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ELECTROFUSION OF ZONA-FREE MOUSE EMBRYONIC CELLS IN ELECTROLYTES AND THEIR DEVELOPMENT IN VITRO

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ABSTRACT

The influence of increasing the physical electrofusion parameters, direct current (DC) pulse strength, pulse duration, pulse number, alternating current (AC) voltage and alignment time, in electrolytes on the rates of fusion, degeneration and development of zona-free mouse 2-cell embryos were examined. Furthermore, the effects of physiological saline and mannitol as fusion media and various mouse strains were also evaluated. Dulbecco's phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum was used as the main fusion solution.

A significant increase in the rate of fusion ($P<0.05$) was obtained by increasing pulse strength from 30 to 300 V/mm. The embryos fused at the pulse strengths of 30 to 70 V/mm had significantly higher development rates to blastocysts compared with those fused at 100 to 300 V/mm ($P<0.05$). There were no significant differences in the rates of fusion, degeneration and development to blastocysts when the pulse duration was increased from 30 to 90 μsec. Although fusion rates were increased ($P<0.05$) by increasing the pulse number up to 4, a significant decrease ($P<0.05$) in development to blastocysts was observed when the pulse number was 5. Application of AC voltage prior to the DC pulse tended to increase the fusion rate (89.2–93.8%), compared with fusion with the DC pulse only (75.0%). Prolongation of alignment time from 5 to 15 sec had no effect on the fusion rate. Under the optimum conditions (2 pulses of DC of 70 V/mm, 70 μsec pulse duration and AC of 5 V/mm for 5 sec), no significant difference was obtained in the fusion and development rates in different mouse strains, nor were fusion and development rates significantly different among PBS, physiological saline and mannitol solutions ($P>0.05$).

Key Words : Electrofusion, electrolytes, mouse strain, zona-free embryonic cells.

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INTRODUCTION

Electrofusion is currently used to fuse donor nuclei containing karyoplasts to enucleated cytoplasts in nuclear transplantation experiments[13–15]. This technique can be applied using electrolyte[10,13,17,22] and non-electrolyte solutions[1,5–8,15,16,19]. The use of non-electrolyte fusion media such as mannitol solution has become a common practice[5,8,15,19,20]. Fusion of cells in non-electrolyte fusion media is based mainly on induction of pearl chain formation phenomena (cell chain production) and avoiding convective heat induced by non-uniform alternating current (AC) pulses[13,22,23]. Fusion of mouse blastomeres in phosphate buffered saline (PBS) causes increased cell lysis[21] and the fusion rate of reconstituted bovine embryos in modified Tyrode's medium (electrolyte) is reported to be very low compared with those fused in Zimmermann's fusion medium[14]. In contrast to these reports, a very high fusion rate in PBS was reported by Rickords and White[13]. It was also reported that powerful electrical stimuli in 0.3 M mannitol fusion medium, result in increased karyoplast degeneration[2]. Unexplained cell lysis immediately after placing the micro-manipulated karyo-cytoplast constructs in 0.3 M mannitol fusion medium was observed by Rickords and White[13].

Although there are some reports on the use of PBS as fusion medium for embryonic cells[10,13,14], the pulse strength used in these studies was 100 or 150 V/mm and only Kubiak and Tarkowski[10] examined the effect of the pulse duration. These studies ignored the increase in the intensity of the electrical field strength that may develop due to the very low resistance between electrodes when electrolyte solution is used as fusion medium[12]. This increase in the field intensity may be associated with irreversible breakdown of the cell membrane[22], which results in a reduced fusion rate or development rate. Thus, in this study PBS was used as the main fusion solution to reduce the ionic difference between culture medium (Whitten's medium) and fusion medium (Dulbecco's PBS)[13] and to evaluate the influence of increasing the physical electrofusion parameters in electrolytes based on the proposition that field strengths of less that 100 V/mm could be suitable for fusion of mouse 2-cell embryos in electrolytes. Furthermore, there are no definitive data on the rate of development when PBS is used as fusion medium and the discrepancies between the previously mentioned reports on the use of electrolyte solution as fusion media necessitate further investigation about fusion in electrolytes.

The purpose of this study is to examine the effects of increasing the physical electrofusion parameters, pulse strength, pulse duration, pulse number, alternating current voltage and alignment time in electrolytes on fusion and development rates of zona-free mouse 2-cell embryos.

MATERIALS AND METHODS

Collection of embryos

ICR, ddY and F1 (C57BL/6×CBA) females were given intraperitoneal injections of 5 IU of pregnant mare serum gonadotropin (Serotropin, Teikoku Zoki, Japan) and after 48 hr, 5 IU of human chorionic gonadotropin (hCG, Gonatropin, Teikoku Zoki) for superovulation. Females were mated with males of the same strain. Twenty hours after hCG injection they were examined for the presence of vaginal plugs. Females with vaginal plugs were sacrificed by cervical dislocation 48–50 hr after hCG injection. Two-cell embryos were collected by flushing the oviducts with Whitten's medium[18] containing 100 μM EDTA (WM+EDTA). Zonae were removed from embryos after exposure to 0.5% pronase (Actinase; Kaken Pharmaceutical Co., Tokyo, Japan) in Dulbecco's PBS for 5 min.

Culture of embryos

Zona-free 2-cell embryos were cultured before the electrofusion treatment for 30 to 90 min in 30-μl drops of WM+EDTA under paraffin oil in an atmosphere of 5% CO_2 in air at 37°C as described by Clement and Brem[4] and Kono and Tsunoda[9]. The embryos were separated into 2 groups, fused and non-fused, under a dissecting microscope after a culture period of 2 hr following electrofusion. The fused embryos were further cultured in 25-μl drops of WM+EDTA under paraffin oil in an atmosphere of 5% CO_2 in air at 37°C for 72 hr until assessing the development rate.

Electrofusion

A rectangular fusion chamber, consisting of 2 stainless steel plates electrodes (25.0×15.0×0.5 mm) glued onto a glass slide 0.5 mm apart, was used[2,3]. Two to three hundred microliters of the fusion solution was placed between the electrodes[13] and renewed frequently during experiment. The fusion medium in all experiments was PBS containing 10% fetal calf serum (PBS+10% FCS) except in Experiment 3, in which 0.15 M sodium chloride solution (physiological saline) and 0.3 M mannitol supplemented with 0.05 mM $CaCl_2$ and 0.1 mM $MgSO_4$ were also used. Embryos were washed in several drops of the fusion medium to remove the culture medium and 5 to 8 zona-free embryos were transferred to the fusion chamber connected to the fusion instrument (LF-100, Life Tec Co., Tokyo, Japan). The embryos were oriented manually so that the fusion plain was parallel to the electrodes. An AC field of 0.6 MHz and voltage of 5 V/mm was applied for 5 sec to induce alignment of the blastomeres. The electrofusion parameters were direct current (DC) pulse strength of 70 V/mm, pulse duration of 70 μsec and the pulse number was 2 (1-sec interval). These parameters were fixed during experiments except for those conducted to examine the effects of different parameters.

Experimental designs

Four sets of experiments were designed in the present study. The first set was

designed to examine the effects of electrofusion pulse strength (30–300 V/mm), pulse duration (30–90 μsec) and pulse number (1 to 5) on the fusion and development of zona-free 2-cell embryos after electrofusion in PBS+10% FCS. In the second set, the effects of the AC pulse and alignment time on the fusion and degeneration rates of zona-free 2-cell embryos following electrofusion in PBS+10% FCS were examined. The influence of various voltages and exposure times of an AC field of 0.6 MHz was also examined. The third set was conducted to compare fusion and development rates of embryos fused in different fusion media, PBS+10% FCS, physiological saline and 0.3 M mannitol supplemented with 0.05 mM $CaCl_2$ and 0.1 mM $MgSO_4$. In the fourth set, we investigated the effect of the mouse strain on the rate of fusion and development of zona-free 2-cell embryos electrofused in PBS+10% FCS. ICR zona-free 2-cell embryos were employed in all experiments, except in the fourth set, where ICR, ddY and F1 breeds were used together.

Statistical analysis

Data were analyzed by Fisher's exact probability test. Probabilities of $P<0.05$ were considered to be statistically significant.

RESULTS

Experiment 1

The fusion rate of embryos increased significantly ($P<0.05$) when the pulse strength was increased from 30 to 70 V/mm (Table 1). A further increase of the pulse strength to 100 V/mm or more reduced the rate of development to blastocysts significantly ($P<0.05$). Relatively higher rates of development of fused embryos to blastocysts were obtained when the pulse strength ranged from 30 to 70 V/mm and the highest overall development rate was obtained by a pulse strength of 70 V/mm. The pulse duration did not significantly affect the fusion and developmental rates as shown in Table 2. The fusion rate significantly ($P<0.05$) increased when pulse numbers were increased from 1 to 2 and stayed constantly high when the pulse number was further increased from 2 to 5 (Table 3). However, overall rates of development to blastocysts were not significantly different between 1 and 4 pulse groups and further increasing of the pulse number to 5 significantly ($P<0.05$) decreased the development rate to blastocysts.

Experiment 2

When the embryos were not exposed to an AC field prior to the DC pulse, 75% of the embryos were fused (Table 4). Application of AC voltage of 5 V/mm for 10 sec prior to the DC pulse increased the fusion rate. Increasing the AC voltage and alignment time had no effect on fusion rate.

Experiment 3

There was no difference in the rate of fusion and development to blastocysts among PBS+10% FCS, physiological saline and 0.3 M mannitol (Table 5).

Experiment 4

No significant difference was obtained in the rate of fusion and development to blastocysts among ICR, F1 (C57BL/6×CBA) and ddY (Table 6).

Table 1. The effects of pulse strength on the rates of fusion and development of zona-free mouse 2-cell embryos[a].

DC pulse (V/mm)	No. (%) of embryos			
	Treated	Fused	Developed to blastocysts[b]	Overall development[c]
30	40	8(20.0) [d]	8(100) [d]	8(20.0) [d]
50	40	25(62.5) [e]	24(96.0) [d]	24(60.0) [e]
70	91	91(100) [f]	86(94.5) [d]	86(94.5) [f]
100	89	89(100) [f]	62(69.7) [e]	62(69.7) [e]
150	87	87(100) [f]	53(61.6) [e]	53(61.6) [e]
300	91	91(100) [f]	54(59.3) [e]	54(59.3) [e]

[a] Results were pooled from 4 replicates. Pulse numbers and pulse duration were 2 pulses and 70 μsec (AC 5 V/mm). Fusion medium was PBS+10% FCS.

[b] Based on the number of fused embryos.

[c] Based on the number of treated embryos.

[d, e, f] Values with different superscripts in the same column differ significantly ($P<0.05$).

Table 2. The effects of pulse duration on the rates of fusion and development of zona-free mouse 2-cell embryos[a].

Pulse duration (μsec)	No. (%) of embryos			
	Treated	Fused	Developed to blastocysts[b]	Overall development[c]
30	58	57(98.3)	53(93.0)	53(91.4)
50	60	56(93.3)	47(83.9)	47(78.3)
70	75	70(93.3)	66(94.3)	66(88.0)
90	82	79(96.3)	71(89.9)	71(86.0)

[a] Results were pooled from 4 replicates. Pulse numbers and pulse strength were 2 pulses and 70 V/mm (AC 5 V/mm). Fusion medium was PBS+10% FCS.

[b] Based on the number of fused embryos.

[c] Based on the number of treated embryos.

Table 3. The effects of pulse number on the rates of fusion and development of zona-free mouse 2-cell embryos[a].

Pulse number[b]	No. (%) of embryos			
	Treated	Fused	Developed to blastocysts[c]	Overall development[d]
1	81	74 (91.4) [e]	66 (89.2) [e, f]	66 (81.5) [e]
2	85	80 (94.1) [e, f]	75 (93.8) [e]	75 (88.2) [e]
3	74	74 (100) [f]	64 (86.5) [e, f]	64 (86.5) [e]
4	75	75 (100) [f]	62 (82.7) [f]	62 (82.4) [e]
5	87	87 (100) [f]	36 (41.9) [g]	36 (41.4) [f]

[a] Results were pooled from 4 replicates.

[b] Pulse strength and pulse duration were 70 V/mm and 70 μsec (AC 5 V/mm). Fusion medium was PBS+10% FCS.

[c] Based on the number of fused embryos.

[d] Based on the number of treated embryos.

[e, f, g] Values with different superscripts in the same column are significantly different ($P<0.05$).

Table 4. The influence of AC volage and alignment time on the rate of fusion of zona-free mouse 2-cell embryos[a].

AC voltage (V/mm)	Alignment time (sec)	No. (%) of embryos			
		Treated	Fused	Not fused	Degenerated
0	0	40	30 (75.0) *	10 (25.0)	0 (0)
5	5	40	37 (92.5)	3 (7.5)	0 (0)
5	10	80	75 (93.8) *	5 (6.2)	0 (0)
5	15	30	28 (93.3)	2 (6.7)	0 (0)
10	5	47	42 (89.4)	2 (4.2)	3 (6.4)
10	10	40	36 (90.0)	4 (10.0)	0 (0)
10	15	37	33 (89.2)	4 (10.8)	0 (0)

[a] Results were pooled from 4 replicates. Pulse strength, pulse number, and pulse duration were 70 V/mm, 2 pulses and 70 μsec. Fusion medium was PBS+10% FCS.

* Differ significantly at $P<0.05$.

Table 5. The influences of fusion media on the rates of fusion and development of zona-free mouse 2-cell embryos[a].

Fusion medium	No. (%) of embryos			
	Treated	Fused	Developed to blastocysts[b]	Overall development[c]
PBS+10% FCS	251	241 (96.0)	227 (94.2)	227 (90.4)
physiological saline	90	88 (97.8)	84 (95.5)	84 (93.3)
0.3 M mannitol	100	91 (91.0)	85 (93.4)	85 (85.0)

[a] Results were pooled from 4 replicates. Pulse strength, pulse duration were 70 V/mm, 2 pulses and 70 μsec (AC voltage 5 V/mm).

[b] Based on the number of fused embryos.

[c] Based on the number of treated embryos.

Table 6. The effects of mouse strain on the fusion and development of zona-free mouse 2-cell embryos[a].

Strain	No. (%) of embryos			
	Treated	Fused	Developed to blastocysts[b]	Overall development[c]
ICR	94	94 (100)	83 (88.3)	83 (88.3)
F1[d]	67	67 (100)	63 (94.0)	63 (94.0)
ddY	70	69 (98.6)	61 (88.4)	61 (87.1)

[a] Results were pooled from 4 replicates. Pulse strength, pulse number and pulse duration were 70 V/mm, 2 pulses and 70 μsec (AC voltage 5 V/mm). Fusion medium was PBS+10% FCS.

[b] Based on the number of fused embryos.

[c] Based on the number of treated embryos.

[d] F1 (C57BL/6 × CBA).

Discussion

This study showed that increasing the pulse strength in PBS significantly increased the fusion rates (Table 1). However, the development rates were significantly reduced when the pulse strength was 100 V/mm or more. The results also showed that increasing the pulse duration did not significantly influence the fusion and development rates (Table 2). Furthermore, increasing the pulse number influenced the fusion rates significantly; however, 5 pulses was found to be harmful to development (Table 3). The reduction in the rates of development when we increased the pulse strength to 100 V/mm or more and the pulse number to 5 pulses may be attributed to the increase in the field intensity due to the low resistance between the electrodes in

PBS[12] and the use of a rectangular fusion chamber[3]. The degeneration rate was very low and sometimes there was none, contrasting with the findings of Winkel and Nuccitelli[21] who reported an increased rate of lysis in PBS. The difference in the rate of lysis in the 2 studies may be due to the use of FCS in PBS in our study as it has a protective effect against damage due to the redistribution of membrane proteins and lipids which may occur after an AC pulse application[22]. The highest overall rates of development were recorded when the pulse strength was 70 V/mm and the pulse numbers were 1 to 4.

Zimmermann[22] reported that the AC alignment pulse increases the area of cell-to-cell contact when applied prior to the fusion pulse and in the case of mammalian cell fusion, this degree of contact could be accelerated in the presence of electrolytes. In the present study we obtained a fusion rate of 75% when we used a DC pulse only (Table 4), which contrasts with the findings of Kubiak and Tarkowski[10] who reported no fusion by DC pulse of 60 V/mm without an AC pulse. This difference may be due to the different fusion chambers used in the 2 studies. We also found that increasing the AC pulse from 5 to 10 V/mm and alignment times from 5 to 15 sec did not affect the fusion rates. Clement and Brem[4] reported an increased rate of lysis of mouse blastomeres when the alignment time was extended in Zimmermann fusion medium. In our experiment, increasing the alignment time did not increase the degeneration rate. This difference in the rate of lysis between the two experiments may be due to the difference in the fusion media.

It worth mentioning that the use of DC voltage of 70 V/mm, with a pulse duration of 30 to 90 μsec, an AC voltage of 5 to 10 V/mm and pulse numbers from 1 to 4 were proved to be the parameters that led to the maximum fusion and development rates. Under these conditions there was no significant difference in the rate of fusion and development, whether the fusion medium was PBS, physiological saline or mannitol (Table 5). This finding is in agreement with that of Rickord and White[13] who reported no difference in the in vitro development of pronuclear-stage embryos pulsed in either 0.3 M mannitol containing 0.9 mM Ca^{2+} or PBS. When we employed the proved optimal fusion conditions to fuse 2-cell embryos from 3 different mouse strains, the fusion and development rates obtained were similar (Table 6). This finding agrees with that of Kato and Tsunoda[8] who reported that the mouse strain has no effect on the fusion and development rates of the mouse 2-cell stage embryos.

In conclusion, under the above mentioned fusion conditions the fusion of mouse 2-cell stage embryos can be carried out in PBS+10% FCS, physiological saline or mannitol and can be applied to any strain. However, further experiments are needed to confirm the positive effect of FCS on fusion and development rates.

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