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Electrofusion of *in vitro* produced bovine embryonic cells for the production of isofusion contours for cells used in nuclear transfer

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Introduction

Electrofusion is a reliable method of inducing cell to cell fusion. It can be used for a variety of purposes, including nuclear transfer for the production of identical progeny and hybridoma production for monoclonal antibody production. Electrofusion involves the combination of alternating current (AC) and direct current (DC) pulses to induce membrane fusion. An AC pulse induces cell alignment and dielectrophoresis, which causes tight membrane contact (Mahaworasilpa *et al.*, 1994). The DC pulse results in point membrane fusion and the formation of an unstable flat membrane diaphragm, where spectrin holds lipid fragments together. Although cytoplasm can move between the cells through the pores of a flat membrane diaphragm, large organelles such as mitochondria and nuclei remain contained in the original cell. Under favourable conditions, the flat diaphragm deteriorates to allow cell mixing, indicating true cell to cell fusion (Chernomordik and Sowers, 1991).

Electrical parameters for successful electrofusion rely on the amplitude and duration of the DC pulse. Cell mixing occurs when these parameters are of sufficient magnitude to prevent the flat diaphragm from reforming the membrane lipid bilayers. Other factors that influence cell mixing are temperature, pH and molecules such as cholesterol and ethylmaleimide (A. Sowers, personal communication).

An isofusion contour describes the electrofusion characteristics of a particular cell type and its membranes. A range of DC pulse amplitudes and durations are used to attempt electrofusion of identical cells of equal diameter and of a particular type. The maximum electrofusion result for each pulse duration can be used to construct an isofusion contour. The isofusion contours for different cell types can be compared to predict the optimal electrofusion parameters for fusion between them (Mahaworasilpa, 1992).

This study investigated the electrofusion of identical cell types to produce isofusion contours for cells used for nuclear transfer. The cells used were bovine oocytes matured *in vitro*, oocytes enucleated using two different centrifugation treatments and blastomeres isolated from embryos 3, 4, 5 and 6 days after *in vitro* fertilization (IVF).

Materials and Methods

Bovine ovaries were collected from an abattoir and washed in 0.9% (w/v) saline, before germinal vesicle stage oocytes were aspirated from follicles. Cumulus–oocyte complexes were matured in a humidified atmosphere of 5% CO_2 in air for 24 h in tissue culture medium 199 (TCM 199; Sigma, St Louis, MO) with 10% (v/v) fetal calf serum (FCS; PA Biologicals, Sydney), 0.01 iu bovine pituitary FSH ml^{-1} and 0.01 iu bovine pituitary LH ml^{-1} (NOBL Laboratories, Sioux Centre, IA). Cumulus cells were removed from matured oocytes by vortexing and the zona pellucida was removed from oocytes that had

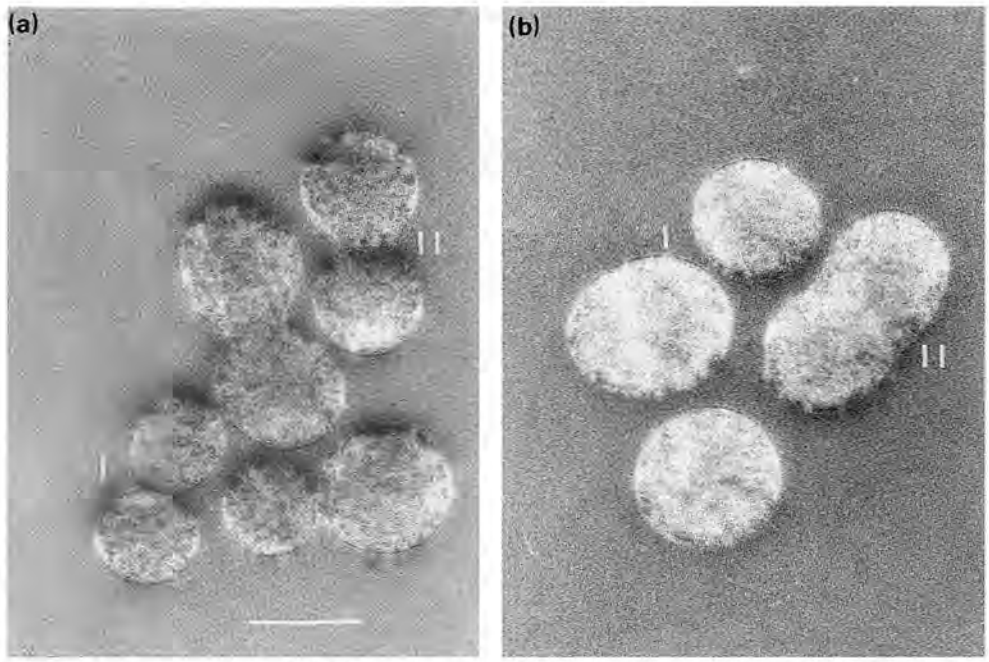

Fig. 1. Electrofusion of coupled blastomeres isolated from bovine embryos produced after *in vitro* fertilization, showing (a) blastomeres with (I) a point membrane fusion, which has progressed to form (II) a flat membrane diaphragm 3 min after pulsation. The membrane diaphragm (b) may either (I) facilitate membrane stabilization, failed fusion and cell separation, or (II) deteriorate to allow cell mixing and true cell fusion 15 min after pulsation. (Scale bar represents 25 μm.)

extruded the first polar body using 0.5% pronase (w/v) (Sigma, St Louis, MO) before being enucleated by centrifugation or used in electrofusion experiments.

Mature oocytes were enucleated with two different centrifugation treatments (Tatham *et al.*, 1995). The zona was removed from oocytes that were placed into a Percoll density gradient of 7.5, 30 and 45% (v/v) isotonic Percoll, containing 10 μg cytochalasin B ml^{-1} (Sigma, St Louis, MO) in a 0.4 ml centrifuge tube (Eppendorf). Oocytes were centrifuged for 4 s at 5000 ***g***. The second treatment involved centrifugation of the matured oocytes with the zona intact for 2 min at 15 000 ***g*** before the zona was removed and the oocytes enucleated as above. Oocyte fragments that did not produce an extrusion cone 6 h after centrifugation were considered enucleated and used for electrofusion experiments (Tatham *et al.*, 1993).

Spermatozoa were prepared using a modified mini-Percoll density gradient (Ord *et al.*, 1990) that consisted of 1.0 ml each of 50, 70 and 90% isotonic Percoll (Sigma, St Louis, MO) in TALP medium (Bavister, 1989) with 10 mmol Hepes l^{-1} (Calbiochem, La Jolla, CA) and 6.0 mg BSA ml^{-1} (Miles Pentex, Kankakadee, IL). Spermatozoa were added to groups of ten oocytes to give a final concentration of 2×10^6 spermatozoa ml^{-1} in a total volume of 50 μl. After fertilization for 22 h, the cumulus was removed and groups of four zygotes were cultured in 30 μl microdrops of modified synthetic oviduct fluid medium (Gardner *et al.*, 1994) covered with mineral oil. The embryos were transferred into fresh 30 μl microdrops of culture medium every 48 h until the embryos were required for electrofusion experiments. The zonae pellucidae of the embryos were removed and the blastomeres disaggregated in TALP with BSA and without Ca^{2+} or Mg^{2+}.

Before electrofusion, the embryonic cells were equilibrated for 10 min with four washes in 50 μl drops of fusion medium covered with mineral oil. Fusion medium consisted of 0.25 mol sucrose l^{-1} buffered to pH 7.4 with 1.0 mol Hepes l^{-1} or 1.0 mol Trisma acetate l^{-1} (Sigma, St Louis, MO). Pairs of identical cells were placed in the fusion chamber, consisting of two parallel stainless steel wires of 0.5 mm diameter and separated by 0.5 mm (BTX microslide 450; BTX, San Diego, CA) and covered with

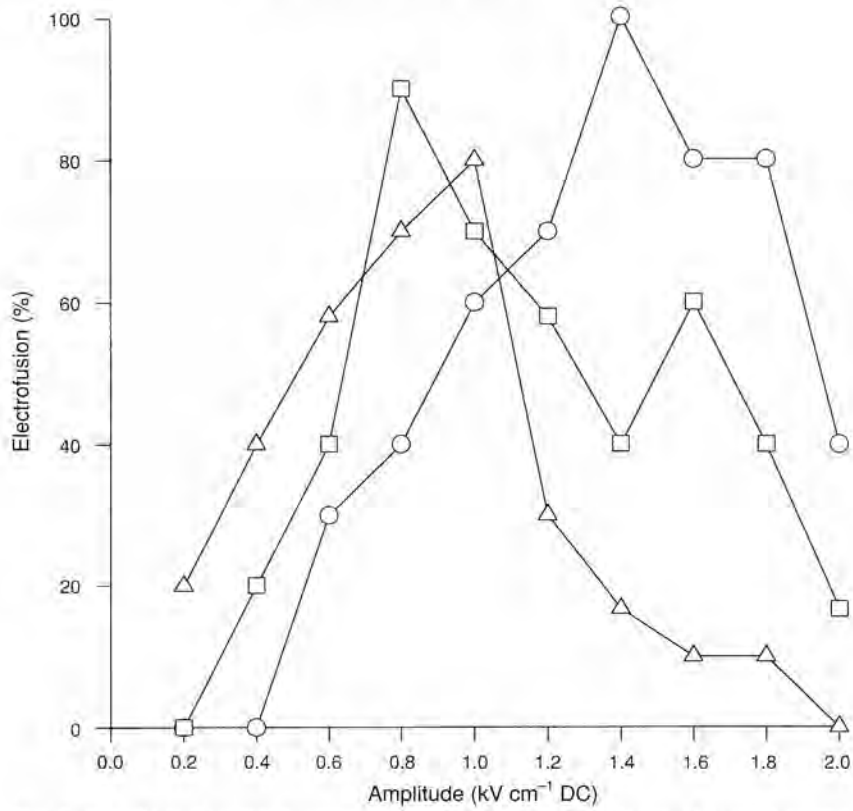

Fig. 2. The effect of amplitude and pulse duration on the electrofusion of coupled *in vitro* matured bovine oocytes that have been enucleated using two centrifugation treatments. Pulse duration of (○) 10 μs; (□) 50 μs; and (△) 100 μs.

fusion medium. Cell alignment and dielectrophoresis were achieved using an AC pulse of 8 volts amplitude for 5 s. Point membrane fusion was then attempted on the electrodes with a single DC pulse of 0.2–2.0 kV cm^{-1} and of 10, 50 and 100 μs duration, using a Zimmerman Fusion Machine (Precision Scientific, Chicago, IL). After electrical treatment, cells were placed on a warm stage and cell fusion assessed 15 min later. Successful fusion was defined as the breakdown of a flat membrane diaphragm that allowed cell mixing. For each cell type, electrofusion was attempted on ten pairs of cells with a minimum of three replicates for the three pulse durations, each with ten different amplitudes.

Electrofusion results were subjected to statistical analysis of variance using the SAS (SAS, 1985) general linear model procedure. The results for each cell type were graphically represented with respect to pulse amplitude and duration. Isofusion contours were then constructed using the optimal electrofusion parameters (i.e., the current at which maximum electrofusion was achieved for a given pulse duration).

Results

The production of isofusion contours for each cell type relied on cell mixing as a measure of successful cell fusion. Point membrane fusion was induced by the DC pulse, and extended to form a flat membrane diaphragm, which then dispersed to allow cell fusion and cell mixing (Fig. 1).

Electrofusion attempts of all different cell types resulted in a significant increase ($P < 0.05$) in fusion when a 10 μs pulse was used compared with a 50 or 100 μs pulse (52% versus 41% and 36%,

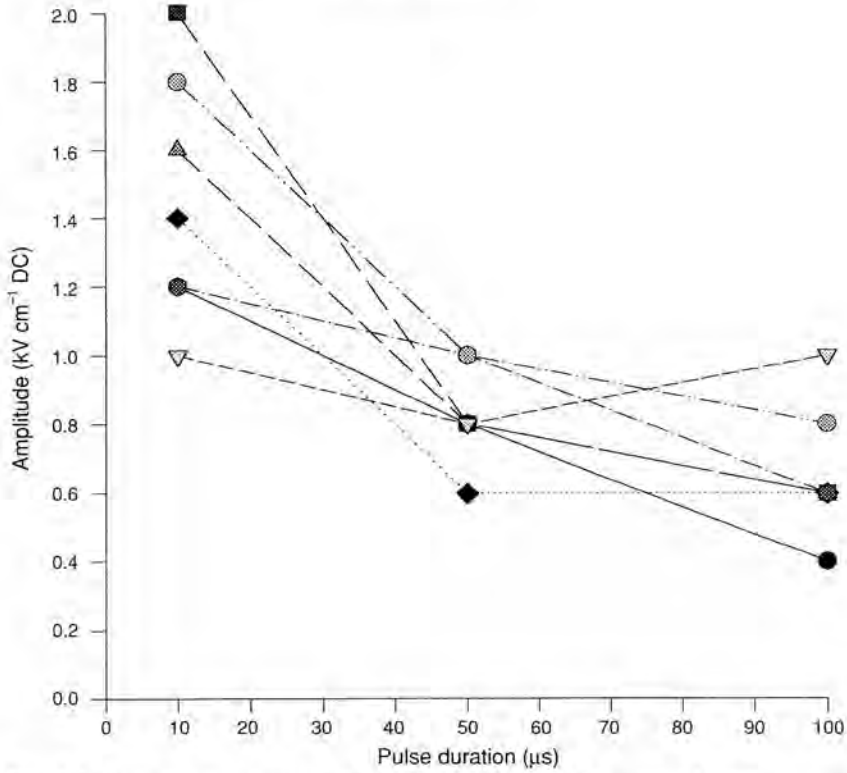

Fig. 3. Isofusion contours produced after electrofusion of (●) coupled bovine oocytes; coupled oocytes enucleated by (■) one and (△) two centrifugation treatments; and, coupled blastomeres from embryos (▽) 3, (◆) 4, (◍) 5 and (◎) 6 days after *in vitro* fertilization.

respectively). The electrofusion of all cell types was significantly increased ($P < 0.05$) with a single 50 μs DC pulse greater than 1.0 kV cm^{-1} than when an amplitude below 1.0 kV cm^{-1} was used (55% versus 33%).

The electrofusion results for each cell type showed a similar pattern (Fig. 2), from which the maximum fusion amplitude for each pulse duration was selected to create an isofusion contour. The slight differences between each contour represented the differences in the electrofusion characteristics of the cells and membranes (Fig. 3), in which the isofusion contours of all the cell types are compared. The optimum parameters for electrofusion of two different cell types can be determined by selecting the point where the isofusion contours for each cell type intersect. When isofusion contours do not intersect, electrofusion parameters can be determined by taking the median at the point where the contours are closest.

Discussion

In this study, the use of a short DC pulse at an amplitude greater than 1.0 kV cm^{-1} resulted in significantly greater electrofusion of identical cells when compared with longer pulse durations of greater amplitudes. This may result from high amplitude DC pulses of short duration creating smaller pores in the opposing cell membranes and causing less damage to membrane integrity (H. Coster, personal communication).

The variation between the isofusion contour gradients reflects the differences between cell types. The different cell types do not have identical membrane composition or diameter, owing to a decreased blastomere diameter with age and changes in lipid and protein composition as development progresses (Barnes and Eyestone, 1990).

Isofusion contours graphically describe the characteristics of cell membranes. Where isofusion contours intersect, the electrofusion properties of different cell types and their membranes approach each other. This provides a simple method for determining the optimal electrofusion parameters for different cell types. The cell types used in this study are used for nuclear transfer in our laboratory, where we are investigating the electrofusion of blastomeres with enucleated oocytes using electrofusion parameters predicted with the aid of isofusion contour interactions.

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References

Barnes FL and Eyestone WH (1990) Early cleavage and the maternal zygotic transition in bovine embryos *Theriogenology* **33** 141–152

Bavister BD (1989) A consistently successful procedure for *in vitro* fertilization of golden hamster eggs *Gamete Research* **23** 139–158

Chernomordik LV and Sowers AE (1991) Physical and ultrastructural evidence that integrin of the spectrin network controls the macroscopic fusion produce morphology following the electrofusion of erythrocyte ghosts *Biophysical Journal* **60** 1026–1037

Gardner DK, Lane M, Spitzer A and Batt PA (1994) Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells: amino acids vitamins and culturing embryos in groups stimulate development *Biology of Reproduction* **50** 390–400

Mahaworasilpa TL (1992) *Cell Electro-dynamics: the Mechanisms of Living Cells in Intense Alternating Electric Fields* PhD Thesis, University of NSW

Mahaworasilpa TL, Coster HGL and George EP (1994) Forces on biological cells due to applied alternating (AC) electric fields. I Dielectrophoresis *Biochimica et Biophysica Acta* **1193** 118–126

Ord T, Patrizio P, Marello E, Balmaceda JP and Asch RH (1990) Mini-Percoll: a new method of semen preparation for IVF in severe male factor infertility *Human Reproduction* **5** 987–989

SAS Institute Inc. (1985) *SAS Users Guide: Basics* Version 5. Cary, NC

Tatham BG, Dowsing AT and Trounson AO (1993) Enucleation by centrifugation for bovine nuclear transplantation *Proceedings of the Australian Society for Reproductive Biology* **25** 31