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Fusion and development of 2-cell bovine embryos to tetraploid blastocyst with different voltages and durations

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Abstract

Background: The values of embryonic stem cell and cloning are evident. Production of clone from embryonic stem cells can be achieved by introduction of stem cell into a tetraploid blastocyst. Tetraploid blastocyst can be produced in vitro by electrofusion of 2-cell embryos.

Objective: The aim of this study was to assess the effect of different voltages and durations on fusion rate of bovine 2-cell embryos and their subsequent development in vitro.

Material and Methods: The in vitro produced bovine 2-cell embryos were categorized into 3 groups: (1) fused group (FG); 2-cell embryos fused by exposure to different voltages (0.5, 0.75, 1, 1.25 and 1.5 kV/cm) and durations (20, 40, 60, 80 and 100 μs), (2) exposed control group (ECG); 2-cell embryos exposed to different voltages and durations but remained unfused and (3) unexposed control group (UCG); embryos cultured without exposure to any voltage. The embryos from each group were cultured and fusion, cleavage and developmental rates were compared in each group.

Results: The results show that increased voltage, increases the fusion rate up to 88% for 1.5 kV/cm; however, the rate of cleavage and blastocyst formation decreases significantly to 18% and 10% respectively ($p<0.05$). Increased duration does not significantly increase fusion rate, however, in high voltage, increased duration decreases cleavage rate and blastocyst formation rate. Blastocyst formation rate in UCG showed a better development (32%) compared to FG (20%) or ECG (22.5%) ($p<0.05$).

Conclusion: It can be concluded that for optimal fusion, cleavage and development, one pulse of 0.75 kV/cm for 60μs should be applied.

Key words: *Bovine, Embryo, Development, Electrofusion, Tetraploid.*

Introduction

Although cloning mammals by transgenic nuclear transfer into oocyte cytoplasts has been performed successfully for nearly a decade, only a very small percentage of cloned embryos have developed to term, with a high incidence of developmental anomalies (1, 2). The impact of this technology has been spectacular, opening up new possibilities for producing recombinant proteins and therapeutic cloning, as animal cells can synthesize these proteins with appropriate post-translation modification (3, 4). Production of transgenic and knock-out mice and cattle is a

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choice for production of tetraploid embryos by aggregation of transgenic embryonic stem (ES) cells with tetraploid morulae (5, 6).

In tetraploid/diploid chimeras, a non-random distribution of cells occurs in the developing conceptus. In these types of chimeras, tetraploid cells readily contributes to extra-embryonic tissue compared to inner cell mass (7) and the offspring from these chimeras are formed solely by embryonic diploid cells. In aggregation of bovine embryonic ES-like cells with day 3 bovine diploid embryos, the ES-like cells have limited presence in various tissues, while in aggregation of bovine ES-like cells with bovine tetraploid embryos, the embryo proper is formed solely by ES-Like cells (8, 10).

Tetraploid embryos are produced by various procedures. One of the most accurate, repeatable and well defined, procedures is electrofusion. During this procedure, embryos are placed between two electrodes in fusion buffer, exposed to fusigenic stimulus for a very short period. This procedure was first reported in mice and in bovine using zona-enclosed blastomeres (11, 12).

Because required and optimum parameters reported for electrofusion by various studies are different, the aim of this study was to evaluate the effect of different voltages (0.5, 0.75, 1, 1.25 and 1.50 kV/cm) and durations (20, 40, 60, 80 and 100 μs) on fusion, cleavage and development of bovine embryos into blastocyst.

Materials and methods

This was an experimental study. All chemicals and reagents were purchased from Sigma chemicals, unless otherwise indicated. Ovaries were collected from local abattoirs from unstimulated heifers and cows, after 30 min of slaughter, transported to the laboratory in sterile 0.9% saline at 25 to 30 ˚C. Ovarian follicles (2 to 6 mm) were aspirated by 18-gauge needle primed with washing medium TCM-199 (Sigma M-2520). In this study, over 1750 high quality (homogene and equal cell size) two-cell embryos were produced 33 to 35 hr post insemination. Nearly 570 two-cell embryo were used for each (control and experimental) group. The results in each group were repeated between 4 to 5 times.

In vitro maturation

High quality cumulus-oocyte-complexes (COCs), having homogenous evenly granulated cytoplasm surrounded by more than three layers compact granulosa cells, were rinsed 3 times in washing medium and twice in maturation medium (MM: washing medium supplemented with 1μg/ml 17β- Estradiol, 0.1 IU/ml HMG, 10% FCS and 1 mM L-glutamine) and subsequently were transferred in groups of 5 into 50μL drops of maturation medium under mineral oil. The COCs were then incubated (LabTec) for 22 to 24h in humidified atmosphere in 5% CO_2 in air at 39˚C (12-14).

In vitro fertilization

After maturation, COCs were rinsed twice in fertilization medium (FM) and were inseminated, with final concentration of 1 to 2 million sperm for ml, in groups of 10 into 50 μL droplets of FM under mineral oil for 18 to 22h. Fertilization media also called Fert-TALP were prepared according to Parrish *et al* (15).

Live spermatozoa from fresh semen of fertile bulls were separated by centrifugation using Percoll gradients (90% and 45%; Seromed, Germany) at 2000 rpm for 25 min. The pellets were washed and diluted to the required concentration in FM. Capcitation of sperms occurred during the IVF culture (13-16).

In vitro Culture

Cleaning of spermatozoa and granulosa cells from inseminated COCs were carried out by vortexing (Ependorf) for 2 minutes in washing medium and subsequently transferred and cultured in SOF_1 (17-21).

Then, 33 to 35h post insemination, 2-cell embryos were selected and categorized into two groups. One group without any exposure to electrical stimulation was taken as control group (UCG = unexposed control group) and was kept in SOF_1 for further 37 to 39h. The 2-cell embryos in the other group were used for electrofusion.

Electrofusion

Two-cell embryos in groups of 5 to 10 were washed for 10-20 seconds in fusion buffer containing 0.3 M mannitol solution containing 0.1 mM $MgS0_4$, 0.05 mM $CaCl_2$, pH=7.2-7.4, immediately transferred between electrodes in fusion chamber containing fusion buffer. Two stainless steel platform electrodes 1mm apart connected to electrofusion machine (CF-150B, Biological Laboratory Service, H-1165 Budapest, Zselyi A. U. 31. Hungary) form this chamber (9, 22). Two-cell embryos with inter-blastomeric axis placed parallel to electrodes were exposed to one pulse of direct current (DC) for certain voltage and duration. Embryos were observed at 60 min post

electrofusion (7). Fused 2-cell embryos were called fused group (FG) and unfused 2-cells were called exposed control group (ECG). These embryos were transferred to SOF_1 for further 37 to 39 h. At this stage embryos from UCG, FG, and ECG were transferred into SOF_2 up to 10th day (SOF_2 was the same as SOF_1 without Na-pyrovate but containing 1%MEM essential amino acids, 10%FCS and 2 mM glucose) (17, 19, 20).

Embryo scoring

Cleavage was evaluated microscopically 72 h post-insemination in each group (FG, ECG, UCG). Morula and early blastocyst were assessed 168 h (7th day) post-insemination. Blastocyst and expanded blastocyst were assessed on the 8th to 10th day post insemination (23). Percentage of cleavage and blastocyst rate were evaluated with respect to the total number of embryos in each group. For cytogentic analysis by Giemsa staining two to eight-cell stage embryos from FG (4n chromosome) were processed according to modified Tarkowski protocol (24, 25). The number of chromosomes was determined under the light microscope at 100x magnification.

Statistical analysis

All statistical calculation including, coefficient of correlation and Chi-square were carried out using statistical package for social studies (SPSS-10) software, and Epi-Info statistical package, respectively.

Results

The results in table I show that, with increase in duration (20 to 100 µs) there is no significant increase in fusion rate in each different voltage (0.5 to 1.50 kV/cm). The results also showed that with increase in voltage, there is a steady increase in fusion rate, in each duration (Table I). Pearson analysis also confirms a high positive correlation between different voltages with fusion rate in different duration. The results in table II showed that with decrease in voltage from 1.5 to 0.5 kV/cm the percentage of cleavage steadily increases in different durations except in 20 µs duration. Pearson analysis also confirms a high negative significant correlation between different voltages with cleavage rate in different duration except 20 µs duration (Table II). Table II also shows that percentage of cleavage in the 1.5 and 1.25 kV/cm groups for 20 µs is significantly greater than that of groups of 40 to 100 µs, suggesting that during high voltage exposure, increase in duration reduces cleavage rate ($p<0.05$). The results also show that maximum cleavage rate was obtained in 0.75 kV/cm in 80 µs duration.

The results in table III show that with increase in voltage from 0.5 to 1.5 kV/cm the blastocyst formation rate decreases steadily during different duration ($p<0.05$), and with increase in duration of exposure during different voltage, blastocyst formation steadily decreases, however this reduction is not significant ($p>0.05$), suggesting that exposure to define voltage does not significantly reduces blastocyst formation rate in different duration. Pearson analysis also confirms a high negative significant correlation between different voltages with blastocyst formation rate in different duration except 60 and 80 µs duration (Table III).Maximum blastocyst formation rate was observed in 0.75 kV/cm in the duration of 60 µs.

The cleavage, morula and blastocyst formation rate in the ECG groups overall followed the same trend as the FG group. Therefore, the data were not shown. Mean cleavage and blastocyst formation rate in the UCG were 80.46 and 32.32% respectively. These values are not significantly higher than the values in the FG and ECG groups with the maximum blastocyst formation rate.

Figure 1 reveals the chromosomal analysis from putative tetraploid embryos produced by electrofusion (0.75 kV/cm for 60 µs).In total 37 (66%) of 56 electro-fused embryos at 2-8 cells could be analyzed and 3 (%8), 6 (%16) and 28 (%76) of 37 were haploid, diploid and tetraploid respectively.

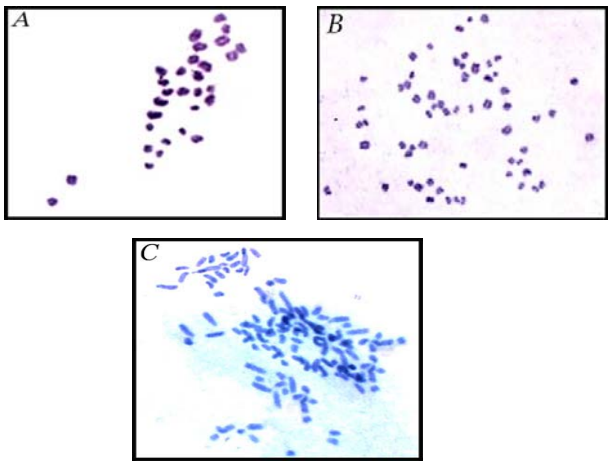

Figure 1. Chromosomal staining by Giemsa, from haploid (A), diploid (B) and tetraploid (C) bovine 2-cell electrofused embryos by voltage of 0.75 kV/cm for duration of 60 µs (magnification ×100).

Discusion

Electrofusion are routinely used for different technologic purposes. During electrofusion due to applied direct current electric field, the membranes are polarized and instabilized, results in attraction of other membrane (point membrane fusion) and formation of unstable flat membrane diaphragm, through reversible pore formation followed by reversible breakdown of the membrane or diaphragm. Under favorable conditions, the flat diaphragm deteriorates to allow cell mixing, indicating through cell-to-cell fusion (27). The obtained results suggest that the fusion rate is voltage dependent and with increases of voltage intensity from 0.5 to 1.5kV/cm, fusion rate increases to 88% (Table I), which is different to fusion rate in study of Lan Li *et al* (77% in goat) (3).

Possibly this difference is due to difference in species. However, increase in duration of electrical pulse to 100 µs did not affect the fusion rate in different voltage (Table I). The results of this study are in concordance with the result of Zhelev *et al* (28) in other cells, which suggest that there is a correlation between pulse intensity and pore formation. Similar results were obtained by Tatham *et al* (29) by fusing enucleated bovine oocytes (by different methods) with blastomeres (with different aged). These authors also showed that increase voltage intensity up to certain threshold level increase the fusion rate, after which fusion rate decreases.

However, they also showed that increasing in pulse duration has no fundamental effect on fusion rate up to the threshold level, which is in agreement with our results.

Table I. The effect of voltage (kV/cm) and duration (µs) on fusion rate of bovine two-cell embryos.

Voltage[b]	Duration[a]				
	20	**40**	**60**	**80**	**100**
0.5	36%	35%	47%	28%	34.5%
0.75	64%	69%	69%	64%	73%
1	64%	62%	64%	73%	72%
1.25	71%	76%	81%	70%	82.5%
1.5	82%	78%	83%	87%	88%
	p=0.026	p=0.04	p=0.031	p=0.043	p=0.045
	r^c=0.921	r=0.845	r=0.912	r=0.890	r=0.880

Duration[a]: Per microsecond (µs)
Voltage[b]: kilovolt per centimeter (kV/cm)
r^c = Pearson correlation between 2 variables (voltage and fusion) in different durations.

Following the fusion of 2-cell bovine embryos, the results of this study suggested that there is a negative correlation between voltage and cleavage rate in all duration, except in duration of 20 µs (Table II). This implying that exposure of bovine 2-cell embryos to high voltages has an inhibitory effect on cleavage rate. This is possibly due to large pore formation (and leakage of cytoplasm) over the two-blastomere membranes.

Table II. The effect of voltage (kV/cm) and duration (µs) on cleavage rate of bovine two-cell electrofused embryos.

Voltag[b]	Duration[a]				
	20	**40**	**60**	**80**	**100**
0.5	64%	64%	72%	70%	74%
0.75	53%	61%	66%	80%	57%
1	52%	49%	56%	51%	58%
1.25	45%	13%	18%	39%	23%
1.5	53%	19%	27%	19%	18%
	p=0.191	p=0.028	p=0.033	p=0.022	p=0.013
	r^c=0.697	r=-0.918	r=-0.930	r=-0.890	r=0.930

Duration[a]: Per microsecond (µs)
Voltage[b]: kilovolt per centimeter (kV/cm)
r^c = Pearson correlation between 2 varriable (voltage and fusion) in different durations.

Therefore it could be suggested that for optimal cleavage rate, exposure of 2-cell bovine embryo to higher than 1kV/cm should be avoided. During this study a negative significant correlation was observed between blastocyst formation rate with increase in voltage in duration of 20, 40,and 100µs. Thus, exposure to high voltage during fusion, decrease blastocyst formation rate. Similar to cleavage rate, the duration of exposure within the range of this study (20 to 100 µs) had no significant effect on blastocyst formation rate. The fact that no significant correlation was observed between blastocyst formation rate and voltage in duration of 60 and 80 µs (Table III) suggest that for optimal developmental competence lower than 0.75kV/cm and higher than 1kV/cm should be avoided, and the best result can be obtained by exposing 2-cell bovine embryo to 0.75 kV/cm for 60 to 80 µs. Cytogenetic analysis of embryo showed that over 76% of fused embryos are true tetraploid. The value obtain in this study is close to value (%78) reported by Iwasaki *et al* (11) and significantly different with value (50%) reported by Prochazka *et al* (30) and by Curnow *et al* (12.5%) (9).

Table III. The effect of voltage (kV/cm) and duration (µs) on blastocyst formation rate of bovine two cell electrofused embryo.
Duration[a]: Per microsecond (µs)

Voltage[b]	Duration[a]				
	20	**40**	**60**	**80**	**100**
0.5	24%	22%	26%	23%	26%
0.75	23%	22%	35%	30%	20%
1	22%	21%	27%	26%	17%
1.25	20%	18%	18%	21%	10%
1.5	18%	13.5%	16%	15%	10%
p-value	0.002	0.030	0.031	0.184	0.006
	r[c]=0.0985	r=-0.9	r=-0.766	r=-0.4	r=-0.971

Voltage[b]: kilovolt per centimeter (kV/cm)
r[c] = Pearson correlation between 2 variable (voltage and fusion) in different durations.

The lower developmental rate in high intensity is likely due to large pore formation and leakage of cytoplasmic material needed for development. The fusion rate obtained in this study when applying 1.5kV/cm for 100 µs was 88%, which is close to value (%76) reported by Curnow *et al* (9) when applied 1.4 kV/cm for 100 µs. For production of tetraploid blastocysts from bovine 2-cell embryos, exposure of 2-cell embryos to 0.75 kV/cm for 60µs is highly recommended. This value is close to value reported by Prochazka *et al* (30) (0.75 kV/cm for 50 µs), and different to Iwasaki *et al* (31) (2 pulses of 100 kV/cm for 10 to 25 µs) and and Xiangyung *et al* (32) (2 pulse of 100 kV/cm for 50 µs). Maybe this difference in electrofusion parameter is related to kind of fusion buffer, electrofusion machine, species of animal and etc. The mean blastocyst formation rate obtained when applying pulse of 0.75 kV/cm for 60 µs (35%) is nonsignificantly close to value in UCG (41%) and significantly different with value (18.8%) reported by Iwasaki *et al* (33). However, the overall developmental rate of embryos in fused group (FG) and exposed control group (ECG) is lower than UCG, suggesting that, alteration in distribution and behavior of microtubules and microfilaments might affect normal formation of mitotic spindle and the contractile ring, respectively (34). The lower developmental capacity of the fused embryo remained unclear, possibly this could be due to electrical stimulation, exposure to non-electrolyte medium or due to chromosomal construction of these embryos.

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