Development of Murine Embryos Following Electroporation

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Purpose: The objective was to establish the parameters for reversible electroporation of murine embryos.

Methods: In Trial 1, murine presumptive zygotes received an electrical pulse of 5, 10, or 20- μ s duration, and one of five voltages (100, 200, 250, 300, or 400 V). In Trial 2, embryo orientation within the electroporation chamber was evaluated with 250 or 400 V at a pulse period of 10 μ s.

Results: Presumptive zygotes that received 400 V at each pulse length and zygotes exposed to 20 μ s at each voltage had the lowest embryonic development ($P < 0.05$). Presumptive zygotes that received 250 V had higher development compared to 400 V, irrespective of orientation ($P < 0.01$), but development was lower than the controls ($P < 0.01$).

Conclusions: Electrical stimulation of presumptive zygotes can have a detrimental impact on early embryo development, but low amounts of stimulation may allow for potential gene transfer in transgenic experimentation.

KEY WORDS: Electroporation; murine; presumptive zygotes; pulse period; voltage.

INTRODUCTION

The application of short, unipolar electric field pulses to cells alters the electrochemistry of the membrane lipid bilayer resulting in the formation of transient aqueous pores through which charged molecules may travel between the intracellular and extracellular environments in either or both directions. When the electrical disruption of the cell membrane is temporary, following electrical stimulation, the technique is termed reversible electroporation. However, the excessive application of electrical pulse or voltage can result in permanent pore formation and cell death, termed irreversible electroporation (1). In order for electroporation to be efficient, several parameters must be identified. Physical parameters include

voltage and pulse interval (2). Pulse period depends on the amount of energy stored in the capacitors of the electroporation apparatus. Voltage or pulse interval settings, where approximately 20–50% of the cells remain viable after electroporation, are thought to be sufficient to allow for DNA transfer in most cells (2,3).

Electroporation has a wide range of applications. Most commonly, electroporation has been applied in vitro to facilitate the transfer of DNA across cell membranes as in the example of bacterial cell transfection with plasmid (1). Novel in vitro electroporation techniques include the transdermal delivery of therapeutic oligonucleotides and proteins (4–6). Examples of recent in vivo electroporation technology include the stimulation of living animal tissues like seminiferous tubules (7), skeletal muscle (8–10), and avian embryos (11) to assist the transfer and establishment of regulated gene expression.

While new techniques for transgenic animal production are continuously emerging, the efficiency of these techniques is still very low and the cost of this inefficiency is high (12). Facilitated gene delivery by

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electroporation of embryos injected with DNA may be useful for improving the gene-transfer efficiency resulting in higher transgene integration rates. However, before transgenesis by electroporation can be studied, criteria for reversible electroporation of murine embryos should be established. The objective of this study was to assess the impact of voltage, pulse period, and embryo orientation on embryo development.

MATERIALS AND METHODS

Weaned female mice (strain CD-1) between 25 and 35 days of age, and weighing 19.5–23.0 g were administered 10 IU of equine chorionic gonadotropin (eCG; Diosynth, Chicago, IL) intraperitoneally (i.p.). After 46–48 h they were administered 5 IU human chorionic gonadotropin (hCG; Sigma, St. Louis, MO) i.p., and placed individually in cages containing a single CD-1 male. They were examined for the presence of vaginal plugs 21–24 h later, sacrificed, and their oviducts removed for embryo harvesting (13).

Presumptive zygotes were harvested at the cumulus stage and placed into M2 medium at 37.5°C (Sigma) at pH 7.4. The cumulus cells were dissociated from the embryos in 2.5 ml M2 medium containing 1 mg hyaluronidase (320 U mg⁻¹, Sigma). Following electroporation the presumptive zygotes were cultured at 37.5°C in 5.0% CO₂ equilibrated CZB droplets for 4 days (14). Approximately 40 embryos were cultured in each 10-μL droplet of CZB medium overlaid with silicon oil (15).

In Trial 1, presumptive zygotes were subjected to a single electrical pulse of 100, 200, 250, 300, or 400 V (DC square pulse) for a period of 5, 10, or 20 μs (ECM[®] 200; BTX Inc., San Diego, CA) in an electroporation microslide chamber (BTX Inc., Part #453). In Trial 2, presumptive zygotes were subjected to a single electrical pulse of either 250 or 400 V for a period of 10 μs, with orientation in either a linear or group formation. The orientation was either linear placement in a single file perpendicular to the electrodes in the chamber or random orientation in a group pattern. The electroporation medium consisted of sterilized 0.22 M D-mannitol (Sigma) with 0.01 % polyvinyl alcohol (Sigma) in HEPES (35 mM; Sigma) at a physiological pH of 7.2. The chamber had a 3.2-mm gap between two steel electrodes, which allowed for a 0.7-ml medium volume. The chamber was sterilized with 70% ethanol prior to use. Approximately 25–80 embryos were electroporated at one time.

The impact of the electroporation was assessed by evaluation of embryo development, 4 days following treatment. In Trial 1, there were four classifications of embryo development. Degenerate and lysed embryos were assigned the score of 1. Embryos that ceased development at the 1-cell to 2-cell stage were assigned the score of 2. Embryos that matured to the 3-cell to 10-cell stage were assigned the score of 3. Embryos that progressed to the morula to hatched blastocyst stages were assigned the score of 4. For Trial 2, development data were classified using an expanded scoring system, where a score of 1 was used for degenerate or lysed embryos, 2 for 2-cell embryos, 3 for 4-cell embryos, 4 for 8-cell embryos, 5 for 16-cell embryos, 6 for morula stage embryos, and 7 for embryos that developed to the blastocyst stage.

Each embryo was assigned one numerical score on day 4 based on its stage of development. The specific treatments tested included control(s), voltage, and duration of exposure to voltage, and in Trial 2, embryo orientation. Development scores at day 4 were analyzed using the general linear model analysis of variance in the Statistical Analysis System (SAS) system, using a model that included pulse interval, voltage, and their interaction (16) in Trial 1, while the model in Trial 2 included group and repetition. The repetition effects were not significant and excluded from final models.

RESULTS

Trial 1

Trial 1 included 1932 embryos. There was a significant effect ($P < 0.05$) of electroporation pulse interval on embryo development. Overall, presumptive zygotes that received the 5-μs electroporation pulse had a development score of 3.0 ± 0.05 , which was not different ($P > 0.05$) from the presumptive zygotes that received 10- or 20-μs pulse periods. However, the presumptive zygotes that received 10-μs electroporation pulse had a development score of 3.1 ± 0.04 , which was higher than the embryos that received the 20-μs electroporation pulse duration (2.9 ± 0.05).

Additionally, there was significant ($P < 0.01$) effect of voltage. Control embryos (3.4 ± 0.05 ; $n = 536$) and those that received 200 V (3.3 ± 0.07 ; $n = 274$) exhibited the highest development scores among treatments ($P > 0.05$). The presumptive zygotes administered 400 V had the overall lowest (2.4 ± 0.07 ; $n = 257$) development following electroporation ($P <$

Table I. Least Squares Mean ($\pm$ SE) Embryo Development Scores^a and Percentage of Murine Morulae/Blastocysts (%) 4 days Following Electroporation at Different Pulse Intervals and Voltages

Voltage	Pulse intervals (μ s)		
	5	10	20
Controls	3.4 ^b $\pm$ 0.10 (62.6)	3.3 ^b $\pm$ 0.08 (71.8)	3.4 ^b $\pm$ 0.07 (71.6)
100 V	2.6 ^c $\pm$ 0.10 (38.9)	3.2 ^b $\pm$ 0.11 (51.5)	2.8 ^c $\pm$ 0.10 (43.8)
200 V	3.5 ^b $\pm$ 0.13 (46.8)	3.2 ^b $\pm$ 0.10 (66.7)	3.3 ^{b,e} $\pm$ 0.12 (56.4)
250 V	3.0 ^d $\pm$ 0.12 (37.3)	3.2 ^b $\pm$ 0.11 (67.0)	2.7 ^{c,f} $\pm$ 0.13 (32.0)
300 V	3.3 ^{b,d} $\pm$ 0.12 (45.6)	3.1 ^b $\pm$ 0.10 (62.3)	3.0 ^{c,e} $\pm$ 0.12 (39.5)
400 V	2.2 ^e $\pm$.011 (20.3)	2.6 ^c $\pm$ 0.11 (33.0)	2.4 ^d $\pm$ 0.15 (13.6)

^a Scores: 1, degenerate and lysed embryos; 2, 1- to 2-cell embryos; 3, 3- to 10-cell embryos; and 4, morula to hatched blastocyst stage embryos.

^{b-f} Means with different superscript differ ($P < 0.05$).

0.05). The groups electroporated with 100 V ($n = 331$) and 250 V ($n = 256$) had mean development scores of 2.9 ± 0.05 and 3.0 ± 0.07 , respectively, and were not different ($P > 0.05$). The group that received 300 V ($n = 278$) had a mean development score of 3.1 ± 0.07 .

There was an electroporation pulse interval by voltage interaction ($P < 0.01$) on embryo development (Table I). In the pulse interval by voltage interaction the control zygotes had similar development ($P > 0.05$) and morula/blastocyst development ranged from 63 to 72%. Development of presumptive zygotes subjected to a 5- μ s pulse period and 200 and 300 V; 10 μ s and 100, 200, 250, and 300 V; and 20- μ s pulse interval and 200 V was not different from controls ($P > 0.05$) and morula/blastocyst development ranged from 46 to 67%. Lowest embryo development score was found with 400 V among all pulse intervals, and morula/blastocyst development was less than 35%. The highest voltage stimulation that did not impact embryo development occurred at 300 V for 5- or 10- μ s pulse periods and at 200 V for a 20- μ s pulse interval.

Trial 2

There was no significant effect ($P > 0.05$) of electroporation on development because of presumptive zygote orientation in the chamber. However, there was a significant voltage effect on presumptive zygote development ($P < 0.01$). Exposure to the D-mannitol medium in the electroporation chamber had no significant effect ($P > 0.05$) on embryo development. Trial 2 included 1528 presumptive zygotes. Presumptive zygotes placed in mannitol medium (4.36 ± 0.18) and nonelectroporated control embryos (4.28 ± 0.12) had the highest development scores ($P < 0.01$) and had about 40% morula/blastocyst development. Pre-

sumptive zygotes exposed to 400 V in the group orientation (2.06 ± 0.12) and to 400 V in the linear orientation (1.97 ± 0.13) had the lowest overall development ($P < 0.01$) and less than 10% morula/blastocyst development. Presumptive zygotes electroporated with 250 V in the group orientation (3.42 ± 0.12) and with 250 V in the linear orientation (3.32 ± 0.12) had the highest development score ($P < 0.01$) of the voltage-treated embryos, but development was lower ($P < 0.01$) than nonelectroporated controls (Table II). Moreover, morula/blastocyst development was about half that of controls.

DISCUSSION

Permeation occurs in the presence of an electric field pulse when the energy applied exceeds the energy holding the lipid bilayer intact (17). Generally, electroporation has been applied to facilitate the movement of molecules across permeated biological membranes. While, this study did not attempt to introduce specific molecules to murine embryos by electroporation, it did examine three pulse durations (5, 10, and 20 μ s) and five voltage (100, 200, 250, 300, and 400 V) impacts on embryonic development. Subsequently, the effects of embryo orientation within the electroporation chamber at two separate voltages (250 and 400 V) were examined using a 10- μ s pulse duration.

In the absence of a selectable marker or dye to visually identify molecular movement across embryonic membranes, and therefore assess membrane permeability, embryo development different from control embryos was the criteria available to assess pulse period and voltage effects. Among groups of electroporated presumptive zygotes that developed similar to controls (5- μ s pulse interval at 200 and 300 V;

Table II. Least Squares Mean ($\pm$ SE) Development Scores^a and Percentage of Murine Morulae/Blastocysts (%) 4 days Following Electroporation at Different Voltages and Orientations

Treatment	Orientation	<i>n</i>	Development score $\pm$ SE	Morulae/blastocysts (%)
Control	—	270	4.28 ^b $\pm$ 0.12	40.0
Mannitol	—	185	4.36 ^b $\pm$ 0.18	38.4
250 V	Group	268	3.42 ^c $\pm$ 0.12	22.7
250 V	Linear	272	3.32 ^c $\pm$ 0.12	23.5
400 V	Group	272	2.06 ^d $\pm$ 0.12	9.9
400 V	Linear	261	1.97 ^d $\pm$ 0.13	6.5

^a Scores 1, degenerate and lysed embryos; 2, 2-cell embryos; 3, 4-cell embryos; 4, 8-cell embryos; 5, 16-cell embryos; 6, morula stage embryos; and 7, blastocyst to hatched blastocyst stage embryos.

^{b-d} Means with different superscript differ ($P < 0.01$).

10- μ s pulse interval at 100, 200, 250, and 300 V; and 20- μ s pulse interval at 200 V), it is difficult to conclude whether or not adequate transmembrane potential was generated to render the membranes permeable. More lysing occurred when presumptive zygotes were subjected to longer pulse intervals (10 μ s at 100 V to 20 μ s at 800 V) in what appeared to be a dose-response effect (18). Embryo lysing was greatest for all voltages when pulse duration was 20 μ s and at 800 V for 10 μ s.

When the electric field pulse is withdrawn, the lipid bilayers immediately restore. However, they may remain permeable for a long time thereafter (17). The electroporated embryos that developed in spite of adverse stimulation (5- μ s pulse period at 100, 250, and 400 V; 10- μ s pulse period at 400 V; and 20- μ s pulse period at 100, 250, 300, and 400 V) may have suffered prolonged membrane permeability that allowed for longer periods of molecular flow into and out of the embryo. The data indicate the following: 1) lowest development was found in embryos electroporated with 400 V, the highest voltage applied and 2) regardless of voltage all embryos electroporated for 20 μ s had lower development than embryos electroporated for 5 or 10 μ s. The development scores reflected the percentage of morulae/blastocysts at day 4. The anomalous development of embryos in the 100-V treatments is difficult to explain.

The detrimental effects of the 400-V exposures may also be exacerbated because of the large cell size of the embryo. Cell diameter contributes to the efficiency of electroporation. Cells with a smaller diameter, as compared with larger diameter cells, can survive electroporation with larger voltage and capacitance (3,19). Therefore, cell diameter plays an important role in selecting voltage and capacitance parameters for electroporation and subsequent

survivability. This is clearly demonstrated as embryos exposed to the 400 V had lower development than all other treatments.

The voltage effects seen in Trial 1 were also demonstrated in Trial 2, irrespective of linear or group orientation. The 10- μ s electroporation pulse period was chosen because of the excessive instability shown in embryos in Trial 1 receiving electroporation for the 20- μ s pulse duration. Embryo grouping appears to have no protective benefit to increase viability, as no significant difference was seen between group and linear orientation at either voltage.

Biological parameters also play an important role in the efficiency of electroporation. These parameters include the ionic strength of the cell suspension medium (20). The optimal medium for transmission of the electrical impulse will have low resistance and high conductivity. The efficiency of electroporation, for some cell types, can be adversely affected by divalent cations found within the media. These divalent cations can act to stabilize the cell membrane, and thus cause resistance to the disruption of the membrane (21). Careful consideration was given to select a medium (D-mannitol) with the proper properties for the specific cell type in use. Consideration of these properties for the embryo allowed for isolation of the effects of voltage and pulse period on embryo development by reducing the significance of the molecules transported across the membranes during electroporation.

In the case of electroporation facilitated transfection, DNA transfer efficiency depends primarily on the length of a pore-forming voltage, not on the duration of the membrane-permeated state. In other words, more DNA enters cells directly through electropores formed and sustained during applied voltage than by electrophoresis during the lingering

permeated state that follows (17). For the purposes of embryo viability, the 20- μ s pulse interval was excessive and probably allowed extreme influx inside the cell membranes, thereby rendering the embryos unstable. Likewise, the application of 400 V may have been excessive and left the membranes permeable for an extended period during which time debilitating electrophoresis may have occurred in both directions.

Electroporation has been shown to be a valuable tool in the transfer of various molecules from the extracellular environment into the cellular matrix. Charged molecules like DNA may cross an electroporation induced permeated cell membrane by electrophoresis, as in the case of transfection (17). This same technology could also be used in the making of transgenic animals. Cytoplasmic injection of DNA is a particularly inefficient technique for producing transgenic animals (22,23). This procedure might be greatly enhanced by the application of embryo electroporation.

These data suggest that while higher voltages of electrical stimulation have a detrimental effect on zygote development, low amounts of electrical stimulation may allow for potential gene transfer in transgenic experimentation. The high cost and low efficiency of the current transgenic animal production techniques clearly demonstrate the need for a more efficient means of transgenic animal production. Further experimentation may reveal those specific combinations of injections and electrostimulation may improve the efficiency of gene transfer and transgene integration.

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