or complete absence of embryonic tissues or membranes. Finally, the culture and karyotype analysis is expensive. Therefore, cytogenetic studies of abortion material are only successful in a minority of cases.

The first chromosomal analysis of spontaneous miscarriage was carried out in 1961. Triploid cells were reported in two abortions (Penrose and Delhanty, 1961). In 1963, the first findings of two XO abortuses were reported (Carr, 1963). Since then numerous studies have been reported. From these it has become clear that the majority of first trimester abortions are caused by chromosome anomalies.

The most common abnormalities are trisomies, arising *de novo* as a result of meiotic non-disjunction during gametogenesis in parents with a normal karyotype. The incidence increases with maternal age. The most frequent abnormality is trisomy 16, representing ~30% of all trisomies. All trisomies have been observed in spontaneous abortion, except trisomy 1. Occasionally, double trisomies and tetrasomies are observed. Autosomal monosomies are practically unknown in human miscarriages. On the other hand, monosomy X is a frequent finding. The abnormality most frequently resulting from abnormal fertilization is triploidy.

Finally, there is a group showing structural chromosome abnormalities, mainly translocations and inversions. About half the number in this group results from karyotypic abnormalities in the father or, more frequently, the mother. This is the most important chromosomal reason for recurrent miscarriage.

Toward the end of the 1970s a number of laboratories initiated systematic studies on the chromosomal status of spontaneous abortions (Boué *et al.*, 1975; Hassold *et al.*, 1980; Kajii and Omaha, 1980).

After 1985, the year the chorion villus biopsy technique was introduced on a large scale for prenatal diagnosis, this problem could be circumvented. This was achieved by direct analysis of well-preserved villous material, evacuated from the uterine cavity in those cases of retained abortion identified by ultrasound examination before any clinical evidence of pregnancy interruption had been obtained (Eiben *et al*., 1987; Guerneri *et al*., 1987; Ohno *et al*., 1991). Recently it has become possible to study spontaneous abortions using CGH, even after unsuccessful culture (Fritz *et al*., 2001).

Table III shows a comparison between the results obtained after culture, direct analysis after chorion villus biopsy and CGH. In all groups, the majority are abnormal. The total percentage of abnormalities is higher in the more recent series than in the older one. The major difference is due to technical improvements. However, a small part of this difference is most probably caused by the inclusion of a number of induced abortions in the early studies. Furthermore, the chance of finding a chromosome abnormality depends on the previous pregnancy history, the frequency of recurrent abortion in the group, maternal age distribution and the duration of pregnancy. In all series, autosomal trisomy, polyploidy and monosomy X constitute ⩾50% of all karyotyped abortions. However, the percentages of each of the different types of abnormalities vary considerably.

Only three autosomal trisomies are regularly observed at birth: trisomies 13, 18 and 21. They have an estimated prenatal survival of 3, 5 and 20% respectively. The other autosomal trisomies have an estimated survival to term of <1 in 1000 (Hassold and Hunt, 2001). Sex chromosome aneuploidies survive normally with the exception of 45,X which is lethal in ~98% of cases (Hassold and Hunt, 2001). There is no difference between the ages of mothers of liveborn trisomic children and those who spontaneously aborted.

The contribution of chromosomal abnormalities to early loss in humans and other organisms

It has become clear that from the moment of fertilization, there is a continuous reduction or 'selection' of conception products showing chromosome abnormalities. Starting from ~38% at conception and ending at 0.6% at birth (Plachot *et al.*, 1988), selection against aneuploid embryos most probably starts at the morula/blastocyst transition (Evsikov and Verlinsky, 1998). It is assumed that ~10% of zygotes do not proceed to cleavage stage embryos, which means that development is stopped immediately after fertilization. Trisomy 1 is probably 100% lethal at the preimplantation stage, because it has only been observed following IVF.

In contrast to humans, meiotic non-disjunction is a rare occurrence in most other species. In the yeast *Saccharomyces cerevisiae* the frequency is as low as 1 in 10 000, and in *Caenorhabditis elegans* the error rate is in the same order of magnitude. In *Drosophila melanogaster* females, estimates vary between 1 in 1700 and 1 in 6000. In the female mouse the frequency is higher and exceeds 1% (Hassold and Hunt, 2001).

Reports of chromosomal abnormalities in mammalian embryos of non-human species at later stages of development are also relatively rare. In 10-day-old pig embryos (Long and Williams, 1982), in 13- to 14-day-old sheep blastocysts (Murray *et al.*, 1986) and in bovine blastocysts obtained after IVF (Iwasaki *et al.*, 1992), polyploidy and in particular mixoploidy, i.e. mosaicism of diploid and polyploid cells, has been reported. However, it is clear that no other species is comparable with the human with respect to the frequency of chromosome abnormalities resulting from non-disjunction.

Early pregnancy loss and infertility

The questions as to whether or not (recurrent) early pregnancy loss may manifest itself as infertility in couples seeking to conceive, or whether subfertile couples are more prone to early loss should they conceive, remain issues of debate. It is possible that similar factors responsible for preventing conception may be detrimental to fetal survival in early pregnancy (Baird *et al.*, 1986). Moreover, the increased risk of miscarriage observed in subfertile women may be due to a detection bias since these women may take pregnancy tests earlier, and thus recognize more pregnancy losses (Baird *et al.*, 1993). Despite these possibilities for over-representation of early pregnancy loss in the subfertile

Table III. Number (%) chromosomal abnormalities in early spontaneous abortions as detected by different methods

Abnormality	Tissue culture[a]	After CVS[b]	CGH[c]
Monosomy	296 (10.1)	24 (5.1)	4 (7.0)
Autosomal trisomy	823 (28.0)	195 (41.2)	28 (49.1)
Double trisomy	32 (1.1)	15 (3.2)	
Polyploidy	380 (12.9)	33 (7.0)	7 (12.3)
Structural abnormalities	66 (2.2)	19 (4.0)	1 (1.7)
Other abnormalities	22 (0.9)	33 (7.0)	1 (1.7)
Total abnormalities	1624 (55.2)	319 (67.5)	41 (71.8)
Normal	1320 (44.8)	154 (32.5)	16 (28.2)

[a]Data obtained after routine tissue culture of abortion material (Boué *et al*., 1975; Hassold *et al*., 1980; Kajii and Omaha, 1980).
[b]Data of direct studies using material obtained by CVS (Eiben *et al*., 1987; Guerneri *et al*., 1987; Ohno *et al*., 1991).
[c]Data of CGH study of abortion material not successfully cultured (Fritz *et al*., 2001).
CVS = chorion villus sampling; CGH = comparative genomic hybridization.

population, a number of studies have shown no such association (Sharp *et al.*, 1986; Liu *et al.*, 1988b, Wilcox *et al.*, 1988). Indeed, in one study, 95% of women with unrecognized early pregnancy loss reported normal pregnancies within 2 years (Wilcox *et al.*, 1988). However, a more recent study is at odds with these findings (Hakim *et al.*, 1995). Women with evidence of subfertility had a rate of early pregnancy loss of 70% compared with 21% in women without fertility problems (relative risk 2.6, 95% confidence interval 1.8–3.8), and this risk increased with advancing age. The discrepancies in these findings with earlier reports (Wilcox *et al.*, 1988) are probably linked to the group considered to be subfertile, and may reflect the more heterogeneous nature of the population studied by Hakim.

Implications for IVF

Despite improvements in laboratory and clinical practice, ongoing pregnancy rates from IVF remain ~20–25% per started cycle. The role of early pregnancy loss in determining clinical outcomes of IVF remains uncertain as there are few studies of the true rate of early pregnancy loss following IVF. In an early study, a pregnancy rate of 23% was recorded, of which only 4% were detected as a transient rise in hCG followed by menstruation, and thus 'occult' (Liu *et al.*, 1988b). This was a considerably lower incidence than observed following spontaneous conception. In a similar study, the rate of premenstrual pregnancy loss was found to be more prevalent following IVF when compared with spontaneous cycles (Lenton *et al.*, 1988). Data from oocyte donation studies suggest that impaired implantation may underlie the high rate of early pregnancy loss observed. Oocyte donation is associated with higher implantation rates than routine IVF (SART/ASRM, 2002) (Table IV, upper panel). A likely contributory factor to this observation is the more physiological endometrial milieu into which embryos are transferred following oocyte donation. Unlike routine IVF, the endometrium of the recipient is not exposed to supraphysiological levels of gonadotrophins in the follicular phase preceding embryo transfer or extremely high luteal phase steroid levels which may adversely affect the chance of implantation (Pellicer *et al.*, 1996; Simón *et al.*, 1998; Macklon and Fauser, 2000).

In a recent study comparing early pregnancy loss following IVF with that following oocyte donation treatment, early pregnancy loss was the outcome in 47% of pregnancies (29% of cycles) following routine IVF and 37.5% of pregnancies (26.1% of cycles) following oocyte donation (Simón *et al.*, 1999) (Table IV, lower panel). While the figures following oocyte donation were comparable with those observed in spontaneous cycles (Wilcox *et al.*, 1988), the rate of loss was greater following routine IVF. While the endometrial environment in which the embryo must implant is probably influenced by factors such as ovarian stimulation, the rate of early pregnancy loss following IVF may also be influenced by the intrafollicular milieu following ovarian stimulation, the physical stress related to oocyte retrieval and the in-vitro culture environment of the pre- and post-fertilization oocyte and embryo.

A history of early pregnancy loss following IVF treatment has been reported to be a positive predictor of future success from IVF treatment (Templeton *et al.*, 1996; Wright Bates and Ginsburg, 2002). It would appear that the ability to achieve implantation is an important basal measure of reproductive competence. However, the course of events following the onset of implantation is likely to be predominantly determined by the quality of the embryo(s). It is becoming clear that morphology does not correlate completely with the chromosomal status of the embryo (Wells and Delhanty, 2000). There is partial selection against chromosomally abnormal embryos by growing them to the blastocyst stage (Sandalinas *et al.*, 2000). The incidence of aneuploidy in the normal IVF patient population remains unclear, since most studies thus far performed have focused on subgroups of women at higher risk of aneuploidy, such as older women (Gianaroli, 2000) or those with recurrent abortion (Vidal *et al.*, 2000). It is possible that fertility rates greater than the maximal rates reported in spontaneous cycles will not be achieved unless the aneuploid embryo is eliminated from the treatment process. The current low implantation rate and high early pregnancy wastage encourages the transfer of multiple embryos in order to increase the chance of an ongoing pregnancy from IVF. When more than one high quality embryo is transferred to the uterus of a young woman, the additional pregnancies obtained as a result have been shown to be predominantly multiple (Hunault *et al.*, 2002). Preimplantation genetic screening offers a possible route to improving embryo selection, thus reducing the need for multiple embryo transfer. Moreover, if aneuploidy is the principal cause of early pregnancy loss following IVF, the application of preimplantation genetic screening could improve success rates from IVF treatment.

Conclusions

Human pregnancy wastage occurs on such a scale that only ~30% of conceived pregnancies will progress to live birth. While the extent and significance of early pregnancy loss have become clearer, the lack of appropriate markers mean that pre- and peri-implantation events continue to elude clinical study. However data from in-vitro and cytogenetic studies indicate that up to 30% of embryos fail to complete implantation, and that the majority of both pre- and post-implantation pregnancy losses are associated

Table IV. Outcome of IVF compared with IVF/oocyte donation cycles. The upper section summarizes outcomes of IVF initiated in 1998 in the United States (SART/ASRM, 2002). The lower section shows data from Simon *et al.*, 1999 which suggest that the higher rate of viable pregnancies associated with oocyte donation may be due to a lower rate of preclinical pregnancy loss. For further explanation see text

Reference	Routine IVF	Oocyte donation
SART/ASRM, 2002		
Rate of clinical pregnancy loss (%)	17.6	15.3
Deliveries per transfer (%)	31.1	41.2
Simon *et al.*, 1999		
Embryo transfers	145	92
Positive implantations (% of embryo transfers)	88 (60.7)	64 (69.6)
Viable pregnancies (% of positive implantations)	30 (34.1)	30 (46.9)
Preclinical pregnancy losses (% of positive implantations)	42 (47.7)	24 (37.5)
Number of miscarriages (% of positive implantations)	16 (18.1)	10 (15.6)

with chromosomal abnormalities in the gametes and embryo. The high incidence of pregnancy loss secondary to embryo aneuploidy not only limits natural human fecundity, but may also define the maximal success rates achievable with IVF treatment. The recent developments in cytogenetics, which have revealed these limits, may now provide the means to overcome them. The application of preimplantation genetic screening to ensure transfer of euploid embryos may open the way to significant improvements in live birth rates from IVF.

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