Developmental Rate of Rabbit Parthenogenetic Embryos Derived Using Different Activating Protocols

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ABSTRACT : The present study compares development of rabbit embryos generated using different oocyte activation protocols and reconstructed with embryonic or cumulus cells as nuclear donor. *In vivo* matured oocytes were collected from New Zealand White rabbits at 16 h after ovulation treatment and were activated at18 h of post-ovulation treatment. The following schemes of oocytes activation were tested: 1) single electric pulse (EP, 3.2 kV/cm, 3×20 μs, 0.3 M mannitol)+5 min culture in the presence of 5 mM Ionomycin, 2) single electric pulse (EP, 3.2 kV/cm, 3×20 μs, 0.3 M mannitol)+1 h culture in the presence of 2 mM 6-DMAP, and 3) three electric pulses 30 min apart. Cleavage rate, percentage of expanded and hatched blastocysts as well as total cell number of blastomeres of parthenogenetic embryos were significantly higher using either EP+6-DMAP or 3×EP schemes, comparing with EP+Ionomycin. Development rate up to hatched blastocyst stage of cloned rabbit embryos using the EP+6-DMAP for activation of nuclei were 19% for embryonic cell nuclei and 36% for cumulus cell nuclei. The best activation protocol optimalized in this study was the combined treatment "EP+6-DMAP", which may be potentially used for nuclear transfer protocol. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 5 : 617-620)***

Key Words : Oocyte Activation, Nuclear Transfer, Development, Rabbit

INTRODUCTION

Recent results from a number of laboratories indicate that one important factor affecting the developmental potential of activated and nuclear-transferred oocytes is the efficiency of oocyte activation (Ha et al., 1998; Inoue et al., 2002; Liu et al., 2002). *In vivo*, the activation of mammalian oocztes is triggered by Ca^{2+} signal in consequence of fertilization process. Activation of egg is characterized by morphological and molecular events that include cortical granule exocytosis, resumption of meiosis, extrusions of the second polar body, formation of pronuclei, DNA synthesis and mitotic cleavage (Ozil and Huneau, 2001). An oocyte can be also activated beside a sperm, by artificial stimulus (electric pulse or chemical reagents) which induce a single intracellular calcium elevation.

The efficiency of activation procedure depends also on the age of oocytes. Aged oocytes can be activated more efficiency than younger oocytes by multiple electrical pulses, but the potential of activated oocytes to develop into blastocysts decreases with age (Collas and Robl, 1990). Mitalipov et al. (1999) reported that the percentage of blastocysts derived from young rabbit oocytes treated with inositol 1,4,5-triphosphate followed by 6-dimethylaminopurine (6-DMAP) was higher than the oocytes activated either with inositol 1,4,5-triphosphate alone or with multiple electrical pulses.

The reagents that increase intracellular calcium (ionomycin) or inhibit protein phosphorylation (6-DMAP) with a combination of modified electric pulse conditions in the mannitol solution with Ca^{2+} could improve the activation and blastocyst development rates in rabbit oocytes (Ju et al., 2002). Numerous research teams search for optimal activation protocol, but their efficiency varies among laboratories and greatly influenced by individual conditions in different laboratories. Therefore, the efforts to futher optimalize procedure of activation are still actual. The subject of our study was to find an optimal protocol for rabbit oocytes activation using either embryonic or somatic cells as nuclear donor.

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MATERIAL AND METHODS

Oocyte and embryo collections

Mature New Zealand White female rabbits were superovulated with single i.m. injection of PMSG (Werfaser), 150 IU. HCG (Werfachor) at a dose of 100 IU was injected i.v. 72 h after the dose of PMSG. At 16 h after injection of HCG, the females were laparotomized and mature MII oocytes were flushed from the oviductwith Dulbecoo's PBS supplemented with 3 mg/ml of BSA (Sigma). The cumulus cells were removed by short exposure to 0.5% of hyaluronidase (Sigma) in CO_2 independent medium (CIM, Gibco BRL) and subsequent pipetting with a small-bore pipette (130-150 μm inner diameter).

The embryos at 8-16-cell stage were flushed from the oviduct of superovulated and mated females at 48 h post hCG using Dulbecoo's PBS supplemented with 3 mg/ml of BSA. Subsequently, embryos were kept in culture medium k-DMEM plus 10% FBS-ES (Gibco BRL) in a humidified

Table 1. *In vitro* development of rabbit parthenogenetic embryos after various activations schemes

Activ. scheme	No oocytes n	Cleavage (n%)	Blastocyst expanded n (%)	Blastocyst hatched n (%)	Total cell number (±SEM)
EP+Ionomycin	50	38 (76 %)[a]	32 (64 %)[c]	25 (50 %)[e]	191±7.63[g]
EP+6-DMAP	50	48 (96 %)[b]	46 (92 %)[d]	44 (88 %)[f]	240±11.48[h]
3×EP	50	46 (92 %)[b]	43 (86 %)[d]	42 (84 %)[f]	244±11.06[h]

[a,b] $p \leq 0.05$, [c,d,e,f] $p \leq 0.001$ (Chi-square). [g,h] $p \leq 0.05$ (t-test).

atmosphere of 5% CO_2 in air at 39°C.

Activation techniques and culture

Oocytes were randomly assigned to group of an activation treatment as follows: 1) electric stimulation (3.2 kV/cm, 3×20 μs, 0.3 M mannitol solution - with 0.1 mM calcium chloride and 0.1 mM magnesium chloride) followed by 5 min culture in the culture medium containing 5 mM Ionomycin (Sigma), 2) electric pulse (3.2 kV/cm, 3×20 μs, 0.3 M mannitol solution) followed by 1 h culture in the culture medium containing 2 mM 6-DMAP (Sigma), 3) three electric pulses 30 min apart. Activated parthenogenetic oocytes were subsequently transferred in the culture medium and cultured 96 h in a humidified atmosphere of 5% CO_2 in air at 39°C up to blastocyst stage.

Preparation of embryonal and somatic donor cells

The embryos, at 8-16-cell stage, were released from zona pellucida by 0.5% pronase treatment (Sigma), 2× rinsed in CIM medium (Gibco BRL) supplemented with 10% FBS-ES (Gibco BRL) and incubated in a PBS - Ca^{++} free medium for 5 min at 38°C. All blastomeres were separated from the embryos using a gauged pipette and kept in CIM medium supplemented with 10% FBS-ES at room temperature awaiting nuclear transfer.

Cumulus cells (CC), were detached from oocytes immediately after flushing of oocytes, dispersed by 4 min treatment with 0.5% hyaluronidase (Sigma) in CIM and incubated in PBS-Ca^{2+} and Mg^{2+} free medium containing about 2% polyvinylpyrolidone (PVP, Sigma) at room temperature until nuclear transfer.

Procedure of cloning (nuclear transfer)

Denuded recipient oocytes were incubated for 30 min in the medium (see before) containing 0.5 μg/ml Hoechst 33342 dye (Sigma). The first polar body and metaphase plate with approximately 5% of adjacent cytoplasm were removed by 10 min treatment of the oocytes with 5 μg/ml of cytochalasine B (Sigma) and subsequent aspirating through a special beveled pipette (20 μm, inner diameter) without any rupture of the plasma membrane.

Single donor cells (blastomere or cumulus cells) were individually introduced into the perivitelline space of enucleated oocytes. Reconstructed eggs were fused by 3 DC pulses of 3.2 kV/cm for 20 μs in a 0.3 M mannitol (Sigma) solution with 0.1 mM calcium chloride and 0.1 mM magnesium chloride.

Fused NT embryos were activated by single electric pulse (3.2 kV/cm, 3×20 μs, 0.3 M mannitol solution) followed by 1h culture in the presence of 2 mM 6-DMAP (Sigma) or by three electric pulses 30 min apart. Treated oocytes were transferred in to the culture medium in a humidified atmosphere of 5% CO_2 in air at 39°C and cultured up to blastocyst stage.

For cell number determination, some blastocysts were stained with Hoechst 33342 (Sigma) at concentration of 1 μg/ml, for 20 min, mounted on slides in Vectashield and analyzed under epifluorescent microscope (Chesne et al., 2002).

Statistics

Data were analyzed using the chi-square test for the developmental potential of activated oocytes at hatched blastosyst stage and the unpaired t-test for the cell numbers of blastocyst.

RESULTS

Parthenogenetic rabbit embryos

Totally 150 *in vivo* matured rabbit oocytes were used to test the development rates of the parthenogenetic embryos using three activation schemes. As shown Table 1, cleavage rate, percentage of expanded and hatched blastocysts as well as total cell number of blastomeres were significantly higher using either EP+6-DMAP or 3×EP schemes, comparing with EP+Ionomycin. Moreover, there were no significant differences between these two schemes.

Therefore in the next series of experiment only these two treatments were used.

Cloned rabbit embryos

Totally 170 *in vivo* matured rabbit oocytes were enucleated and fused with blastomere cells and 230 enucleated oocytes were fused with cumulus cells. *In vitro* development rates of the cloned rabbit embryos using different cell nuclei, activation techniques and number of cells in the embryos at blastocyst stage are summarized in Table 2. When blastomere was used as a donor cell, cleavage rate was significantly higher using 3×EP scheme. Neither proportion of blastocysts nor total cell number of blastomeres were affected by either activation scheme.

Table 2. Development of rabbit cloned embryos from using two activation protocol and source of NT donors

Activ. scheme	Source of NT donor	No font embroys	Cleavage rate n (%)	Blastocyst		Total cell number (±SEM)
				Expanded n (%)	Hatched n (%)	
EP+6-DMAP	blastomere	40	8 (25%)[a]	8 (25%)[a]	6 (19%)[a]	121±7.02[c]
3×EP	Blastomere	130	45 (41%)[b]	32 (29%)[a]	25 (23%)[a]	132±6.26[c]
EP+6-DMAP	Cumulus cell	139	85 (87%)[a]	37 (38%)[a]	35 (36%)[a]	130±4.46[d]
3×EP	Cumulus cell	91	47 (84%)[a]	21 (37.5%)[a]	17 (30%)[a]	145±6.99[e]

[a, b] $p<0.01$ (Chi-square), [d, e] $p<0.05$ (t-test), differences were evaluated within groups at donor cells separately.

When cumulus cells were used as a donor cells no influence of any activation protocol on cleavage rate and blastocyst yield was observed excepting total cell number, which was higher using 3×EP protocol. All parameters were significantly higher when cumulus cells were taken as nuclei donor (versus blastomere) using both activation schemes.

DISCUSSION

The artificial activation of rabbit oocytes has been induced by various stimuli, such as cooling (Chang. 1954), electrical pulses (Ozil. 1990). sperm factor (Stice and Robl. 1990). inositol 1.4.5-triphosphate (Mitalipov et al., 1999). ethanol (Ju et al., 2002; Liu et al., 2002), combination of electric and chemical stimuli (Ha et al., 1998; Yin et al., 2000; Dinnyes et al., 2001; Liu et al., 2002). Success of above-mentioned inductions varied among laboratories from 25 to 85%. In our study we used three different activation protocols. which are based on electrical pulse but are differed by the presence or absence of chemical inducers such as Ionomycin (well known as inducer of intracellular calcium (Ca^{2-}) oscillations) and 6-DMAP. The latter is cytokinesis inhibitor (extrusion of polar body), which induces oocyte activation. affects differentiation of embryonic teratocarcinoma cells to somatic cells and influences reprogramming of somatic cell nuclei after or during activation (Wakayama and Yamagimachi. 2001).

Our experiments were conducted to test three commonly used protocols for rabbit oocyte activation related to their effect on the blastocyst yield. Parthenogenetic experiments showed that two of three activation treatments (EP+6DMAP and 3×EP) were more effective concerning cleavage rate and blastocyst development rates comparing with earlier results (Ha et al., 1998; Dinnyes et al., 1999; Mitalipov et al., 1999; Yin et al., 2000; Liu et al., 2002). Possible cause for lower developmental competence of Ionomycin treatment is the ploidy of activated parthenotes. Liu et al. (2002) reported no polar body extrusion when rabbit oocytes were activated with 6-DMAP. Is generally agreed that activated haploid oocytes are less competent than diploid parthenotes. Second possible cause for the low developmental competence of Ionomycin treatment is a single rise calcium transient, which may reduce histone H1 kinase or MPF activity temporally. Multiple calcium stimulation is required to keep this kinase activity at basal level. which is the prerequisite for a complete parthenogenetic development in rabbit oocytes (Liu et al.. 2002).

Ozil and Huneau (2001) obtained 93-100% of 8-cell stage rabbit embryos using EP treatment. which corresponds to our results at this development stage using EP+6-DMAP protocol. Our results with 2 mM 6-DMAP are comparable with the observation of Ha et al. (1998). concerning cleavage rate and blastocyst yield. We confirmed also the observation (Liu et al., 2002). that concentration of 6-DMAP and durations of treatment are important factors influencing activation efficiency of rabbit oocytes. Prolonged incubation (more then 2 h) in 6-DMAP in combination with IP3-mediated Ca^{+-} rise induced parthenogenetic development of MII rabbit oocytes to the blastocyst stage (50% efficiency). but longer exposure to 6-DMAP can avoid the risk of chemical-induced chromosomal abnormalities in donor nucleus (Mitalipov et al., 1999).

It is known that cloned rabbit embryos are characterized by their poor postimplantation development. Recent findings clearly demonstrate that the oocyte activation protocol influence the postimplantation development of nuclear-transferred rabbit embryos (Chesne et al.. 2002; Inoue et al.. 2002).

We observed that better results of preimplantation embryo development in case of both activation schemes were obtained using somatic cell (cumulus cells) as a donor of nuclei comparing with embryonic blastomere. This observation is in concern with previous findings (Heyman at al.. 1990; Chesne et al., 2002; Ju et al., 2003).

Our protocol of activation with electrical pulses and 6-DMAP allowed obtain cleavage and blastocyst yield comparable with results reported earlier.

REFERENCES

Chang, M. C. 1954. Development of parthenogenetic rabbit blastocyst induced by low temperature storage of unfertilized ova. J. Exp. Zool. 125:127-149.

Chesne, P., P. Adenot, C. Viglietta, M. Baratte, L. Boulanger and J. P. Renard. 2002. Cloned rabbits produced by nuclear transfer from adult somatic cells. Nature Biotechnology 20:366-369.

Collas, P. and J. M. Robl. 1990. Factors affecting efficiency of nuclear transplantation in the rabbit embryo. Biol. Reprod. 43:877-884.

Dinnyes, A., L. Liu, Y. Dai, J. Xu, P. Zhou, V. A. Velde and X. Yang. 1999. Development of cloned embryos from adult rabbit fibroblast cells. Theriogenology 51:199 (Aabstr.).

Escriba, M. J. and F. Garcia-Ximenez. 2000. Use of a variable electrical pulsing sequence in rabbit oocytes activation. Reprod. Nutr. Dev. 40:261-269.

Fissore, R. A. and J. M. Robl. 1992. Intracellular Ca response of rabbit oocytes to electrical stimulation. Mol. Reprod. Dev. 32:9-16.

Ha, R. J., X. J. Yin, D. W. Kang, J. K. Park, S. Y. Song, I. K. Kong, H. J. Lee and C. S. Park. 1998. Parthenogenetic activation and development of rabbit oocytes following electric stimulation or ionomycin plus 6-dimethylaminopurine. Theriogenology 49:153 (Abstr.).

Heyman, Y., P. Chesne and J. P. Renard. 1990. Full-term reprogramming of frozen embryonic nuclei after nuclear transfer in rabbit species. C. R. Acad. Sci. Paris. 311:321-326.

Inoue, K., N. Ogonuki, Y. Yamamoto, Y. Noguchi, S. Takeiri, K. Nakata, H. Miki, M. Kurome, H. Nagashima and A. Ogura. 2002. Improved postimplantation development of rabbit nuclear transfer embryos by activation with inositol 1,4,5-trisphosphate. Cloning Stem Cells 4(4):311-317.

Ju, J. C., T. H. Chen, J. K. Tseng, C. Tsay, S. P. Yeh, P. C. Chou, C. H. Chen and C. T. Liu. 2002. Cytoskeletal patterns, *in vitro* maturation and parthenogenetic development of rabbit GV oocytes. Asian-Aust. J. Anim. Sci. 15(12):1695-1701.

Ju, J. Ch., J. S. Yang, Ch. T. Liu, Ch. H. Chen, J. K. Tseng, P. Ch. Chou and S. P. Cheng. 2003. Differential influences in sizes and cell cycle stage of donor blastomeres on the development of cloned rabbit embryos. Asian-Aust. J. Anim. Sci. 16:15-22.

Liu, C. T., C. H. Chen, S. P. Cheng and J. C. Ju. 2002. Parthenogenesis of rabbit oocytes activated by different stimuli. Anim. Reprod. Sci. 15, 70 (3-4):267-276.

Mitalipov, S. M., K. L. White, V. R. Farrar, J. Morrey and W. A. Reed. 1999. Development of nuclear transfer and parthenogenetic rabbit embryos activated with inositol 1,4,5-triphosphate. Biol. Reprod. 60:821-827.

Ozil, J. P. 1990. The partenogenetic development of rabbit oocytes after repetetive pulsatile electrical stimulation. Development 109:117-127.

Ozil, J. P. and D. Huneau. 2001. Activation of rabbit oocytes: the impact of the Ca signal regime on development. Development 128:917-928.

Stice, S. L. and J. M. Robl. 1990. Activation of mammalian oocytes by a factor obtained from rabbit sperm. Mol. Reprod. Dev. 25(3):272-80.

Yin, X. J., T. Tani, Y. Kato and Y. Tsunoda. 2000. Development of rabbit parthenogentic oocytes and nuclear transferred oocytes receiving cultured cumulus cells. Theriogenology 54:1469-1476.

Wakayama, T. and R. Yamagimachi. 2001. Effect of cytokinesis inhibitors, DMSO and the timing of oocyte activation on mouse cloning using cumulus cell nuclei. Reproduction 122:49-60.