—Review—

Chromosomal Abnormalities and Embryonic Development into the Blastocyst Stage in Mammalian Embryos Derived In Vitro

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Introduction

The recent advance of many technologies in assisted reproduction in experimental and domestic animals has been remarkable. Boerjan *et al.* [1] described that Assisted Reproductive Technologies (ART) have been introduced 1) to overcome reproductive failures in the humans, 2) to increase the number of offspring from selected females and 3) to reduce generation intervals in livestock. However, many technical problems remain, for example, the rates of implantation and pregnancy of embryos produced by assisted reproductive technologies are not high in cattle [2] and humans [3].

Assurance of cytogenetic quality of the embryos is essential to get higher conception rates. Especially, cytogenetic normality is a very important issue in respect of generation effects in humans. Many reports concerning chromosomal abnormalities have been published [4–10], and some of them have made mention of the relation between chromosomal abnormalities and the maturity of oocytes and sperm concentration, however the evaluation of cytogenetic safety in ART is insufficient. It has been shown that chromosome analysis of spare human embryos may have a predictive value for their transferred sibling embryos, and detection of chromosomally normal embryos for transfer is integral for improving the success rate in human *In Vitro* Fertilization (IVF) [11].

The present review looks at the incidences of chromosomal abnormalities in embryos of several species of experimental and domestic animals, and the suspected relationship between the development to the blastocyst stage in mammalian embryos and chromosomal abnormalities in the resulting embryos, including human embryos.

Other reviewers introduce the establishment of culture methods and the improvement of the culture condition of mammalian embryos derived from IVF, and the clinical uses of the embryos developed into blastocysts.

Condition of Gametes used in *In Vitro* Fertilization in Some Mammals

Although IVF techniques have been used in many kinds of mammals, there is great variance in the results of IVF among the species. The variance results from numerous differences in IVF techniques: circumstance of experiments, conditions of the gametes used, medium and procedure, etc. For example, in mice, ovulated oocytes and epididymal spermatozoa capacitated after *in vitro* culture are used (Table 1). In cattle and pigs, follicular oocytes, which are obtained from ovaries without hormone treatments in slaughterhouses and incubated for many hours, are available from females of both species. On the other hand, frozen-thawed spermatozoa, which are ejaculated and diluted, are used for insemination in cattle, but frozen-thawed spermatozoa, which are obtained from epididymis to eliminate accessory genital gland fluid, are used in pigs. In humans, oocytes obtained from Graffian follicles in ovaries treated with hormones and fresh or frozen-thawed ejaculated spermatozoa are used.

We have to bear in mind that these differences might

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Table 1. Conditions of gametes used in mammalian IVF

Species	Male gametes	Female gametes
Mouse	Epididymal spermatozoa	Ovulated oocytes
Pig	Frozen-epididymal spermatozoa	Follicular oocytes
Cattle	Ejaculated-frozen spermatozoa	Follicular oocytes
Humans	Ejaculated-fresh or frozen spermatozoa	Stimulated-follicular oocytes

Table 2. Development of (BALB/c × C57BL/6) F_1 and ICR mouse eggs fertilized and cultured *in vitro*

	No. and developmental rates[1)] of embryos at each cleavage stage				
	1-cell	2-cell (24 h)[2)]	4-cell (48 h)[2)]	morula (72 h)[2)]	blastocyst (96 h)[2)]
F_1	103 (85%)	87 (99%)*	86 (94%)*	81 (99%)*	80
ICR	141 (77%)	108 (62%)*	67 (85%)*	57 (56%)*	32

[1)] Percentage of embryos that developed in each interval of cleavage stages. [2)] Examination time in hours after the beginning of insemination. *Significant difference ($P<0.001$) from the corresponding value in ICR (This table is from Yoshizawa *et al.* 1993 [24]).

cause cytogenetic abnormalities in the resultant embryos, since there are some reports detailing the relation of maturation of oocytes and chromosomal abnormalities in IVF embryos in mice [12, 13], cattle [14, 15], and humans [16].

Embryonic Development to the Blastocyst Stage and Deviation of Incidences of Chromosomal Abnormalities at Several Developmental Stages in Mouse Embryos Derived from IVF

The incidence of numerically chromosomal abnormality is discussed. Polyploidy and aneuploidy were low in fertilized golden hamster eggs which were obtained by natural ovulation and mating [17], and similar also to 1-cell stage mouse embryos which were derived from superovulation with 5 IU of PMSG and hCG each and sequential mating with males [18–21], and other doses [22]. Takagi and Sasaki [23] reported that superovulation with 10 IU each of PMSG and hCG induced diploid eggs, resulting in triploid embryos after fertilization. Several groups of researchers [24, 25] demonstrated that 7.5 IU of PMSG caused an increase in triploids by polyspermy in mouse IVF. This data indicates that superovulation with adequate doses of PMSG and hCG does not effect the incidence of triploidy by polygeny. The same reports also noted a similar low tendency for the incidence of aneuploidy. Gras *et al.* [26] determined that the rate of aneuploidy in non-stimulated human oocytes was 20%, which was not significantly different from the 34% of superovulated oocytes. They also concluded that the superovulation protocols used in IVF might not be responsible for the higher rate of aneuploidy in human oocytes.

The developmental rates to the blastocyst stage in mouse embryos derived from IVF are high in the cases in which the medium, experimental conditions and the IVF techniques are optimized. Table 2 shows a typical set of results for mouse IVF in the author's laboratory [27]. The fertilization rate is generally evaluated from eggs extruding the second polar body such as fertilized eggs in mouse IVF. However, extrusion of the second polar body is a result of parthenogenesis as well. Although parthenogenesis is considered to be low in IVF under proper conditions, high incidences of parthenogenesis have been shown in mouse IVF of aged oocytes [13]. In view of these problems, we determined the fertilization rates by evaluating the numbers of pronuclei and chromosomes at the first cleavage metaphase in the chromosome samples. The fertilization rates were about 90% [13, 27, 28], which is typical of mouse IVF. Approximately all of the fertilized eggs divided into 2 cells and almost all of the 2-cell embryos developed to the blastocyst stage, showing a developmental rate to the blastocyst stage of about 92% [27].

An increase of chromosomal abnormalities caused by polyspermy was reported in mice [25, 27–29]. Especially, incidence of triploids depended on the strain used in IVF, i.e. the incidence of triploid embryos caused by dispermy was higher in hybrid females than

in ICR [27], and it was increased by administration of caffeine into the IVF medium [28]. Only a portion of the tripronuclear mouse eggs derived from IVF developed into triploid embryos and the remainder showed normal diploidy, while some tripronuclear human eggs developed into triploids or mosaics, while others changed to normal diploids [30, 31]. It has also been shown that chromosomal aberrations of n/2n and 2n/3n mosaics, the results of polyspermic penetration, were found in mouse blastocysts derived from microinjection of multiple spermatozoa under the zona pellucida [32]. However, no mosaics were observed in not only the embryos derived from *in vivo* fertilization but also those derived from conventional IVF, although high incidences of triploids have been commonly reported in IVF in mice. It was concluded that the injection of multiple spermatozoa under the zona pellucide of mouse oocytes significantly increased the frequency of parthenogenesis and mosaicism but did not affect aneuploidy. Post-ovulatory aging resulted in elevated levels of parthenogenetical haploids [13] and premature centromere separation in mouse oocytes and aneuploidy in zygotes [33].

Embryonic Development to the Blastocyst Stage and Deviation of Incidences of Chromosomal Abnormalities at Several Developmental Stages in Bovine Embryos Derived from IVF

Diagnosis of successful fertilization in bovine IVF is usually carried out on the division of eggs because the observation of pronuclei or the second polar body is very difficult due to the darkness of oocytes which contain granular structures and the remains of cumulus cells around the eggs after the fertilization. Some methods for visualizing pronuclei in living bovine zygotes derived from IVF have been introduced [34, 35], however they are not in general use.

The high incidence, 50.6%, of haploid blastomeres in the early developmental stages of 5 to 10 cells [36] derived from IVF indicated that abnormal bovine zygotes also divided. The presence of the Y chromosome in the haploids showed that many of haploids were derived from sperms although some of them might have been the result of parthenogenesis. It has also been reported that androgenetic bovine embryos at 2- to 32- cell stages were caused by the arrest of female pronucleus formation after the penetration of a Y chromosome-bearing sperm [37].

In bovine oocytes maturated *in vitro* for 26 to 28 h, 89% of all oocytes resumed meiosis and 72% reached the metaphase II (MII) stage. Furthermore, the non-disjunction rate and diploidy rate were 5.8% and 10.7%, respectively [14]. High incidences of diploids in MII bovine oocytes, 11 and 12.4%, were shown in *in vitro* maturation for 24 h [38]. However, in IVF of bovine immature oocytes incubated for only 12 h, a significant increase of chromosomal aberrations, haploids, polyploids and mosaics, was caused by sperm penetration [15].

At advanced developmental stages, incidences of chromosomal anomalies were 15.4% in bovine blastocysts [39] and 18.2% to 22.2% in the inner cell mass of bovine blastocysts derived from IVF, and both the 2n/3n and 2n/4n anomalies were also observed in two-cell embryos [40]. There was no definite change in the incidence of anomalies in bovine embryos derived from *in vitro* fertilization in connection with the development prior to the blastocyst stage, and the anomalies observed were mainly caused by abnormal fertilization [41]. Viuff *et al.* [42] revealed, however, that 72% of blastocysts produced *in vitro* were mixoploid, i.e., were a mixture of normal, diploid, and polyploid cells, using multicolor fluorescence *in situ* hybridization with chromosome-specific probes. Furthermore, the polyploid cells in whole bovine embryos derived from *in vitro* fertilization were located in the trophectoderm rather than the ICM or the embryonic disc, and the level of polyploidy on days 7–8 was significantly higher than on day 12 as assessed by fluorescence *in situ* hybridization [43]. These results might reflect en route developmental arrestation of polyploids.

The incidence of total ploidy and mixoploidy on day 7 bovine embryos, especially mixoploidy, could be better studied by a Feulgen staining procedure measuring the DNA content of the individual nuclei of an embryo, and it was suggested that the presence of hyperdiploid and hypodiploid nuclei might indicate the frequent occurrence of mitotic segregation failures during mitosis in bovine embryos [44].

Incidences of Chromosomal Abnormalities in Human Embryos Derived from IVF

Martin *et al.* [45] compared the frequency and distribution of aneuploidy in 11,615 karyotyped human sperm and 772 karyotyped human oocytes to determine if all chromosomes are equally likely to be involved in aneuploid events or if some chromosomes are particularly susceptible to non-disjunction. In general, hypohaploid chromosome complements were more

frequent than hyperhaploid complements in sperm and oocytes. A conservative estimate of aneuploidy (2 × hyperhaploidy) was approximately 3–4% in the human sperm and 18–19% in human oocytes. The results not only indicate that all chromosomes are susceptible to non-disjunction but that chromosome 21 is particularly prone to aneuploidy in both human sperm and oocytes. Martin *et al.* also demonstrated that sex chromosome aneuploidy was common in human sperm but not in human oocytes. Furthermore, Nicolaidis and Petersen [46] reviewed the origin and mechanisms of non-disjunction in human autosomal trisomies.

It was indicated that superovulation protocols used in IVF did not have an impact on aneuploidy rates in failed fertilized human oocytes [26, 47]. However, the higher incidence of meiotic aberrations and prematurely condensed sperm chromosomes (PCC) in the unfertilized population indicated that some retrieved oocytes exhibited incomplete nuclear and cytoplasmic maturation after superovulation [47]. Although the mechanism of the formation of second meiotic errors in human oocytes remains open to speculation, the situation with respect to first meiotic-division non-disjunction is somewhat clearer in relation to the maternal age affect [48]. No difference was found in the rate of aneuploidy or in the incidence of mosaicism involving chromosomes X, Y, and 18 in two groups of embryos derived from mature and immature oocytes by fluorescence *in situ* hybridization analysis [49].

Although there is no report to our knowledge of chromosomal analysis in morphologically abnormal embryos or developmentally delayed embryos in mice and domestic animals, many results of chromosomal analysis in unfertilized human eggs have been reported [7, 26, 47, 50]. However, it is evident that the incidence of chromosomal anomalies in humans is calculated from abnormal eggs or remaining excessive embryos, which are not transferred to the uterus, while all of the embryos produced by IVF are analyzed in mice and domestic animals. Also it must be considered that many chromosomally anomalous individuals are found among sterile patients treated by ART.

Triploidy occurred in 1% to 3% of recognized conceptions *in vivo*, while *in vitro* fertilization (IVF), which placed large numbers of sperm in proximity to the oocyte, might increase the risk of triploidy [51]. Plachot *et al.* [7] proposed a model of natural selection of normal conceptuses according to the results of a cytogenetic analysis performed on 151 unfertilized oocytes, 22 fertilized eggs at the pronuclear stage, and 108 cleaved embryos obtained in the course of IVF. Human polyploidy conceived *in vitro* conferred an immediate developmental disadvantage; one third of polyploid embryos failed in cleavage, and those which divided demonstrated more asynchronous divisions [52]. Furthermore, fertilization abnormalities (premature chromosome condensation of spermatozoa: PCC, triploidy, haploidy) were analyzed in order to determine their origin [9]. In human embryos fertilized *in vitro*, 19.1% aneuploids, 2.2% mosaic embryos and 1.1% structural anomalies were reported on day 2 and day 3 of culture [53].

Cytogenetic Abnormalities in Human ICSI

High incidences of chromosomal abnormalities derived from polyspermy are well-known in IVF [9, 51], while ICSI can not cause polyspermy, therefore it can block the appearance of polyploidy. Diploidy and aneuploidy of oocytes and aneuploidy originating from patients with chromosomal abnormalities are of concern in ICSI. Occurrence of premature chromosome condensation (PCC), which is caused by sperm injection into the ooplasm at inadequate stages of oocyte maturation, is a difficulty in ICSI. Chromosomes of the G-group [21, 22] were identified with the majority of the anomalies by cytogenetic and fluorescent *in situ* hybridization (FISH) chromosomal studies on *in vitro* fertilized and intracytoplasmic sperm injected 'failed-fertilized' human oocytes [54]. The rate of aneuploidy was similar in both oocyte groups of IVF and ICSI. In the ICSI 'failed-fertilized' oocytes, however, there was a significant increase in incidence of PCC.

There was a statistically significant increase in sex chromosome aberrations (0.83%) in 1082 prenatal diagnoses in pregnancies after ICSI, compared with the aberration percentage (0.19–0.23%) described in the literature with regard to the neonatal population [55]. An increased percentage of de novo chromosomal aberrations might result from the ICSI procedure itself, or it might be linked to a defined subgroup of males with impaired semen samples. Bonduelle *et al.*, however, also examined all children born after ICSI in a prospective study, and the incidence of major malformation, 2.3%, was comparable to figures known for children born after IVF, ICSI or natural conception.

In a comparison of the rates of numerical chromosome abnormalities in biopsied blastomeres of embryos on day 3 of culture, derived from two pronuclear zygotes produced by ICSI and conventional IVF using FISH with specific probes for some chromosomes, there were no statistical differences

between the rates of numerical chromosomal abnormalities detected in the IVF and ICSI embryos analyzed [10].

Human embryos, which were derived from *in vitro* matured oocytes using ICSI and FISH techniques, showed high incidences of multinuclear blastomeres (43.3%) and chromosomal anomalies (78.5%), suggesting abnormal cytokinesis or genetic abnormalities [56].

Causes of Polyploid and Mosaic Embryos

It is clear that the rate of polyspermy, which causes polyploidy, is affected by the number of spermatozoa used for IVF [4, 28, 29], and the maturity of the oocytes [4, 15].

At the first cleavage division in zygotes of almost all mammals including humans [57] and cattle [58], functional centrioles are derived from spermatozoa except in mice [59]. Furthermore, tripolar spindles are detected in dispermic human embryos and tripolar spindles altered the first cleavage division to 3 cells [9, 57], and multiple mitotic spindles were also observed in polyspermic bovine zygotes [58]. If supernumerary sperms penetrate a bovine oocyte, the first cleavage division of the resultant zygote will become abnormal because an extra sperm aster works also as a centriole. Kola *et al.* [30] reported that most tripronuclear human zygotes divided into 3 cells directly and that they also showed severely abnormal chromosomal complements. The tripronuclear zygotes that developed into 2 cells were triploids. Kole *et al.* indicated that a correlation exists between the pattern of the first cleavage division and the subsequent chromosome complements of these zygotes.

In human embryos derived from IVF, incidences of binucleate blastomeres containing two nuclei of equal size were 17% at the 2- to 4-cell stages and 65% at 8- to 16-cell stages, but the majority of these embryos arrested at these stages. The blastomeres arose from a failure of cytokinesis between the second and fourth cleavage divisions [60].

The effect of triploidy on the development of bovine embryos was examined by the injection of 2 sperms into mature oocytes, and the results suggested that triploidy was not the sole cause of arrested development before the 8-cell stage in cleaved embryos, although two-sperm penetration and pronucleus formation strongly influenced oocyte cleavage [61].

Attempts to Decrease Incidences of Chromosomal Abnormalities in Embryos Derived by ART

It was suggested in bovine IVF that chromosomal abnormalities were associated with developmental delay in embryos, i.e. the development rates (as evidenced by cell numbers) were the slowest in haploid and polyploid embryos, intermediate in aneuploid embryos, and the fastest in mixoploid and diploid embryos [62]. Furthermore, Viuff *et al.* [63] reported that in bovine IVF embryos at days 2–5 post-insemination, the development of polyploid embryos was slow and was apparently arrested during the third cell cycle, whereas the mixoploid embryos seemed to continue development. On the other hand, the occurrence of males among advanced embryo stages and of females among less advanced stages could be used as an easy non-invasive method for prenatal sexing of embryos produced *in vitro* [64]. Furthermore, early cleaving embryos (24 and 30 h post insemination) were more likely to have developed to the 8-cell stage or beyond (52.2% vs. 20%), contained more cells (22 vs. 17), and were more likely to be male (3.6:1 vs. 0.93:1) when compared with late cleaving embryos (40–62 hpi) [65].

It was proposed to select human embryos of 'early cleavage' as a simple and effective non-invasive method for selection and evaluation of embryos prior to transfer because there were significantly more clinical pregnancies in the early cleavage group compared with the later cleavage group [66], although the suggestion in bovine embryos described above was not confirmed. The development *in vitro* of human spare embryos was investigated to assess the criteria commonly used to select embryos for transfer: the cleavage rate and gross morphology according to the degree of fragmentation and shape of the blastomeres [67]. It was confirmed by chromosome analysis of human spare embryos that improving detection of chromosomally normal embryos for transfer was important [68]. The rate of chromosomal abnormality for apparently good quality human embryos was 23.5% and for poor of fragmented embryos 83.3% [69]. The majority (77.3%) of the readable metaphase plates for polypronuclear 1-cell human embryos cleaved after showing grossly abnormal chromosome complements, however 19% of the cleaved polypronuclear embryos contained sets of normal diploid chromosomes. Triploid human embryos can develop to cleavage stages indistinguishable from those generated by diploid embryos. Thus, identifying

and discarding embryos with > 2 pronuclei is a vital function of an IVF laboratory [51]. Moreover, it was shown that PN stage morphology (the criteria of the size, number and alignment of pronuclei and nucleoli, cytoplasmic halo effect, the presence of vacuoles and granularity of ooplasm) was related to blastocyst development [70]. However, Gras and Trounson [71] reported a birth resulting from transfer of a blastocyst observed to have one pronucleus at the time of check on fertilization, and concluded that observation of a single pronucleus at the normal time of fertilization assessment would not appear to be an absolute indicator of developmental incompetence.

There have been some attempts to improve implantation rates in human IVF, i.e the effectiveness of blastocyst culture and transfer in a prospective randomized trial in patients having a moderate to good response to gonadotrophin stimulation [3], and the addition of the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) to embryo culture media to improve the yield of implantation-competent blastocysts [72]. However, day 3 aneuploid embryos revealed that a biopsied blastomere developed into expanded and hatching blastocysts showing mosaicism of aneuploidy in ICM cells [73]. Furthermore, a significant appearance of monozygotic twinning in the prolonged culture of embryos was also reported [74]. This is considered to be a warning for a cautious approach to adopting new techniques in IVF.

Furthermore, it has been suggested that phenotypic manifestation of paternal genomic abnormalities might not occur prior to implantation, and blastocyst transfer might not prevent the inheritance of genetic defects involving 'male factor' loci in ICSI [75].

Conclusion

Because of the high incidence of polyspermy under *in vitro* conditions, it seems to be important to routinely examine the oocytes at the pronuclear stage. Reduction of the number of spermatozoa used for IVF and the exact timing of insemination according to the maturity of the oocyte might reduce the occurrence of polyspermic fertilization. To eliminate polyploids and mosaics, the number of pronuclei should be diagnosed accurately. Especially in humans, we can avoid using many chromosomally abnormal embryos derived from polyspermy for transfer because it is comparatively easy to observe the pronucleus.

There have been some attempts to eliminate the embryos having chromosomal abnormalities by morphology, developmental speeds or other criteria. Although incidences of haploidy are naturally reduced with advancing embryonic development, morphologically normal embryos are not guaranteed to be normal cytogenetically because polyploidy, mosaicism and aneuploidy remain at the blastocyst stage. It appears to be true that there is a high incidence of cytogenetic abnormality in morphologically abnormal embryos. Therefore, we have to avoid the transfer of the embryos, which are morphologically abnormal and/or delayed developmentally.

To decrease the incidence of aneuploidy, it is important also to consider the risk of aged patients and eggs aging.

Furthermore, technical perfection in preimplantation genetic diagnosis (PGD) of embryos, for example, diagnosis of oocyte aneuploidy performed by polar body sampling using micromanipulation techniques [76], and cell fusion using bovine oocytes for diagnosing normality of human embryos [77], should be pursued because PGD may be accepted in Japan in the near future.

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