Comparison of different electrofusion treatments to be used in rabbit somatic cloning

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SUMMARY

The present study examined the efficiency of different electrofusion treatments (A, B and C), in terms of fusion and lysis rates when fibroblasts or cumulus cells were used as nuclear donors. Regardless of nuclear donor cell used, treatment B (a pulse of 2.8 kVcm⁻¹ for 60 μsec, applied three times 30 min apart) gave acceptable fusion rates (fibroblasts: 59 %; cumulus cells: 69-95 %) with low lysis rates (fibroblasts: 1.6 %; cumulus cells: 0 %). Additionally, treatment A (two pulses of 1.5 kVcm⁻¹ for 60 μsec, applied three times 30 min apart) presented a high disconnection rate (39 %), while treatment C (three pulses of 3.6 kVcm⁻¹ for 20 μsec, repeated once 90 min apart) supposed an excessively high lysis rate (19-22 %). Hypo-osmolarity in the B-pulsing medium reduces fusion rate (0.25 M: 81 % vs. 0.3 M: 95 %, P < 0.05), as well as cleavage rate (0.25 M: 23 % vs. 0.3 M: 70 %, P < 0.05). Thus, the isosmolar treatment B is an efficient electrofusion treatment in rabbit somatic cloning when these cellular types are used as nuclear donors. On the other hand, the pulse order in which the fusion occurs determines differences on further cleavage rates.

Key words: somatic cloning, electrofusion, osmolarity, fibroblast, cumulus cells, rabbit.

INTRODUCTION

There is an increasing interest in somatic nuclear transfer, mainly for several agricultural and biotechnological purposes, since it offers new opportunities for genetic engineering (Chan, 1999) and genome preservation of breeds of either economic or ecological interest (Wells et al., 1998). Furthermore, it could enable the multiplication of elite livestock (Wells et al., 1999).
The list of species from which viable offspring have been produced by adult somatic nuclear transfer is rapidly increasing (Pennisi and Vogel, 2000). Rabbit has been appeared as a really difficult species to be cloned by this technology. In addition to their interest as meat producers, rabbit constitutes a good model for other species and will help to elucidate the fundamental mechanisms involved in the nuclear transfer process (Dinnyés et al., 2001).

In general, two somatic cell types are considered the best candidates as nuclear donors in somatic cloning technology: epithelial fibroblasts and cumulus cells (Pennisi and Vogel, 2000), although the latter are only present in sexually mature females. On the other hand, adult skin fibroblasts are easy to obtain without the limitations of animal age, sex and physiological state. Moreover, skin samples cryopreservation (Silvestre et al., 2002) and in vitro fibroblasts culture (Stanyon and Galleni, 1991) are not difficult.

In the rabbit species and for somatic cloning, the required micromanipulation techniques are similar to those applied in embryo cloning (Mitalipov et al., 1999; Yin et al., 2000; Dinnyés et al., 2001; Chesné et al., 2002; Li et al., 2002). However, electrofusion treatment should be redefined, mainly due to the different type and size of cells used as nuclear donors. Unfortunately, the literature referring to electrofusion in rabbit somatic cloning is scarce and inconclusive. Therefore, our goal was to develop an efficient electrofusion treatment both for nuclear transfer and activation in this species.

MATERIAL AND METHODS

Nuclear donor cells

Two cellular types were used as nuclear donors: epithelial fibroblasts and cumulus cells.

Epithelial fibroblasts

In the first experiment (see below), fibroblasts used as nuclear donors were obtained as follows. Very thin (0.25-1.0 mm²) tissue pieces were obtained from the ear of 4 to 4 1/2 month-old rabbits in modified PBS (Dulbecco’s Phosphate Buffered Saline, Cat N.º D-5773, Sigma, Madrid, Spain) supplemented with 20 % (v/v) FCS (hereafter: S-PBS). Tissue pieces were immediately vitrified in S-PBS containing 3.58 M (20 %; v/v) ethylene glycol (EG) and 2.82 M (20 %; v/v) DMSO, following the procedure described by Silvestre et al. (2002). After thawing, four to six pieces were seeded in a 2 hr pre-equilibrated Nunc 4-well multidish containing Ham’s F-10 (N-6635, Sigma, Madrid, Spain) supplemented with 20 % (v/v) FCS (hereafter: F-Ham’s) and incubated in 5 % CO₂ in air at 95 % relative humidity at 39 °C. When tissue pieces attached to the substratum, serum content was reduced to 10 % (v/v) for the rest of the primary culture period, changing the culture medium every 4 d for 28 days as maximum. After a sufficient outgrowth, the explants were removed and the culture plate trypsinized. Dissociated cells were plated in a Nunc 4-well multidish and allowed to proliferate in the same medium.

Fibroblasts used for nuclear transfer were from passage 2 to 5 of culture.
Cumulus cells

In experiments 2 and 3 (see below), fresh cumulus cells were used as nuclear donors. Cumulus cells from oviductal metaphase II oocytes were fully disassembled by a brief incubation in hyaluronidase solution (2 mg/ml; H4272, Type IV-S, Sigma, Madrid, Spain) assisted by gentle pipetting. Then, cumulus cells were washed twice in Hepes-buffered Ham’s F-10 supplemented with 0.15 % (w/v) BSA (hereafter: H-Ham’s), and maintained at room temperature until use.

Enucleation of metaphase II oocytes and nuclear transfer

Cumulus-oocyte complexes (hereafter: COCs) were recovered in S-PBS from oviducts of adult mixed-breed females around 12 hr to 14 hr after the ovulation induction treatment (20 μg im GnRF Fertagyl, Intervet, Spain).

COCs were incubated in hyaluronidase solution for 10 min to remove cumulus cells before oocyte denudation by gentle pipetting. Obtained oocytes were held in F-Ham’s in 7 % CO₂ in air at 39 °C until use.

Groups of approximately 10-12 oocytes were put into a droplet of H-Ham’s containing 7.5 × 10⁹ g/ml cytochalasin B (CCB) under mineral oil (M8410, Sigma, Madrid, Spain). A sufficient number of donor cells were added to the manipulation drop. Oocytes were enucleated by aspirating the first polar body and a small amount (25 %-30 %) of the surrounding ooplasm (presumably containing the metaphase II plate) with a 40° beveled 25-27 μm outer-diameter glass pipette. After blind enucleation (Yang et al., 1992; Piotrowska et al., 2000), the same pipette was used to insert the donor cell into the perivitelline space. Donor cell was wedged between the zona and the ooplast to facilitate close membrane contact for subsequent fusion. After micromanipulation, ooplast-donor cell complexes (hereafter: ODCs) were held in F-Ham’s without CCB for 30 to 90 min, in 7 % CO₂ in air at 39 °C until electrofusion-activation treatment.

Fusion and activation

Electrofusion was performed at approximately 16 hr post-GnRF injection.

Three electrofusion procedures were assayed in this work:

Treatment A was derived from that proposed by Du et al. (1999), and consisted of a train of two consecutive DC pulses of 1.5 kV/cm⁻¹ for 60 μsec in 0.3 M mannitol, 100 μM CaCl₂ and 100 μM MgSO₄ with 0.01 % PVA as electrofusion medium. Pulse train was repeated twice at 30 min intervals on non-fused ODCs.

In treatment B, based on Dinnyés et al. (2001), ODCs fusion was induced by a pulse of 2.8 kV/cm⁻¹ for 60 μsec applied in 0.3 M mannitol solution containing 100 μM CaCl₂ and 100 μM MgCl₂. On non-fused ODCs only, the pulse was repeated twice as maximum at 30 min intervals.

In treatment C (Adenot et al., 1997), a train of three consecutive DC pulses of 3.6 kV/cm⁻¹ for 20 μsec were applied to ODCs. The electrofusion medium was that described for treatment B. Pulse train was repeated once on non-fused ODCs 90 min apart.

In all cases, fusion treatment was performed at room temperature. The electrical pulses were delivered by a BTX Electrocell Manipulator 2001 (BTX, San Diego, CA).

Experimental Design

Experiment 1

The efficiency of the A and B electrofusion procedures was compared (using fibroblasts as nuclear donors) by evaluation of both ODCs fusion and cell lysis (either in cell and/or oocyte) rates.

Experiment 2

Since in experiment 1 electrofusion treatment A was discarded due to the high disconnection rates, using epithelial fibroblasts as nuclear donors, in this experiment only the electrofusion treatment B was tested with cumulus cells. Moreover, as current trends in somatic cloning technology favour obtaining the ODCs fusion without repeating the electrical fusing stimulus, treatment C was also considered. In the latter case, and in order to increase the degree of oocitary stimulation, a hypo-osmolar fusion medium was also assayed (0.18 M).

Experiment 3

Finally, the efficiency of treatment B was evaluated by both cleavage rate and in vitro development ability to morula or blastocyst stage of reconstructed embryos. In order to avoid disconnections between donor cells and ooplasts observed in 0.3 M mannitol, this treatment was also applied using a slightly hypo-osmolar pulsing medium (0.25 M mannitol).

In this experiment, electrofusion train was repeated three times on every fused ODCs for both pulsing media. Reconstructed embryos were cultured in F-Ham’s in 7 % CO₂ in air at 39 °C in Nunc 4-well multidish. In vitro development was assessed every 24 hr throughout the culture period of 5 days. Nuclear status of reconstructed embryos was not evaluated.

Statistical Analysis

At least three replicates were performed in all experimental groups. The fusion, cell lysis, cleavage and embryo development rates were recorded and the results within each experiment were analysed by the Chi-square test. When a single degree of freedom was involved, the Yates’ correction for continuity was carried out.

RESULTS

Experiment 1

Treatment A resulted in a lower fusion rate (38 %) than treatment B (59 %) when frozen-thawed fibroblasts were used as nuclear donors (Table 1). The differences did not reach levels of significance, due to the reduced effectiveness in group A as a consequence of the unacceptably high rate of disconnections in ODCs when they were exposed to the pulsing medium (39 %, 17/43-data not shown in tables). On the other hand, lysis rate was very low in both treatments (A: 0 % and B: 1.6 %; P > 0.05).
The obtained results show the necessity of applying all three fusion trains in order to obtain an acceptable fusion rate (Table 1).

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Treatment A</th>
<th>Treatment B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial ODCs</strong></td>
<td>26</td>
<td>63</td>
</tr>
<tr>
<td><strong>Fused at 1st pulses train (%)</strong></td>
<td>4 (15)</td>
<td>17 (27)</td>
</tr>
<tr>
<td><strong>Fused at 2nd pulses train (%)</strong></td>
<td>6 (23)</td>
<td>9 (14)</td>
</tr>
<tr>
<td><strong>Fused at 3rd pulses train (%)</strong></td>
<td>0 (0)</td>
<td>11 (17)</td>
</tr>
<tr>
<td><strong>Total fused (%)</strong></td>
<td>10 (38)</td>
<td>37 (59)</td>
</tr>
<tr>
<td><strong>Lysed ODCs (%)</strong></td>
<td>0 (0)</td>
<td>1 (1.6)</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Treatment B</th>
<th>Treatment C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial ODCs</strong></td>
<td>35</td>
<td>62</td>
</tr>
<tr>
<td><strong>No. pulse trains</strong></td>
<td></td>
<td>1st 0.3 M</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.18 M</td>
</tr>
<tr>
<td><strong>Total fused (%)</strong></td>
<td>1st + 2nd</td>
<td>24 (69) a</td>
</tr>
<tr>
<td></td>
<td>33 (53) a</td>
<td>40 (59) a</td>
</tr>
<tr>
<td><strong>Lysed ODCs (%)</strong></td>
<td>0 (0) a</td>
<td>12 (19) b</td>
</tr>
<tr>
<td></td>
<td>15 (22) b</td>
<td></td>
</tr>
</tbody>
</table>

a,b Data within the same row with different superscripts are statistically different (P < 0.05).
1 In treatment C, ODCs received one pulse and 90 minutes later non-fused ODCs received the second pulse.
2 In treatment C, lysis rate was evaluated after receiving the second pulse.

Experiment 2

In treatment C, and whatever the osmolarity of the pulsing media used, the application of only one train of pulses resulted in significantly lower fusion rates than those obtained in treatment B (C-0.3 M: 34 % and C-0.18 M: 44 % vs. B: 69 %; P < 0.05. Table 2), even when treatment C was repeated once again after 90 minutes, although in this case the observed differences did not reach levels of significance (C-0.3 M: 53 % and C-0.18 M: 59 % vs. B: 69 %; P > 0.05). Moreover, treatment C implies a very high lysis rate regardless of mannitol concentration (C-0.3 M: 19 % and C-0.18 M: 22 % vs. B: 0 %; P < 0.05).
Experiment 3

Disconnection rates (before the pulse was done) were significantly different between both osmolarities tested (0.3 M: 24 % vs. 0.25 M: 4 %; P < 0.05 -data not shown in tables). Although fusion rates were high regardless of mannitol concentration, significant differences were observed between them (0.3 M: 95 % vs. 0.25 M: 81 %; P < 0.05. Table 3), but in the 0.25 M group, postfusion lysis rate calculated as a percentage of reconstructed and cultured embryos was high (0.3 M: 0 % vs. 0.25 M: 26 % (8/31); P < 0.05 -data not shown in tables). Furthermore, the cleavage rate of embryos reconstructed in 0.3 M mannitol was significantly higher than that in 0.25 M mannitol (70 % vs. 23 %; P < 0.05).

The highest development rates were reached in the 0.3 M group, although the differences did not reach levels of significance (morulae: 37 % vs. 14 % and blastocyst: 5 % vs. 0 %; P > 0.05).

Finally, and only in the 0.3 M mannitol group, the effects of the temporal relationship between fusion and activation were evaluated. Cleavage rates were significantly lower when ODCs fused at first pulse (1st: 50 % vs. 2nd plus 3rd: 88 %; P < 0.05. Table 4), although no effects on development rates to morulae were detected (1st: 43 % vs. 2nd plus 3rd: 34 %; P > 0.05).

### Table 3

<table>
<thead>
<tr>
<th>Mannitol concentration</th>
<th>Fused (%)</th>
<th>Cultured</th>
<th>Cleaved (%)</th>
<th>Morulae/ cleaved (%)</th>
<th>Morulae/ fused (%)</th>
<th>Blastocysts/ cleaved (%)</th>
<th>Blastocysts/ fused (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 M</td>
<td>35/43 (81)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31</td>
<td>7 (23)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.3 M</td>
<td>61/64 (95)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61</td>
<td>43 (70)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16</td>
<td>16</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Data within the same columns with different superscripts are statistically different (P < 0.05).
DISCUSSION

Fusion rates reported in rabbit somatic cloning (Mitalipov et al., 1999; Lagutina et al., 2000; Dinnyés et al., 2001; Li et al., 2002) range from 43% to 95%. However, in general these authors do not take into account the disconnection rate between donor cell and recipient ooplasm, nor the lysis rate, when the overall efficiency of the process is evaluated.

In the case of treatment A, the very high disconnection and the low fusion rates discourage its use in rabbits, although in our hands it has proven highly efficient in pig somatic cloning assays (Silvestre et al., 2000).

In relation to fusion and from experiment 1 emerges the need to apply multiple pulses to accomplish a sufficient fusion efficiency. Most authors (rabbit: Mitalipov et al., 1999; Dinnyés et al., 2001; Chesné et al., 2002; Li et al., 2002; bovine: Stice et al., 1994; Cibelli et al., 1998; ovine: Loi et al., 1998; porcine: Koo et al., 2000) pursue fusion with only one pulse, as this enables control of the beginning of activation and the possibility of combining electrical fusion and chemical activation stimuli. In experiment 2, our aim with treatment C was to attempt a high fusion rate applying only one pulse train. This was not successful, essentially due to high lysis rate as well as a low fusion rate that possibly reflects an excessive stimulation (Robl et al., 1992). This excess of stimulation would be due only to the intensity of the electric field used (3.6 kVcm⁻¹), the highest that could be applied on this species (García-Ximénez et al., 1995). Although the osmolarity of fusion medium has a dramatic effect on activation (Escribá and García-Ximénez, 2000), no differences were observed on fusion rate in this same experiment with 0.18 M, and in the third experiment, 0.25 M mannitol reduced fusion rate, and more specially cleavage rate, showing an effect of the osmolarity on both fusion and activation, at the same electric field intensity.

The low fusion efficiency obtained throughout all the experiments when only one pulse was applied, could be explained by the oocyte ageing at fusion (Collas and Robl, 1990). In fact, fusion rates obtained by various authors using younger oocytes (Mitalipov et al., 1999, Dinnyés et al., 2001) are higher than ours (13-14 hr after ovulation treatment).
Therefore, if repetition of pulses is needed to accomplish acceptable fusion rates, the fusion may occur as a consequence of any of them. This implies that the activation process can begin prior to or simultaneously with fusion. Activation prior to fusion has positive effects on further embryo development (Wells et al., 1999; Hill et al., 2000); in our case, cleavage rates were higher when activation occurred before fusion. Activation reduces the high levels of MPF activity in the recipient cytoplasm (Smith and Wilmut, 1989; Campbell et al., 1993, 1996; Adenot et al., 1997), so that nuclei introduced a few hours later do not undergo PCC (Premature Chromosome Condensation), and further embryo development is consequently improved (Campbell et al., 1996; Adenot et al., 1997; Piotrowska et al., 2000).

The different fusion rates obtained with treatment B between the different assays realized (experiment 1, with fibroblasts: 59%; experiment 2, with cumulus cells: 69%; experiment 3, with cumulus cells: 95%) could be due to both cellular type and donor origin (Dominko et al., 1999). In spite of that, it may be concluded that, in our conditions, the best fusion treatment is that based on a 2.8 kV/cm–1 pulse for 60 μsec in 0.3 M mannitol with 100 μM CaCl₂ and 100 μM MgCl₂ applied three times at 30 min intervals.

FINAL NOTE: Once this work had been finished and the manuscript had been sent for publication, the obtention of the first cloned rabbits by somatic cloning has been published (Chesné et al., 2002).

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RESUMEN

Comparación de diferentes tratamientos de electrofusión para la clonación somática en conejo

El presente trabajo estudia la eficacia de tres tratamientos de electrofusión, cuando se utilizan fibroblastos y células del cúmulo como donantes de núcleos. Con independencia del tipo celular utilizado, el tratamiento B (un pulso de 2.8 kV/cm–1 durante 60 μsec repetido dos veces cada 30 min), ofrece tasas de fusión adecuadas (fibroblastos: 59%; células del cúmulo: 69-95%) con muy bajas tasas de lisis (fibroblastos: 1.6%; células del cúmulo: 0%). Además, el tratamiento A (dos pulsos de 1.5 kV/cm–1 durante 60 μsec repetido dos veces cada 30 min), presenta elevadas tasas de desconexiones (39%), mientras el tratamiento C (tres pulsos de 3.6 kV/cm–1 durante 20 μsec repetido una vez 90 min después) supone tasas de lisis excesivamente altas (P < 0.05). Con el tratamiento B, una leve hipoosmolaridad reduce las tasas de fusión (0.25 M: 81% vs. 0.3 M: 95%, P < 0.05), y las de segmentación (0.25 M: 23% vs. 0.3 M: 70%, P < 0.05). Siendo el tratamiento B el que mejores resultados ha mostrado, debe señalarse que el pulso en que se produce la fusión condiciona las tasas de segmentación posteriores.

Palabras clave: clonación somática, electrofusión, osmolaridad, fibroblasto, células del cúmulo, conejo.
REFERENCES


