Proceedings of the

4-7 july **2000** – Valencia Spain

These proceedings were printed as a special issue of WORLD RABBIT SCIENCE, the journal of the World Rabbit Science Association, Volume 8, supplement 1

ISSN reference of this on line version is 2308-1910
(ISSN for all the on-line versions of the proceedings of the successive World Rabbit Congresses)

Escribá M.J., Silvestre M.A., Saeed A.M., García-Ximénez F.

EFFECT OF 8-CELL HAPLOID EMBRYOS DONOR NUCLEI STORED AT 4ºC FOR 24H TRANSFERRED UPON PRESUMED ANDROGENETIC RABBIT HEMIZYGOTES

Volume A, pages 369-373

EFFECT OF 8-CELL HAPLOID EMBRYOS DONOR NUCLEI STORED AT 4ºC FOR 24H TRANSFERRED UPON PRESUMED ANDROGENETIC RABBIT HEMI-ZYGOTES

Escribá M.J.*, Silvestre M.A., Saeed A.M., García-Ximénez F.

Laboratorio de Reproducción, Departamento de Ciencia Animal,
Universidad Politécnica de Valencia, Spain

*Corresponding author: Laboratorio de Reproducción. Departamento Ciencia Animal. Universidad Politécnica de Valencia. Camino de Vera 14, 46071 Valencia. Spain. Telephone: +(9)63879433; Fax: +(9)63877439; e-mail: mescriba@dca.upv.es

ABSTRACT

Two types of 8-cell parthenogenetic rabbit haploid blastomeres were used as donors in haploid nuclear transplants upon androgenetic haploid zygotes: fresh or stored at 4ºC for 24h. Transferring these nuclei to zygotes which contained only the paternal nucleus would reconstitute the normal heteroparental ploidy. Retrospective comparison of developmental data in vitro reconstructed embryos from reached significant differences after transferring both types of blastomeres, obtaining a 21% blastocysts only after transferring stored blastomeres.

INTRODUCTION

In the mouse, the combination of haploid nuclei from 2- to 16-cell parthenogenetic haploid embryos with recipient eggs that contain a resident pronucleus of the opposite parental type would reconstruct the normal heteroparental ploidy (Surani et al.,1987; Howlet et al., 1987). To test this possibility in rabbits would open new application approaches since each ova, as its parthenogenetic haploid development proceeds every nucleus would be combined to the parental counterpart and in turn, being the female genotype represented into more offspring.

Storage at low temperatures above freezing stops the cell cycle (Heyman et al., 1990) which may be relevant as a possible method for synchronizing cellular cycles between

donor nucleus and recipient cell (Smith and Wilmut, 1989 ; Kato et al., 1998). At this respect, the aim of the present study is to test whether storage of haploid nuclear donor at +4ºC for 24 hours affects the efficiency of the above referred nuclear transfer technique, assessed by the in vitro developmental ability of reconstituted zygotes.

MATERIALS AND METHODS

Nuclear donor collection and handling

Young MII oocytes (14-15 h after hCG injection) were activated by electrical pulses (Escribá and García-Ximénez, 1999). Briefly, oocytes were placed between two stainless-steel round wire electrodes approximately 0.5-mm apart and overlaid with 0.18M mannitol containing 100µM CaCl2 and 100µM MgCl2. A maximum of eight square wave electrical DC pulses of 0.6 kV/cm for 60 µsec each were applied at regular intervals over 270 min (38 min apart). Before each pulse, positive PB2 extruded oocytes did no receive more pulses and were cultured in Ham's F10 medium plus 20% (v/v) fetal calf serum (s-Ham's) under a 7% CO_2 atmosphere at 39ºC. After activating treatment, only the haploid eggs were selected and cultured for 24h.

Two types of 8-cell haploid embryos blastomeres were used as donor nuclei in separate assays: In fresh group (assay A), approximately at 23 to 25h after the first electroactivating pulse, parthenogenetic 8-cell haploid embryos nuclei were isolated by a chemical dissaggregation procedure (Yang et al., 1990; García-Ximenez and Escribá, 1996), whereas in stored group (assay B), 8-cell embryos were collected from culture every two hours, commencing at 23 to 29h after first electroactivation stimulus. Their development was arrested at this stage by storage at +4º C in Dulbecco's phosphate-buffered saline plus 20% (v/v) 72h-ovulated-doe serum (s-PBS) (García-Ximénez and Escribá, 1996). Following 24h storage, arrested haploid 8-cell embryos were rewarmed at room temperature during 15 min, then dissagreggated and used as donors.

Source of zygotes

Mature does were mated twice with fertile males at the time of 25 I.U. of

endovenous hCG injection. Around 12-12.5h after mating, early zygotes were recovered with s-PBS immediately after euthanasia of the donor female. Then, zygotes were incubated in hyaluronidase solution (1mg/ml; Type IV-S) to remove the corona cells.

Reconstruction of embryos and in vitro culture

Early zygotes and haploid donor blastomeres were incubated in modified Ham's F-10 (m-Ham's) containing 25mM Hepes, 0.15% (w/v) BSA and cytochalasin B (7.5mg/mL) for 30min at room temperature before manipulation.

Presumed removal of the maternal genetic material was performed by aspiration of one-fourth of the zygotic cytoplasm close to the position of the PB2 by a single enucleation-transfer pipette (32-35μm outer and 30-32μm inner diameter) with a sharpened and 45º-beveled tip (McGrath and Solter, 1983). Then, each single donor blastomere was transferred to the perivitelline space of previously manipulated zygote.

Obtained couplets were washed in s-Ham's and equilibrated in electrofusion medium (0.3M mannitol + 100μM CaCl2 + 100μM MgCl2; 320mOsmkg^{-1}) for 3min and aligned manually. Fusion was induced with three square DC pulses of 2.2 kVcm-1 for 60μsec duration each, 30min apart. After each fusing pulse, couplets were cultured in s-Ham's. Before each next pulse, fusion was assessed and positive couplets received no more pulses.

In assay A, couplets were submitted to fusion pulses immediately after micromanipulation, whereas in assay B, couplets were rinsed and cultured in s-Ham's for 1h before the application of fusing pulses.

The reconstructed zygotes were cultured in s-Ham's, without discerning the nuclear condition of the resulted embryos, primary due to the darkness of their cytoplasm at the fusion time. In vitro reconstructed-embryo developmental ability was checked every 24 h for 5 d.

Retrospective comparison between assays A and B was realized by a Chi-square test. When a single degree of freedom was involved, the Yate's correction for continuity was carried out.

The efficiency of female pronucleus removal was evaluated by mounting some manipulated, but non-nuclear transplanted zygotic recipients. Their nuclear configuration was examined after manipulated eggs had been fixing in ethanol : acetic acid (3 : 1 mixture)

for 24h.

RESULTS

Of 174 fertilized eggs on which presumed removal of female pronucleus was performed, 130 (75%) survived the enucleation procedure (data non-shown in tables).

After fixing, only one pronucleus was present in 44% (34/78) of surviving manipulated zygotes. Due to the technical procedure, it may be the male pronucleus. The rest of analyzed and manipulated eggs were either non-enucleated (i.e. two pronuclei were present: 35%: 27/78) or wholly enucleated (no pronuclei were present: 22%: 17/78) (data non-shown on tables).

In both assays similar fusion rates were observed (81% and 72%, $p>0.05$; table 1). Comparison of developmental data from reconstructed embryos indicates that differences in the cleavage rates (89% and 97%, respectively) did not reach statistical level of significance, nor in the morulae rates (30% and 45%, respectively). On the contrary, whereas fresh blastomeres did not support in vitro development to the blastocyst stage in any case, 21% (7/33) of blastocysts were obtained after transferring 24h-preserved haploid blastomeres.

Table 1. Efficiency of technical process on reconstructing eggs by nuclear transfer with different types of blastomere donors.

Type of * Blastomere	Nº of manipulated zygotes/ Nº (%) of survival couplets		Fused (%)‡	Nº of cleaved eggs	Morula stage (%)§	Blastocyst stage (%)§
Fresh	99/57	(58)	46 (81)	41 (89)	14 (30)	0 (0) [a]
Stored	75/46	(61)	33 (72)	32 (97)	15 (45)	7 (21) [b]
Total	174/103	(59)	79 (77)	73 (92)	29 (37)	7 (9)

*Fresh blastomere: 8-cell haploid nuclei. Stored blastomeres: 8-cell haploid nuclei stored at +4º C for 24 h.

‡ As percentage of cells survived manipulation.

§ As percentage of fused couplets.

[a, b] Values in the same column with different superscripts differ ($P<0.05$).

DISCUSSION

Parthenogenetic haploid embryo 8-cell nuclei derived from electroactivating rabbit ova and in vitro culture would be used as maternal genome donors.

In the present work, the developmental ability of reconstructed zygotes was only evaluated in vitro, and some blastocysts (21%) were obtained. However, since no relationship between the nuclear condition (diploid or triploid) and developmental potential has been found either in mice or rabbit (McGrath and Solter, 1986; Surani et al., 1987; Yang et al., 1990; Ozil, 1990); the obtained blastocysts could come not only from correctly reconstructed diploid zygotes, but also from reconstructed triploid eggs.

Storage at low temperatures above freezing induces depression of cellular metabolism, eventually stopping the cell cycle (Anderson and Foote, 1975; Heyman et al., 1990). In vivo-produced 8-cell rabbit embryos were stored at +4ºC for 24h and no effect on their subsequent in vitro development was shown (García-Ximénez and Escribá, 1996), indicating cold-arresting reversibility. In other experiments, involving transplantation of nucleus from preserved embryos (at +4ºC or cryopreserved) to fresh enucleated eggs, it has been indicated that the nucleus retains the ability to cleave, with the environmental cytoplasm being more decisive in subsequent development (Heyman et al., 1990; Nakamura and Tsunoda, 1986; Niwa et al., 1991).

The different developmental ability of reconstructed embryos observed when stored or fresh nuclei were used as donors may be due to the phase of the cell cycle at which nuclei were transferred (Collas et al., 1992; Smith and Wilmut, 1989). Since asynchronous cell cycles amongst blastomeres appear in earlier stages of normal embryo development, cooled 8-cell embryos could contain blastomeres in different phases of the cell cycle (Heyman et al., 1990; Smith and Wilmut, 1989), probably at more advanced cell-cycle phase than those from fresh 8-cell haploid donor nuclei. However, when stored nuclei were transferred, due to delay on fusion, the more advanced cell cycle phase of Hemi-zygotes recipient would also affect developmental ability of reconstructed eggs.

From these preliminary results we suggest to consider this technique as feasible for amplifying the female genetic representation into more offspring.

ACKNOWLEDGMENTS

The authors thank Mr. Luis García Valero for his excellent technical assistance in the laboratory and with animal care, and Mr. Neil Macowan for revising the English version. This work was supported by CICYT (AGF 97-0803) and by Consellería de Educación y Ciencia de la Comunidad Valenciana.

REFERENCES

Anderson GB, Foote RH, 1975: Development of rabbit embryos after storage at 10º C. *J. Ani. Sci., 40: 900-904.*

Collas P, Balise JJ, Robl JM, 1992: Influence of cell stage of the donor 4 nucleus on development of nuclear transplant rabbit embryos. *Biol. Reprod., 46: 492-500.*

Escribá MJ, García-Ximénez F, 1999: Electroactivation of rabbit oocytes in an hypotonic pulsing medium and parthenogenetic in vitro development without cytochalasin B-diploidizing pre-treatment. *Theriogenology, 51: 963-973.*

García-Ximénez F, Escribá MJ, 1996: Effects of storage at 4ºC and chemical-enzymatic denudation on in vitro development of 8-cell-stage rabbit embryos for applying to nuclear transplantation. *6th World Rabbit Congress. Toulouse, 2: 171-173.*

Heyman Y, Chesné P, Renard J-P, 1990: Reprogrammation complète de noyaux embryonnaires congelés, après transfert nucléaire chez le lapin. *CR. Acad. Sci. Paris, t.311, Série III: 321-326.*

Howlett SK, Barton SC, Surani MAH, 1987: Nuclear cytoplasmic interaction following nuclear transplantation in mouse embryos. *Development, 101: 915-923.*

Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y, 1998: Eight calves cloned from somatic cells of a single adult. *Science, 282(5396):2095-8.*

McGrath J, Solter D, 1986: Nucleocytoplasmic interactions in the mouse embryo. *J. Embryol. Exp. Morphol., 97: 277-289 suppl.*

Nakamura K, Tsunoda Y, 1986: Investigation on the low temperature resistance of mouse pronuclei by using nuclear transplantation technique. *Jpn. J. Anim. Reprod., 32: 134-137.*

Niwa K, Funahashi H, Ohmae K, Kattoh M, 1991: First cleavage of enucleated rat eggs following transplantation of karyoplast removed from pronuclear eggs stored at 2 to 6 ºC for various durations. *Theriogenology, 36: 411-417.*

OZIL JP, 1990: The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. *Development 109, 117-27.*

Smith LC, Wilmut I, 1989: Influence of nuclear and cytoplasmic activity on the development in vitro sheep embryos after nuclear transplantation. *Biol. Reprod., 40: 1027-1035.*

Surani MAH, Barton, SC, Norris ML, 1987: Experimental reconstruction of mouse eggs and embryos: an analysis of mammalian development. *Biol. Reprod., 36: 1-16.*

Yang X, Zhang L, Kovacs A, Tobback C, Foote RH, 1990: Potential of hypertonic medium treatment for embryo micromanipulation: II. Assessment of nuclear transplantation methodology, isolation, subzonal insertion, and electrofusion of blastomeres to intact or functionally enucleated oocytes in rabbits. *Mol. Reprod. Dev., 27: 118-129.*