Published in final edited form as:

Theriogenology. 2013 January 15; 79(2): 234–241. doi:10.1016/j.theriogenology.2012.08.007.

Biopsy of embryos produced by in vitro fertilization affects development in C57BL/6 mouse strain

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Abstract

Preimplantation genetic diagnosis (PGD) is considered highly successful in respect to its accuracy in detecting genetic anomalies but the effects of embryo biopsy on embryonic/fetal growth and development are less known, particularly in conjunction with in vitro fertilization (IVF). Here, we compared biopsied (B) and non-biopsied (NB) mouse embryos for their developmental competence. Embryos C57BL/6 (B6) and B6D2F2 (F2) generated by IVF were subjected to single blastomere biopsy at the 4-cell stage, and were either cultured for 120 h and subjected to differential inner cell mass (ICM) and trophoblast (T) staining, or were transferred into the uterine tubes of surrogate mothers after 72 h of culture, to examine their pre- and post-implantation development, respectively. Non-biopsied embryos from the same IVF cohorts served as controls. Embryo biopsy negatively affected preimplantation development to blastocyst in C57BL/6 (69 vs 79%, $P<0.01$) but not in B6D2F1 mice (89 vs 91%, $P=NS$). Although B6 embryos had lower total cell number than F2 (B6: 47 and 61 vs. F1: 53 and 70; B and NB, respectively, $P<0.05$) there were no differences between B and NB blastocysts in %ICM (B6: 19.8 vs 19.8; F2: 20.9 vs 20.4, $P=NS$) and ICM:T ratio (B6: 4.7 vs 4.7; F2: 4.4 vs. 4.7) in both mouse strains. Post-implantation development to live fetuses of B embryos as compared to NB counterparts was impaired in C57BL/6 (6 vs 18%, $P<0.001$) but not in B6D2F1 mice (26 vs 35%, $P=NS$). We conclude that blastomere biopsy impairs embryonic/fetal development in mice known to be sensitive to in vitro culture and manipulations. Such mice model infertile couples with poor quality gametes seeking help in assisted reproduction technologies (ART) clinics.

Keywords

embryo manipulation; assisted reproductive technology; development; in vitro fertilization; embryo biopsy; C57BL/6

1. Introduction

Preimplantation genetic diagnosis (PGD) is a method of genetic screening of embryos conceived with assisted reproduction technologies (ART) [1,2]. Most often it involves biopsy of one or more blastomeres from an early cleavage stage embryo and subsequent

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Conflict of Interests

The authors declare no competing interests.

Authors Contributions

AS performed all experiments, participated in data analysis and interpretation and paper drafting. MAW conceived, designed and coordinated the study, acquired funding, participated in data analysis and interpretation, and wrote the paper. Both authors read and approved the final manuscript.

molecular analysis of these cells by polymerase chain reaction, fluorescent in situ hybridization, and other methods. The genetic status of the embryo is inferred from these analyses and only unaffected embryos are transplanted to mother. Preimplantation genetic diagnosis has been successfully applied to test for numerical and structural chromosomal abnormalities that can result in recurrent miscarriage, to identify embryo sex to prevent transmission of X-linked diseases, and for the detection of specific serious monogenic disorders [3].

Preimplantation genetic diagnosis is typically performed on human embryos derived from either in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), and cultured until Day 3 post fertilization [4,5]. The classic study that followed shortly after the introduction of the PGD concept has shown that biopsy of the 8-cell stage human embryo does not impair preimplantation development, and observed reduction of embryo metabolism and cell number is proportional to the loss of embryo cellular mass [2]. However, a more recent study provided evidence that blastomere biopsy results in delayed compaction and altered hatching [6] and a similar impairments were reported in mice [7–9].

Here, we examined the effects of embryo biopsy on pre- and post-implantation development, using the 4-cell stage embryos generated by IVF and two mouse strains, vigorous hybrid B6D2F1 and sensitive inbred C57BL/6. This is the first mouse study assessing embryo biopsy effects in conjunction with IVF.

2. Materials and methods

2.1. Animals

Mice were obtained at six weeks of age; B6D2F1 (C57BL/6 x DBA/2) and C57BL/6 from the National Cancer Institute (Raleigh, NC, USA) and CD-1 from Charles River Laboratories (Wilmington, MA, USA). Mice B6D2F1 and C57BL/6 were used as sperm and oocytes donors and CD-1 mice were used as recipients and vasectomized males for embryo transfer. Mice were fed *ad libitum* with a standard diet and maintained in a temperature and light-controlled room (22 °C, 14 h light/10 h dark), in accordance with the guidelines of the Laboratory Animal Services at the University of Hawaii and guidelines presented in National Research Council's (NCR) "Guide for Care and Use of Laboratory Animals" [10]. The protocol for animal handling and treatment procedures was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii.

2.2. Reagents and media

Equine Chorionic Gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) were purchased from Calbiochem (Spring Valley, CA, USA). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise stated. Media T6 [11] and HTF [12] were used for IVF, HEPES-buffered CZB (HEPES-CZB [13,14]) and HEPES-KSOM [15] for gamete handling and embryo transfer, CZB [13,14] and mKSOM^{AA} [15] for embryo culture, and Ca²⁺- and Mg²⁺- free CZB and Ca²⁺- and Mg²⁺- free mKSOM^{AA} for incubation preceding blastomere biopsy. Media CZB, mKSOM^{AA}, T6, and HTF were maintained in an atmosphere of 5% CO₂ in air, and HEPES-CZB, HEPES-KSOM were maintained in air. In vitro fertilization and embryo culture were done exclusively in T6/CZB media for B6D2F1, and in either T6/CZB or HTF/mKSOM^{AA} for C57BL/6 mice.

2.3. In vitro fertilization (IVF)

Sperm capacitation and IVF were performed as reported by us before [16]. Briefly, the oocytes were collected from females induced to superovulate with injections of 5 iu eCG and 5 iu hCG given 48 h apart. Epididymal sperm were collected by release from caudae

epididymides directly into T6 or HTF medium, and were capacitated for 1.5 h at 37 °C in a humidified atmosphere of 5% CO₂. The gametes were co-incubated for 4 h. After gamete co-incubation, the oocytes were washed with HEPES-CZB or HEPES-KSOM, followed by at least one wash with CZB or mKSOM^{AA} medium. Only morphologically normal oocytes were selected for culture.

2.4. Embryo culture and biopsy

Fertilized oocytes (the oocytes with two well developed pronuclei and extruded 2nd polar body) were cultured in 50 µL drops of CZB or mKSOM^{AA} medium pre-equilibrated overnight with humidified 5% CO₂ in air. After ~48 h of culture, 4-cell embryos were transferred into Ca²⁺- and Mg²⁺- free culture medium for 10–20 min to disrupt cell adhesion, and were then transferred to microdrops of Ca²⁺- and Mg²⁺ free manipulation medium on the micromanipulation dish (Fisher, Los Angeles, CA, USA). Blastomere biopsy was performed as reported by us before [17] using Eppendorf Micromanipulators (Micromanipulator TransferMan, Eppendorf, Hamburg, Germany) with a Piezo-electric actuator (PMM Controller, model PMAS-CT150, PrimeTech, Tsukuba, Japan). The zona pellucida was penetrated with a micropipette (20 µm ID; Sutter instrument, Novato, CA, USA) and one blastomere was aspirated from each manipulated embryo. Control (non-biopsied) embryos were from the same IVF cohorts and were cultured under the same conditions as their biopsied counterparts but were not micromanipulated.

2.5. Assessment of preimplantation development

To assess preimplantation development and blastocyst quality biopsied and non-biopsied (control) embryos were cultured for up to 120 h. After 96 h the proportions of developed blastocysts were calculated, and after 120 h some of the blastocysts were processed for chemical staining. Total, trophoblast (T) and inner cell mass (ICM) cell number of biopsied and non-biopsied were stained with propidium iodide and bisbenzimidazole (Hoechst 33258) as described previously [18]. The zona-intact blastocysts were first incubated in 500 µL of BSA-free HEPES-CZB with 1% Triton X-100 and 100 µg/mL propidium iodide for up to 10 s or until trophoblast visibly changed color to red and shrank slightly under a dissecting microscope. The blastocysts were then immediately transferred into 500 µL of fixative solution (100% ethanol, Pharmco-Aaper, Brookfield, CT, USA/Shelbyville, KY, USA, 25 µg/mL Hoechst 33258) and stored at 4 °C overnight. Blastocysts were mounted on glass slides (Fisher Scientific, Pittsburgh, PA) in a drop of glycerol, and cell counting was performed from images obtained from an inverted microscope (IX-71; Olympus, Tokyo, Japan) fitted with an ultraviolet lamp and excitation filters (410 nm and 560 nm for blue and red fluorescence, respectively).

2.6. Assessment of post-implantation development

To assess post-implantation development, biopsied embryos were cultured for subsequent 24 h until they developed to morula/early blastocyst stage (72 h of culture), and were then taken for embryo transfer. The biopsied and non-biopsied embryos were transferred into the uterine tubes (seven to nine per uterine tube) of separate CD-1 females mated during the previous night with vasectomized CD-1 males. Caesarian section was performed on Day 18 and numbers of live fetuses and implantation sites were scored.

2.7. Experimental design

Embryos B6D2F2 and C57BL/6 generated by IVF were subjected to single blastomere biopsy at the 4-cell stage, and were either cultured for up to 120 h or were transferred into the uterine tubes of recipients after 72 h of culture to examine their pre- and post-implantation development, respectively (Fig. 1). Total, ICM and T cell numbers of biopsied

and non-biopsied blastocysts after 120 h of culture were also assessed using propidium iodide and bisbenzimidazole staining. Non-biopsied embryos from the same IVF cohorts served as controls.

2.8. Statistical analyses

Blastocyst cell number and pregnancy rate were analyzed with Student's unpaired t-test. Chi-square and Fisher exact probability tests were used for analyzing all other data. The differences were considered to be statistically significant at $P < 0.05$. The analyses were done by Statcel2 software (OMS, Saitama, Japan; <http://www.oms-publ.co.jp/exl01/howto01.html>).

3. Results

3.1. Effect of blastomere biopsy on preimplantation embryo growth and development

Embryos B6D2F2 and C57BL/6 generated by IVF and subjected to single blastomere biopsy at the 4-cell stage were cultured for up to 120 h to examine their preimplantation development. Fewer embryos developed to blastocyst stage after biopsy of C57BL/6 embryos, as compared to non-biopsied counterparts (69 vs 79%, $P < 0.01$) while preimplantation development of biopsied B6D2F2 embryos remained not affected (89 vs 91%, $P = \text{NS}$, Table 1).

When single blastomeres are removed from the 4-cell stage embryos, it is expected that the blastocysts developed from such embryos will have 75% of total cells compared to blastocysts originating from not manipulated embryos. In our study biopsied embryos had total cell number constituting 74.8% and 76.6% of non-biopsied, for B6D2F2 and C57BL/6 strains, respectively. Differential cell staining assessing the ICM and TE contribution to each blastocyst (Table 2, Fig. 1) revealed that on both genetic backgrounds there were no differences between biopsied and non-biopsied embryos in respect to % ICM and T:ICM ratio. Blastocysts C57BL/6 had significantly lower cell number (total, ICM and T) than B6D2F2 embryos, in both biopsied and non-biopsied and groups (Table 2).

3.2. Effect of blastomere biopsy on postimplantation embryo development

Embryos B6D2F2 and C57BL/6 generated by IVF and subjected to single blastomere biopsy at the 4-cell stage were transferred into the uterine tubes of surrogate mothers after 72 h of culture to examine their post-implantation development. In these experiments we initially used T6/CZB, which is our routine media combination for IVF and culture [16,19,20], and which we have used to assess preimplantation development in this study (Table 1&2). This resulted in satisfactory fetal rates with B6D2F1 mice. However, the first three experiments with C57BL/6 mice yielded very low fetal rates, both with and without biopsy (3% and 9%, respectively, Table 3). Therefore, we continued experiments with C57BL/6 mice using HTF/mKSOM^{AA} media combination, which we knew worked well with inbred strain embryos [21,22]. As expected, post-implantation development improved with HTF/mKSOM^{AA} (Table 3).

Overall, fewer recipients became pregnant after transfer of biopsied than non-biopsied embryos in C57BL/6 (61%, 11/18 vs 93%, 14/15; $P = 0.04$) but not in B6D2F1 mice (100%, 11/11 vs 86%, 12/14, $P = \text{NS}$). Post-implantation development of biopsied C57BL/6 embryos was impaired as compared to non-biopsied controls, and yielded less fetuses (6 vs. 18%, $P < 0.001$) and implants (36 vs 65%, $P < 0.001$; Table 3, pooled C57BL/6 data). This developmental impairment was observed under both culture conditions except that the difference in fetal rates between biopsied and non-biopsied groups cultured in T6/CZB did not reach significance (Table 3). In B6D2F2, there was no effect of biopsy on post-

implantation development and similar proportions of fetuses (26 vs 35%) and implants (58 vs 63%) were obtained after transfer of biopsied and non-biopsied embryos, respectively (Table 3).

4. Discussion

In this study, we examined the developmental potential of mouse embryos generated by in vitro fertilization and subjected to single blastomere removal at the 4-cell stage. We demonstrated that biopsy negatively affected embryonic and fetal development in C57BL/6 inbred, but not in B6D2F2 hybrid mice.

Our work is novel because, as far as we know, all but one ([23], article in Japanese) preceding mouse studies focusing on blastomere removal effects were done using embryos conceived in vivo, and flushed from the uterine tubes. In humans, only in vitro generated embryos are subjected to PGD. Thus, our work models the situation in human ART/PGD more closely. Moreover, there are only two previous papers describing the use of inbred C57BL/6 mice to study the effects of embryo biopsy and the biopsy in these studies was performed at the blastocyst stage [24,25].

Biopsy of preimplantation embryos can be performed at any stage from the 2-cell to the blastocyst. In humans, biopsy is routinely performed in the morning on Day 3 after fertilization, at which time embryos are at the 6–8-cell stage [5]. In this study we performed biopsy at the 4-cell stage to avoid interfering with embryo compaction, which in mice begins at the 8-cell stage [26]. Performing biopsy during or after compaction is likely to have negative effects on developing embryos because it would interfere with cell-cell adhesion, gap and tight junctions, and cytoplasmic polarization, which all first appear at the 8-cell stage in the mouse. In humans compaction does not occur before the 16- to 32- stage [27]. The biopsy at the 4-cell stage in mice represents well biopsy at the 6–8-cell stage in humans also because of the timing in embryonic genome activation, which is initiated at the 2-cell in mice [28] and the 4–8-cell stage in humans [29].

When we assessed the efficiency of pre- and post- implantation development in hybrid B6D2F2 mice, we did not observe any impairment resulting from a single blastomere biopsy. Similar proportion of morula/early blastocysts after embryo culture, and fetuses and implants after embryo transfer, were obtained in biopsied and non-biopsied groups. The majority of previous studies on the 4-cell stage biopsy performed on naturally conceived embryos from hybrid mice also reported no biopsy effects [30–33].

Contrary to B6D2F2, we have found that C57BL/6 embryos were significantly affected by biopsy yielding less blastocysts and fetuses. C57BL/6 mice are known to have gametes and embryos extremely sensitive to manipulation in vitro. Sperm from this strain survive cryopreservation poorly yielding low fertilization rates [34]. Although different approaches have been successfully used to enhance the chance of successful fertilization with frozen-thawed C57BL/6 sperm, such as partial zona dissection [35], sperm selection prior to IVF [22] or inclusion of various chemicals in fertilization medium [36–38], C57BL/6 sperm cryopreservation as a mean to archive of genetic resources remains very inefficient. The oocytes from C57BL/6 females are also susceptible to manipulations and were shown to be fragile during ICSI due to decreased oolemma healing capacity [39] and respond poorly to pronuclear microinjection [40]. Finally, C57BL/6 embryos are sensitive to in vitro culture and often require modified culture conditions [41]. We have previously shown that C57BL/6 embryos develop in vitro with lower efficiency than embryos of other strains and yield blastocysts with a lower cell number [21,22]. The comparison of non-biopsied C57BL/6 and B6D2F2 embryos in this study agrees well with these previous findings. Because of sensitivity to in vitro culture, we usually transfer embryos at the 2-cell stage to generate live

offspring after assisted fertilization [19,42]. The decrease in post-implantation development of non-biopsied C57BL/6 embryos transferred at morula/early blastocyst stage in this study could be due to prolonged embryo culture.

Impairment in pre- and post-implantation development of biopsied C57BL/6 embryos could be due to slower cell cycle in this strain. It is possible that these embryos have been caught by the biopsy at an unfavorable time when they were more sensitive, i.e. during S-phase, and required more time to recover. Constant ICM:T ratio also points to a general delay of the cell cycle at an early cleavage stage. Interestingly, DBA2/J mice used to produce B6D2F1 and then F2 carry a fast *Ped* (preimplantation embryonic development) allele while B6 mice may carry a fast or slow *Ped* allele depending on the B6 substrain [43].

It has been shown that mouse embryos developed from blastomeres separated at the 4-cell stage could implant but only sporadically formed small egg cylinders, most probably due to the reduced number of cells at cavitation [44]. When these 'quarter' embryos were aggregated with carrier tetraploid blastomeres that tend to colonize in the trophoblast, they survived beyond the early egg cylinder stage and resulted in birth of normal and fertile adults [45]. Therefore, a certain number of all cells, and a certain number of trophoblast cells, may be required for full term development. In our work, the removal of 25% of the cell mass in C57BL/6 embryos subjected to biopsy might have reduced the cell number dangerously close to a threshold for successful development in this strain. However, it cannot be excluded that manipulation per se played a role. Literature review revealed that three types of controls were previously used in studies on the effects of blastomere biopsy: (1) 'intact controls', represented by embryos cultured without interruptions in parallel to biopsied embryos; (2) 'CMF-exposed', represented by embryos which, similarly as biopsied embryos are transiently exposed to Ca^{2+} - and Mg^{2+} - free medium but are not manipulated; and (3) 'sham-biopsied' controls represented by embryos manipulated in exactly the same way as biopsied embryos but without the blastomere removal. In our study we used as controls the 'CMF-exposed' embryos. Thus, it cannot be differentiated whether the observed impaired development of biopsied C57BL/6 embryos was due to manipulations (biopsy procedure) or removal of the blastomere. In our recent study with B6D2F1 mice the comparison of biopsied and non-biopsied embryos yielded similar results to those obtained from the comparison of biopsied and sham-biopsied counterparts, both showing that embryo biopsy deregulates placental steroid metabolism [8]. However, the delay in preimplantation development and decrease in fetal body weight, which could either be a remnant of previous developmental delay or a result of impaired development *in utero*, perhaps as a consequence of insufficient steroid action, were observed in biopsied group only when compared to non-biopsied controls, supporting that the zona pellucida manipulation contributes to developmental delay and fetal weight decrease of biopsied embryos [8].

Fertilization in vitro and embryo culture decrease the number of trophoblast cells and leads to misexpression of a number of genes, most of which are involved in pathways regulating proliferation, apoptosis and development [46]. In our study embryos were produced by IVF and cultured for 72 h before embryo transfer. Although we did not carry out a comparison with naturally conceived and in vivo developing preimplantation embryos, we can suspect that in vitro manipulations could have placed embryos, both biopsied and non-biopsied, at a significant developmental disadvantage resulting from the alteration of gene expression and/or the reduction of trophoblast cell number. Here, again, C57BL/6 strain, would suffer more due to its extreme sensitivity. In support of this, C57BL/6 embryos developed better in mKSOM^{AA} medium, which was previously reported to cause less severe changes in global patterns of gene expression in blastocysts as compared to other, simpler, culture media [47]. Altered gene expression during early development may have profound effects for offspring well being. Indeed, it has been shown that mouse embryo biopsy results in changes in

postnatal growth and physiological and behavioral activities, such as memory decline, increased weights at adulthood, and altered expression of proteins associated with neurodegenerative diseases [48].

To summarize, we have shown that removal of one blastomere from the 4-cell stage mouse embryos generated by IVF negatively impacts sensitive inbred C57BL/6 embryos and fetuses but does not impair development in hybrid mice. Hybrid B6D2F1 mice are strong and vigorous, and have excellent reproductive performance. As such they hardly model infertile couples, with poor quality of gametes, which are the usual target ART and PGD. Inbred C57BL/6 mice, although fertile in vivo, have lower reproductive abilities than hybrid mice, and as discussed earlier have gametes and embryos sensitive to in vitro manipulations. As such, C57BL/6 mice seem to be a better model for human ART clinic patients. Assisted reproduction is successful in overcoming most human infertility cases, and it can also be used successfully in C57BL/6 mice. The success in obtaining viable offspring, however, does not preclude the possibility that certain epigenetic changes occurred during manipulations.

Our study reporting on developmental impairment in C57BL/6 mice subjected to ART adds to an ongoing debate about the negative effect of ART overall, and accumulating evidence pointing to several problems that may be associated with these techniques. It emphasizes the need for future investigations of ART and PGD effects, which could focus on characterization of molecular causes leading developmental impairment of C57BL/6 mice, particularly focusing in changes in developmental gene and protein expression and epigenetic modifications.

Considering an ongoing debate about the negative effect of ART overall, and accumulating evidence pointing to several problems that may be associated with these techniques, it seems prudent to continue to investigations of ART and PGD effects. One such effort could focus on characterization of molecular causes leading developmental impairment in C57BL/6 mice subjected to ART, particularly focusing in changes in developmental gene and protein expression.

Acknowledgments

This study was supported by NIH RR024206 (Project 2) grants to MAW.

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Fig. 1. Experimental Design

Embryos B6D2F2 and C57BL/6 generated by in vitro fertilization (IVF) were subjected to single blastomere biopsy at the 4-cell stage, and were either transferred into the uterine tubes of recipient CD-1 females after 72 h of culture or were cultured for up to 120 h and subjected to differential inner cell mass (ICM) and trophoblast (T) staining. Intense pink color in ICM/T staining represents the chromatin in nuclei of lysed T cells that had been stained both red (propidium iodide) and blue (Hoechst) while blue cells are ICM cells, which were not permeabilized. Scale = 50 μ m.

Table 1

The effect of blastomere biopsy on preimplantation embryo development.

Mice	No. oocytes inseminated (no. exp.)	No. 2-cell embryos obtained (%) ¹	No. 4-cell embryos developed (%) ²	No. 4-cell embryos biopsied	No. 4-cell embryos non-biopsied	No. embryos survived (%) ³	No. blastocyst developed (%) ^{3,4}
B6D2F1	554 (8)	360 (65)	355 (99)	184	-	174 (95)	155 (89) ^a
				-	171	-	156 (91) ^a
C57BL/6	628 (9)	380 (61)	343 (90)	171	-	170 (99)	117 (69) ^b
				-	172	-	136 (79)

Percentage calculated from

¹ oocytes inseminated;

² 2-cell embryos;

³ 4-cell embryos;

⁴ 4-cell embryos survived biopsy. IVF and embryo culture was done in T6/CZB media.

Statistical significance:

^a $P < 0.05$ vs. respective group in C57BL/6;

^b $P < 0.01$ vs. non-biopsied within strain.

Table 2

The effect of blastomere biopsy on blastocyst quality.

Mice	Biopsy	No. blastocysts	Cell number (mean $\pm$ SEM)			% ICM of total cells	T:ICM ratio
			Total	ICM	TE		
B6D2F1	+	49	53 $\pm$ 2 ^a	11 $\pm$ 1 ^a	42 $\pm$ 1 ^a	20.9	4.4
	-	50	70 $\pm$ 2 ^a	14 $\pm$ 1 ^a	56 $\pm$ 2 ^a	20.4	4.7
C57BL/6	+	43	47 $\pm$ 2	9 $\pm$ 1	37 $\pm$ 1	19.8	4.7
	-	43	61 $\pm$ 2	12 $\pm$ 1	49 $\pm$ 2	19.8	4.7

Statistical significance:

^ap<0.05 vs. respective group in C57BL/6. IVF and embryo culture were done in T6/CZB media.

ICM = inner cell mass; T = trophoblast

Table 3

The effect of blastomere biopsy on postimplantation embryo development.

Mice	Culture conditions	No. oocytes inseminated (no. exp.)	No. 2-cell embryos (%) ¹	No. 4-cell embryos (%) ²	No. 4-cell embryos biopsied	No. 4-cell embryos non-biopsied	No. biopsied embryos survived (%) ³	No. M/EB (%) ^{3,4}	No. M/EB transferred	No. fetuses at Day 18 (%) ⁵	No. implants at Day 18 (%) ⁵
B6D2F1	T6/CZB	573 (10)	524 (91)	523 (100)	212	-	186 (88)	184 (99)	169	44 (26)	98 (58)
					-	311	-	311 (100)	196	69 (35)	124 (63)
C57BL/6	T6/CZB	221 (3)	203 (92)	200 (99)	103	-	101 (98)	99 (96)	97	3 (3) ^C	26 (27) ^{C,a}
					-	97	-	96 (99)	42	5 (12) ^C	23 (55)
C57BL/6	HTF/mKSOM ^{AA}	624 (5)	534 (86)	454 (85)	255	-	248 (97)	228 (89)	184	15 (8) ^{C,b}	75 (41) ^{B,c}
					-	199	-	188 (94)	150	31 (21) ^B	109 (73)
C57BL/6	Both culture conditions	845 (8)	737 (87)	654 (88)	358		349 (98)	327 (94)	281	18 (6) ^{C,c}	101 (36) ^{C,c}
						296		284 (96)	192	36 (19) ^C	132 (69)

Percentage calculated from

¹ oocytes inseminated;

² 2-cell embryos;

³ 4-cell embryos;

⁴ 4-cell embryos survived biopsy;

⁵ M/EB transferred. M/EB = morula/early blastocyst.

Statistical significance:

^a P<0.05,

^b P<0.01,

^c P<0.001 vs. non-biopsied within strain;

^B P<0.01,

^C P<0.001 vs. respective group in B6D2F1.