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ARTICLE

Increased incidence of mosaicism detected by FISH in murine blastocyst cultured *in vitro*

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Abstract The majority of in-vitro-derived human preimplantation embryos are chromosomally abnormal but whether the same pattern exists *in vivo* is unknown. This would be impossible to demonstrate in humans. Hence we chose murine embryos to study this difference owing to their ease of manipulation and compared the incidence of mosaicism between *in-vivo*- and in-vitro-cultured embryos. Two groups of embryos were analysed. Group A (*in vitro*) were obtained 48 h following superovulation and cultured *in vitro* until the blastocyst stage. Fluorescent in-situ hybridization (FISH) was performed at different stages that included the cleavage, morula and blastocyst stage. Group B (*in vivo*) were obtained on day 2 or day 5 and FISH was performed immediately without culture. There was an increase in chromosomal mosaicism seen from the cleavage stage up to the blastocyst stage in the in-vitro culture group. Overall chromosomal abnormality from day 3 to day 5 was found to be 30% (28/94) in group A. The incidence of chromosomal abnormalities in blastocysts from group B was significantly lower than group A blastocysts (8% (3/40) and 31% (20/64) respectively; $P < 0.05$). These data show that in-vitro cultured embryos had a significantly higher incidence of mosaicisim in comparison with the in-vivo group.

KEYWORDS: FISH, in-vitro culture, morphology, mosaicism, murine embryos

Introduction

In-vitro fertilisation has been successful in treating human infertility (Steptoe and Edwards, 1978). Approximately 3 million babies have been born as a result of IVF. Britain alone sees 35,000 women undergoing IVF each year. Despite its success, the safety of IVF technology is still an issue. This concern is further exacerbated by the lack of: (i) long-term

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follow-up studies of the children born; and (ii) thorough validation of existing/new methods.

The IVF environment plays a pivotal role in the development and maturation of the embryos (Gardner et al., 2005) but the conditions that the gametes are subjected to during IVF procedures do not resemble the natural human cycle which can thus potentially give rise to various errors. There is evidence that variation in in-vitro conditions can have drastic effects on growth potential of the preimplantation embryo and the development of the embryo prenatally as well as postnatally (Fernandez-Gonzalez et al., 2007; Natale et al., 2001). It has been noted that in-vitro cultured embryos have darker cytoplasm, lower buoyant density (Pollard and Leibo, 1993), flimsy zona pellucida (Duby et al., 1997), swollen blastomeres (Van Soom et al., 1992) and reduced intracellular communication (Boni et al., 1999). Recent studies on animal models show that expression of imprinted genes can be affected by the constitution of preimplantation culture media (Khosla et al., 2001; Lucifero et al., 2004). Further it has been shown that maternal undernutrition during the preimplantation stage could give rise to various defects including abnormalities of the blastocyst (Kwong et al., 2000). In addition, any subtle changes in the culture medium such as oxygen tension may have an impact on gene expression and metabolic activity of the embryo (Bean et al., 2002). Various studies have also raised questions about the long-term effects of in-vitro embryo culture on development, growth, physiology and behaviour in the resulting offspring (Ecker et al., 2004; Fernandez-Gonzalez et al., 2004). Watkins et al. (2007) demonstrated that in mouse, in-vitro culture to blastocyst resulted in reduced cell number and increased systolic blood pressure in offspring when compared with that of embryos that developed *in vivo*.

Karyotyping of preimplantation embryos from various species, including human, have shown chromosome abnormalities (Bond and Chandley, 1983; Clouston et al., 2002; Curlej et al., 2010; Glenister et al., 1987; Iwasaki and Nakahara, 1990; Jamieson et al., 1994; Liu et al., 2008; Maudlin and Fraser, 1977; Papadopoulos et al., 1989). However, karyotyping of preimplantation embryos has some technical difficulties, the most important being that not all the cells can be analysed in the embryo as it is difficult to arrest the cells in metaphase (Harper et al., 1995b). Application of fluorescent in-situ hybridization (FISH) has overcome some of these difficulties. In the human, FISH has been efficiently employed to analyse chromosomes from single isolated blastomeres, polar bodies and trophectoderm cells, as well as from the intact embryo (Delhanty et al., 1997; Harper et al., 1994, 1995a; Munne, 2006; Ruangvutilert et al., 2000; Sandalinas et al., 2001; Veiga et al., 1999). Dupont et al. (2009) using five-colour FISH on 8-cell embryos from rhesus macaques showed that 46% of embryos were chromosomally abnormal. Two studies on bovine embryos used FISH to analyse the chromosome constitution of in-vivo- and in-vitro-derived embryos (Slimane et al., 2000; Viuff et al., 1999). These have shown that embryos derived through in-vitro fertilization are mixoploid (up to 70%) in contrast to embryos derived from in-vivo maturation, fertilization and development (25%) (Slimane et al., 2000; Viuff et al., 2002). Similar results were also reported by Hyttel et al. (2000) using FISH on bovine and porcine embryos, who reported a three fold increase in the incidence of polyploidy cells in embryos produced by IVF.

One major drawback of investigation of human aneuploidy has been the paucity of a reliable aneuploidy model system (Dupont et al., 2009; Hassold et al., 2007). Due to ethical, legal and experimental limitations, all the present knowledge about human preimplantation embryos has been from untransferred embryos retrieved from IVF and preimplantation genetic diagnosis (PGD). As a result, it is difficult to achieve a true estimation of mosaicism and aneuploidy that exists *in utero*. Hence, there is a need to establish a mammalian model for the study of chromosomal abnormalities. Non-human primates and other non-primate models which exhibit aneuploidy are expensive and difficult to maintain (Dupont et al., 2009). Mouse preimplantation embryos have been an excellent source to study and gain insight into preimplantation development mainly due to the ease of obtaining a large number of oocytes and embryos. Additionally the mouse genome is closely related to humans and the majority of the genes have corresponding functional counterparts. The frequency of aneuploidy in non-primate mammals like mouse is estimated to be not more than 1–2% (Bond and Chandley, 1983).

The present study employed FISH for chromosomes 2 and 11. The aims of the study were set as follows: (i) to establish if murine embryos (MF1 strain) could be used to study aneuploidy; and (ii) to compare the incidence of mosaicism between the in-vivo and in-vitro cultured embryos.

Materials and methods

Control slides

Control slides were made using murine (MF1 strain) liver and kidney tissue which had been cryopreserved at −80°C. Tissue was dabbed without prior treatment. These slides were flooded with fixative (3:1 methanol/acetic acid) for 10 s and air dried. Slides were then flooded with 70% acetic acid for 10 s, air dried and dehydrated through ethanol (70%, 90% and 100%) for 5 min each. All the slides were examined under light microscopy to ensure the presence of adequate interphase nuclei. Following FISH on the control tissue, the efficiency of probes was calculated as the percentage of nuclei in interphase which showed expected signals out of the number of nuclei analysed.

Embryo procurement and culture

The mice were approximately 5–7 weeks old. The standard superovulation protocol was as follows; MF1 females (Charles River, UK) were injected with 7 IU of pregnant mare serum gonadotrophin (Intervet) and 5 IU of human chorionic gonadotrophin (HCG; Intervet) 48 h later. Following this, females were immediately mated with MF1 males.

Group A in-vivo- and in-vitro-cultured embryos

Approximately 48 h post HCG injection (day 2), the female mice were killed and 1–2-cell stage embryos were flushed from the oviducts into HEPES-buffered potassium simplex optimization medium (KSOM) (Lawitts and Biggers, 1993;

Summers et al., 2000). These were subsequently cultured in non-sequential KSOM containing amino acids (Millipore, Billerica, MA, USA) at 37°C in 5% CO_2 and atmospheric O_2. All embryo manipulations were carried out under oil. Embryos were cultured until they reached different preimplantation stages of 6–8-cell stage (72 h post HCG; day 3), morula (96 h post HCG; day 4) and blastocyst (120 h post HCG; day 5) then spread on poly-L-lysine slides.

Group B and in-vitro culture embryos

Approximately 48 h post HCG (day 2) and 120 h post HCG (day 5), the female mice were killed and embryos were flushed from the oviduct and uterus. These embryos were washed in HEPES-buffered KSOM media and spread on poly-L-lysine slides. The analysis of group A and B embryos was not conducted 'blind' as the spreading and FISH were performed by the same person.

Morphology assessment

Prior to spreading, each embryo was graded. Cleavage embryos were graded as described by Hardarson et al. (2001). Grade I embryos had evenly sized blastomeres with or without fragments of <20% and grade II embryos had unevenly sized blastomeres with or without fragments of <20%. Embryos of both these grades were considered as good-quality embryos. Grade III embryos had 20–50% fragmentation and were average-quality embryos whereas grade IV embryos had >50% fragmentation and were considered poor-quality embryos. Although no grading system was applied, fragmentation was also scored for at the morula stage according to the above criteria.

For blastocysts, a modification of Dokras et al. (1993) was applied. Blastocysts were graded as follows. Grade A or good-quality blastocysts were expanded with a distinct trophectoderm and eccentrically located inner cell mass (ICM). Grade B or average-quality blastocysts had poor expansion and/or less defined trophectoderm and ICM cells but did not show any signs of degenerative cells. Grade C or poor-quality blastocysts exhibited poor morphology with degenerative foci in the ICM and/or trophectoderm.

Embryo spreading

For the in-vivo embryos, spreading of embryos was carried out on the same day as embryo procurement to reduce the errors due to suboptimal culture conditions and avoid the need of cryopreservation. Whole embryos were spread according to the procedure described in Harper et al. (1994) and Coonen et al. (1994). Embryos were spread on poly-L-lysine slides to minimize loss of nuclei. Following the spreading procedure, the location of the nuclei was mapped using an England finder (Graticules, UK).

Fluorescent in-situ hybridization

The FISH method was performed as described by Coonen et al. (1994) and Harper et al. (1994) with some modifications.

Briefly, the slides were incubated in 0.01 mol/l HCl (BDH, UK) containing 10 mg/ml pepsin (Sigma, Germany) for 20 min at 37°C to remove remnants of cytoplasm and make the nuclei accessible for hybridization to probes. After subsequent rinses in bi-distilled water and phosphate-buffered saline (PBS), slides were fixed using 1% paraformaldehyde (Sigma, UK) in PBS for 10 min at 4°C. This was followed by rinses in PBS and bi-distilled water and dehydration through an ascending ethanol series (70%, 90% and 100%) for 3 min each.

Initially, co-denaturation and separate denaturation were both employed to test the efficiency of the probes but separate denaturation of slides and probe was more effective. For seperate denaturation, 100 μl denaturing mixture (70 μl of de-ionized formamide (Sigma) and 30 μl of 2× saline sodium citrate buffer (SSC)) was applied and the slides were denaturated at 75°C for 5 min. Denaturation was arrested by incubating the slides for 5 min in 70% ice cold ethanol and dehydrating through 90% and 100% ethanol for 3 min in each. The probe was denatured separately at 75°C for 5 min, incubated at 37°C for 30 min and applied onto air-dried slides. The slides were incubated overnight at 37°C.

Commercially supplied, ready-to-use dual-colour LSI FISH probes for chromosome 2 (2qH3) and chromosome 11 (11qE2) (Kreatech, Netherlands) was added directly after denaturation to the slides. The probes were direct-labelled with Platinum Bright 550 and Platinum Bright 495. The probe for chromosome 2 (2qH3) bound to the AurKa gene while the probe for chromosome 11 (11qE2) bound to the gene TK near to the telomeres. The initial testing revealed that the suppliers' protocol was not optimal for the tissue being examined and hence there were modifications made to post washes and probe quantity. The amount of probe (2–4 μl) used was optimized and altered according to number of cells in the embryo. For cleavage-stage embryos, 2–3 μl of probe was used and for blastocyst stage 4 μl of probe was used.

Slides were washed in 60% formamide/2× SSC for 5 min and 5 min in 2× SSC both at 42°C, followed by 2 × 5 min washes in 4× SSC/0.05% Tween 20 at root temperature.

After post hybridization washes slides were dehydrated and mounted in 10 μl Vectarshield (Vectar Laboratories, CA, USA) containing 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma, UK).

Visualization and scoring

Slides were visualized under an epifluorescence microscope (Olympus BX40) coupled with a Photometrics cooled charged coupled device camera that employs Smartcapture II software (Digital Scientific, UK) for the purpose of capturing imaging. The analysis were carried out using single band pass filters and separate images of DAPI, fluorescein isothiocyanate and rhodamine fluorescence were captured and subsequently amalgamated using Smartcapture II software.

An attempt was made to score all the embryos on which FISH was performed. The criteria suggested by Hopman et al. (1988) were used which state that signals should not overlap and should not be covered with cytoplasm. In interphase, a chromosome having two chromatids will appear as doublets (split signals), the signals will be equal in size and smaller than a normal signal. The split signal is separated by a width that is less than a normal signal so that it is classified as one and not two chromosomes. Stretched, diffused

signals or minor hybridization spots of low intensity were not counted. When nuclei had unreadable signals or absence of any signals they were scored as failure of FISH. For control slides, 200 interphase nuclei per slide were scored for each of the probes that were employed and the number of signals was recorded. This was used to calculate probe efficiency before the probe was used on embryos.

Embryo classification

After scoring all the nuclei and removing the background FISH error rate (see Results), embryo classification was carried out as described by Delhanty et al. (1997): (i) nuclei with uniformly diploid signals were classified as normal; (ii) embryos were classified as normal/diploid when more than 90% of the nuclei showed uniformly diploid signals for both the chromosomes; (iii) embryos were classified uniformly abnormal due to meiotic error if the abnormality was seen in at least 90% of the cells; (iv) if the embryos showed two different cell lines, they were classified as mosaic; (v) if the majority of nuclei were diploid with some being haploid, aneuploid or tetraploid, it would be described as diploid mosaic, while if the majority were mosaic with a few being normal it was described as abnormal mosaic; and (vi) if all the cells were affected, the embryo was classified as fully chaotic, alternatively it may be partially diploid or aneuploid and partially chaotic.

Mechanism of mosaicism

Classification of chromosomal mosaicism and associated mechanisms were carried out as described in Daphnis et al. (2005). The three mechanisms that could give rise to diploid mosaic embryos with aneuploid cells were (i) when the embryo had cells with monosomies, the mechanism was classified as chromosome loss; (ii) when the embryo had cells with trisomies, the mechanism was termed as chromosome gain; and (iii) when the embryo had monosomies and trisomies of the same chromosome(s) in different cells, it was classified as mitotic non-disjunction.

Data analysis

For statistical analysis, the chi-squared test and Fisher's exact test were used. For optimum results, the selection of the test was based on the assumption that the expected number of abnormal embryos for each group was at least five. If this assumption was satisfied then the chi-squared test was conducted. Otherwise, for comparisons with a lower sample size, the Fisher's exact test was conducted as this test is best suited for small sample sizes. A cut off for significance of 0.05 was used for important comparisons while a cut off for significance of 0.0001 was used elsewhere to avoid spuriously significant results due to multiple testing.

The number of embryos analysed was dependent on their availability during the research phase.

The grouping of data was carried out to understand the abnormality rate at different time intervals of in-vitro culture or stages of embryo development. In group A, comparison of day-3 versus day-5 abnormality was to identify the abnormality difference in the cleavage stage to the final blastocyst stage. Day-3 + 4 versus day-5 grouping was to compare the abnormality rate before and after the blastocyst stage. Lastly, day 3 versus day 4 + 5 was to investigate the embryo abnormality rate at the end of the cleavage stage as compared with the end of the blastocyst stage.

Similarly in group B, day-2 versus day-5 comparison was to identify if there is any increase in chromosomal abnormality from the cleavage to blastocyst stage when embryos are grown *in vivo*.

Group A and group B blastocysts were compared to identify whether in-vitro culture gives rise to chromosomal abnormality.

Results

A total of 227 embryos were analysed — 119 in group A and 108 in group B — and 184 yielded results. The efficiency of the control slides was 94.5%. Of the control nuclei that gave signals, 94.5% (189/200 nuclei) showed the expected diploid number of chromosomes. Therefore 5.5% was taken as the background FISH error rate. Any embryos showing up to 5.5% abnormal cells were classified as normal.

Chromosomal abnormalities and mosaicism

The results of the FISH analysis are summarized in **Table 1**. In group A, 94 embryos were successfully analysed on either day 3, 4 or 5. In group B, 90 embryos were successfully analysed on day 2 or day 5. In group A, the level of normal embryos decreased from day 3 (83%) to day 5 (69%) with an overall diploid rate of 70%. In group B, 96% of in-vivo embryos were chromosomally normal.

The most common chromosome abnormality seen was mosaicism. In group A, there was an increase in chromosomal mosaicism seen from the cleavage stage up to the blastocyst stage (**Table 1**). In this group the overall chromosomal mosaicism was 30% (28/94). At the morula and blastocyst stage, the incidence of mosaicism increased strikingly and was almost double that of the cleavage stage. For day 3 versus day 5 (17% versus 31%), the increase in mosaicism was not significant (Fisher's exact test). When chromosomal mosaicism at day 3 + 4 versus day 5 was compared, the increase was non-significant (26% versus 31%; chi-squared test) (**Table 1**).

Group B consisted of in-vivo-obtained day-2 embryos and blastocysts. The incidence of chromosome abnormality was only 2% in day-2 embryos and 8% in blastocysts. The increase in abnormality from day 2 to day 5 was insignificant in this group (**Table 1**). The proportion of morphologically good blastocysts (grade A) was higher in group B versus group A (87.5% versus 68.8%, $P < 0.05$, Fisher's exact test) while the proportion of poor embryos (grade C) was lower in this group as compared with group A blastocysts (15.6% versus 2.5%, $P < 0.05$, Fisher's exact test) (**Table 2**).

Analysis of mosaicism in group A (blastocysts) versus group B (blastocysts) demonstrated a significant difference (31% versus 8%, $P < 0.05$, chi-squared test) suggesting that the in-vitro culture resulted in an increased level of mosaicism. Moreover there was no significant difference noted in the incidence of diploid/aneuploid mosaics between group

Table 1 Classification of chromosomal abnormalities of group A and group B embryos.

Group	*Day (Days in culture)*	*Stage*	*Total number of embryos*	*Total Cells analysed (Mean/ embryo ± S.D)*	*Embryo classification*						
					Diploid (%)	*Diploid/ Aneuploid mosaic*	*Diploid/ polyploid mosaic*	*Diploid/ chaotic mosaic*	*Aneuploid*	*Abnormal embryos (%)*	***p-value***
Group A (in vitro)	Day 3 (24 hrs)	Cleavage	**12**	89 (7.4 ± 0.9)	10 (83%)	2	—	—	—	2 (17%)	Day 3 vs 5[*]
	Day 4 (48 hrs)	Morula	**18**	416 (23 ± 6.3)	12 (67%)	4	—	2	—	6 (33%)	Day 3+4 vs. 5 [*]
	Day 5 (72 hrs)	Blastocyst	**64**	4189 (65 ± 14.8)	44 (69%)	8	3	9	—	20 (31%)	Day 3 vs. 4+5 [*]
		Total	**94**		66 (70%)	14	3	11	—	28 (30%)	
Group B (in vivo)	Day 2	Cleavage stage	**50**	141 (2.8 ± 0.98)	49 (98%)	—	—	—	1	1 (2%)	Day 2 vs. 5 [*]
	Day 5	Blastocyst	**40**	2867 (74 ± 16.7)	37 (93%)	3	—	—		3 (8%)	Day 5 (Group A vs. group B[∞])
		Total	**90**		86 (96%)					4 (4%)	

[*] $p < 0.05$, increase in abnormality was not statistically significant.
[∞] $p < 0.05$, increase in abnormality was statistically significant.

Table 2 Morphology of group A and group B embryos.

Group	Stage	Total number f embryos	Morphology of all embryos			Total number of abnormal embryos	Morphology of abnormal embryo		
			Good	Average	Poor		Good	Average	Poor
Group A (in vitro)	Day 3	12	2	5	5	2	1	—	1
	Day 4	18	4	12	2	6	1	5	—
	Day 5	64	44 (68.8)[a]	10	10 (15.6)[b]	20	13	3	4
	Total	94	50	27	17	28	15	8	5
Group B (in vivo)	Day 2	50	41	7	2	1	—	1	—
	Day 5	40	35 (87.5)[a]	4	1 (2.5)[b]	3	3	—	—
	Total	90	76	11	3	4	3	1	—

Values in parentheses are %.
[a]day-5 group A vs. day-5 group B, $p < 0.05$, proportional of good quality embryos was statistically significant.
[b]day-5 group A vs. day-5 group B, $p < 0.05$, proportional of poor quality embryos was statistically significant.

A (blastocysts) versus group B (blastocysts) (Fisher's exact test). Group B (blastocysts) did not show any embryos that had diploid/polyploidy or diploid/chaotic configuration which was pronounced in group A blastocysts (**Table 1**). Fifty-six percent (18/32) of abnormal/mosaic embryos in both groups at different stages were morphologically good quality (**Table 2**).

When the mechanism of chromosomal mosaicism was examined, group A showed a total of 44 post-zygotic errors and 15.9% (7/44) were due to mitotic non-disjunction, 29.5% (13/44) were due to chromosome gain and 54.5% (24/44) were due to chromosome loss. The majority of mitotic non-disjunction (12/13) events were seen in blastocysts in this group. In group B, six post-zygotic errors were seen in the abnormal embryos, of which 66.7% (4/6) were due to chromosome loss and 33.3% (2/6) were chromosome gain and there were no mitotic non-disjunction events seen in this group. **Figure 1** shows results from control slides and embryonic nuclei.

Discussion

The first aim of this study was to establish if murine embryos could be used to study aneuploidy. This was achieved using dual-colour FISH with good efficiency (94.5%). In the human, up to 12 chromosomes can be analysed at one time using FISH (Munné et al., 2010) and all of the chromosomes using metaphase (Wells and Delhanty, 2000) or array comparative genomic hybridization (Harper and Harton, in press; Hellani et al., 2008; Hu et al., 2004). Dupont et al. (2009) used five-colour FISH to examine rhesus monkeys but the current study was unable to find more than two FISH probes that could be used in mouse interphase nuclei. An important point is that even using two probes, an estimation of chromosome abnormalities can be achieved. In the human, the overall level of chromosome abnormalities in embryos has not changed significantly from the early studies where just two chromosomes were analysed (Delhanty et al., 1993) to the recent studies where all of the chromosomes have been analysed (Fragouli et al., 2010).

Approximately 50% of human embryos are chromosomally mosaic, mainly due to post-zygotic errors (Wells and Delhanty, 2000). It has been suggested that aneuploid cells could be detrimental to embryo development but high levels of mosaicism and chaotic embryos can still be detected at the blastocyst stage in human embryos (Magli et al., 2000; Sandalinas et al., 2001). It is uncertain as to whether this phenomenon is unique to in-vitro-created embryos or it is common to all human embryos irrespective of their environment.

The second aim of this study was to compare the incidence of mosaicism between in-vivo- and in-vitro-cultured embryos. In both groups the embryos were obtained by mating of the mice to exclude any stress/mosaicism caused to embryos by IVF and thus depicting a true picture of chromosomal abnormalities resulting solely from culture and development of embryos *in vitro*. It must be stressed here that this study does not examine the equivalent situation as occurs in humans since the mouse embryos were not fertilized *in vitro*.

In group A (*in vitro*), from 94 embryos that gave results, 28 were found to be abnormal. There was a high incidence of chromosomal abnormality found in day-4 and day-5 embryos in this group and these were mainly due to post-zygotic errors resulting in diploid mosaicism. This study supports the fact that, like human preimplantation embryos, high levels of mosaicism are also a common finding in in-vitro-cultured murine preimplantation embryos.

In group A, 30% of all the classified embryos were diploid mosaic. Among the diploid mosaic, diploid/aneuploid, diploid/polyploid and diploid/chaotic were seen (**Table 1**). These are similar patterns as seen in human embryos (15–75% are diploid mosaic) (Delhanty et al., 1997; Harper et al., 1995a; Munne, 2006; Ruangvutilert et al., 2000; Sandalinas et al., 2001; Veiga et al., 1999). There were no embryos that were completely aneuploid, mosaic abnormal or had a completely chaotic chromosomal complement. In human embryos, variable levels of specific abnormalities have been reported, including up to 47% being completely abnormal (Sandalinas et al., 2001), 25% being mosaic abnormal (Voullaire et al., 2000) and 26% embryos being chaotic

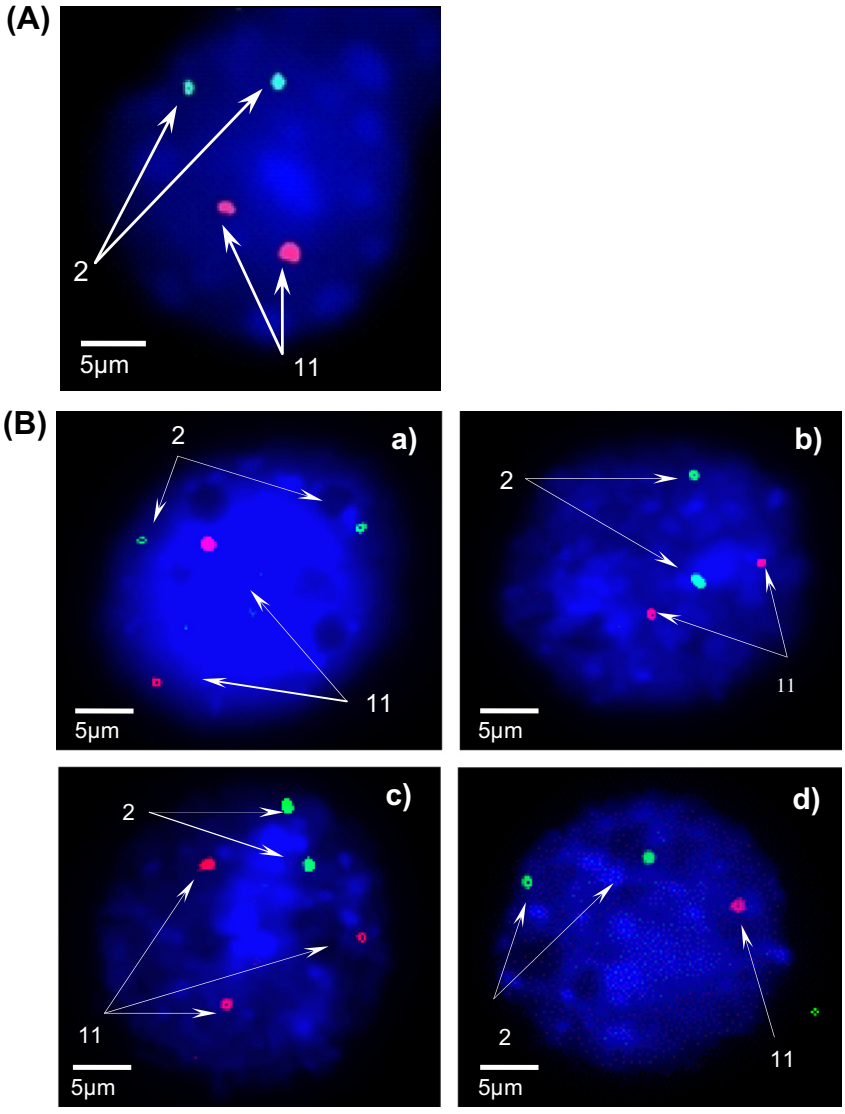

Figure 1 (**A**) Murine kidney nuclei, 100×. The expected/normal signals are two green signals representing the presence of two copies of chromosome 2 and two red signals representing the presence of two copies of chromosome 11. (**B**) Mouse embryonic nuclei, 100×. Blue colour indicates DAPI-stained nucleus. The expected/normal signals are two green signals representing the presence of two copies of chromosome 2 and two red signals representing the presence of two copies of chromosome 11. Other combinations would be considered as abnormal: (a, b) normal/diploid murine embryonic nuclei for chromosomes 2 and 11 obtained after fluorescent in-situ hybridization; (c) trisomy 11; and (d) monosomy 11 obtained after fluorescent in-situ hybridization.

(Delhanty et al., 1997; Ruangvutilert et al., 2000). Comparison between group A and group B, diploid/aneuploid mosaic revealed no statistical difference.

The predominant mechanism leading to diploid/aneuploid mosaicism was chromosomal loss in both groups, implying chromosome loss is an event that leads to mosaicism even in in-vivo embryos. This finding in murine embryos was in accordance with Daphnis et al. (2005, 2008) and Coonen et al. (2004) which reported chromosomal loss as the major mechanism leading to mosaicism in IVF derived human embryos. Chromosome loss is assumed to result from anaphase lag, although in the current study there was no evidence of micronucleation in the group with chromosome loss. This makes the mouse a potential model to study the origin and causes of this mechanism.

An interesting finding is that mitotic non-disjunction was not seen in group B, suggesting that mitotic non-disjunction probably gives rise to mosaicism in in-vitro embryos and is less likely to arise in in-vivo embryos and could be extrapolated to the culture systems used *in vitro*.

Several studies support that mosaic embryos can form good-quality blastocysts (Bielanska et al., 2005; Fragouli et al., 2008; Sandalinas et al., 2001). This is consistent with the present study where a considerable proportion of blastocysts (31% in group A and 8% in group B) were mosaic. Evsikov and Verlinsky (1998) and Ruangvutilert et al. (2000) observed that there was a decrease in the number of abnormal cells in mosaic embryos at the blastocyst stage compared with cleavage-stage embryos. Most abnormal cells at the blastocyst stage are tetraploid (Ruangvutilert et al., 2000).

Fragouli et al. (2008) demonstrated that many aneuploidies involving the larger chromosomes can develop to the blastocyst stage and could survive until implantation. In the same study, abnormality of chromosome 2 was shown to be one of the most common errors. Further it is suggested that since FISH-based studies use probes only for smaller autosomes and sex chromosomes, the larger chromosomes have been ignored. It would be interesting to study the fate of such embryos as it is believed monosomies of such a large chromosome would have a detrimental effect on development and would be arrested subsequently (Sandalinas et al., 2001). Lightfoot et al. (2006) studying the mouse, revealed that although mosaic embryos are able to implant and initiate gastrulation (day 6.5), they quickly degrade by day 8 of development, explaining reasons for the absence of mosaic karyotypes at fetal stages (day 12) even when there is a high presence at blastocyst stages as supported by this study. Currently it is not known if the data for chromosomes 2 and 11 are representative of the other chromosomes in terms of their relative susceptibility in the mouse.

In group A, no differences were seen in morphology of embryos at different days suggesting that extended in-vitro culture does not necessarily affect morphology of embryos. About half (15/28) of the abnormal embryos in group A and the majority (3/4) of abnormal embryos in group B exhibited 'good' morphological characteristics. In addition, 65% (13/20) of mosaic blastocysts in group A exhibited good morphology. Bielanska et al. (2005) reported that in a study of human IVF embryos evaluating chromosome abnormalities in relation to blastocyst morphology, they found that 65% of mosaic blastocysts had good morphology. Thus, the current study adds to the existing data that states that morphological examination by itself is incapable of selecting against chromosome abnormalities and perhaps is a poor predictor of embryo viability (Munne, 2006). This has important implications for assisted reproduction treatment as presently the major approach to choose embryos for transfer is morphology, which does not correlate with the 'chromosomal normality' of the embryo selected.

It has been suggested that embryos produced by different stimulation protocols (Ma et al., 1997; Takagi and Sasaki, 1976) and under different culture conditions have very diverse mosaicism and aneuploidy rates (Munne et al., 1997). There have been studies that also suggest that hormonal stimulation in some mouse strains could give rise to aneuploidy (Hansmann and El-Nahass, 1979; Maudlin and Fraser, 1977). In both groups the same superovulation protocol was used, hence we can conclude that the difference in abnormality *in vivo* and *in vitro* is not attributable to the superovulation protocol. The effect of superovulation *per se* on overall incidence of abnormality seen in this study cannot be fully excluded. It is presumed that, since the only difference in both the groups was culture environment post day 2, in-vitro culture condition(s) are responsible for the rise in mosaicism. It has been suggested that chromosomal imbalances can originate at fertilization as meiotic or mitotic errors which could be the consequence of chromosomal misalignment due to abnormal spindle and malfunctioning of cell cycle check points (Harrison et al., 2000; LeMaire-Adkins et al., 1997). These abnormalities related to the mitotic spindle could also arise due to suboptimal conditions during in-vitro culture (A'arabi et al., 1997; Pickering et al., 1990). Since fertilization was *in vivo*, the former possibility is excluded and it is assumed the latter is the cause of the abrupt increase in mosaicism in group A blastocysts.

Possible parameters of *in-vitro* culture conditions that might have contributed to the increased incidence of chromosomal anomalies during later stages of development could be non-sequential culture conditions, concentration of different components in the culture medium, concentration of O_2 and susceptibility of the strain to chromosomal abnormality. Use of 20% oxygen tension (atmospheric) instead of 5% is found to be related to increased chromosomal abnormalities in gametes and embryos (Dumoulin et al., 1995; McKiernan and Bavister 1990; Pabon et al., 1989). Supporting this, Bean et al. (2002) reported an increase in mosaic sex chromosome aneuploidy in embryos cultured at 5% CO_2 and atmospheric oxygen. This experiment utilized 5% CO_2 and atmospheric O_2 (20%) for culturing murine embryos. Perhaps increased aneuploidy could be attributed to this parameter and hence this should be further evaluated.

There are a number of studies reporting high chromosome abnormalities observed using FISH in preimplantation embryos from macaques (Dupont et al., 2009), bovine (Viuff et al., 1999, 2002; Slimane et al., 2000) and porcine (Hyttel et al., 2000). There is insufficient data on the incidence of chromosomal abnormality of mouse embryos. The majority of previous studies on mouse have used karyotyping (**Table 3**) to analyse the chromosomal constitution at cleavage stages and did not consider morula- and blastocyst-stage embryos (Bond and Chandley, 1983; Glenister et al., 1987; Liu et al., 2008; Maudlin and Fraser, 1977).

The overall efficiency of the protocol was high. It was seen that Tween/HCl maintained the quality of nuclei for FISH analysis which was in agreement with other studies (Coonen et al., 1994; Harper et al., 1994; Xu et al., 1998) that have reported high efficiency using this technique.

While performing the FISH analysis, there were a few technical obstacles that included failure of hybridization, overlapping signals, diffused and split signals yielding incon-

Table 3 Studies analysing chromosomal constitution of mouse preimplantation embryos.

Study	*Aneuploidy rate (%)*	*Stage of developments*	*Method of analysis/comments*
Maudlin and Fraser (1977)	1.43—2.10	First-cleavage division (2-cell stage)	Karyotyping (Tarkowski, 1966)
Bond and Chandley (1983)	1—2	1-Cell stage to 4-cell stage	Karyotyping
Glenister et al. (1987)	3.3—3.4	First-cleavage division (2-cell stage)	Karyotyping (Tarkowski, 1966)
Bean et al. (2002)	3	2-Cell stage to 16-cell stage	FISH/chromosome X and Y
Liu et al. (2008)	4—1.3	2-Cell stage to blastocyst	Karyotyping (Roberts et al., 2005)

clusive results (Munne et al., 1998; Ruangvutilert et al., 2000). A few embryos did not yield results due to high background and some of the embryos failed to produce signals in any of the nuclei in spite of good staining with DAPI, perhaps due to failure of hybridization. In some cases, incomplete metaphases were obtained which made the scoring procedure difficult. The study was designed such that in-vitro culture could enable us to identify the stage that exhibited maximum mosaicism and compare it to its in-vivo counterpart. Day 4 and day 5 of the in-vitro development was identified as exhibiting maximum mosaicism. In addition, the blastocyst stage was chosen to be studied as in the human IVF setting this is the stage when the majority of embryos are transferred back to the patients.

If in-vitro culture does give rise to mosaicism, is it possible to identify those factor(s) giving rise to this phenomenon? Perhaps each of the in-vitro culture parameters should be studied independently in relation to mosaicism. Further questions arising include whether these mosaic embryos, when of good morphology (as shown in the current study), implant? If yes, do they go to full term in which case the embryo has a mechanism to deal with mosaicism but is it affecting the health of IVF babies? Or, by choosing these good-quality mosaics, are implantation rates being lowered if they do not develop beyond blastocyst stage? Is there a better way to select embryos that are not chromosomally compromised rather than morphology? This study noted an increase in chromosomal abnormality, although not significant, due to extended culture from day 3 to day 5. Have clinicians moved on to blastocyst culture prematurely, thus subjecting the embryos to an environment less conducive for their chromosomes?

Animal studies, including this study, demonstrate that embryo development *in vivo* is significantly better than *in vitro*. Using a mouse model, further studies can be conducted examining the effects of variation in culture conditions on embryo development and aneuploidy.

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