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Methodology article

Optimization of ectopic gene expression in skeletal muscle through DNA transfer by electroporation

Jordan Taylor[1], Charlie F Babbs[1], Mohammed Borhan Alzghoul[2], Aaron Olsen[1], Mickey Latour[3], Amber L Pond[1] and Kevin Hannon*[1]

Address: [1]Department of Basic Medical Sciences, Purdue University, W. Lafayette IN 47907, USA, [2]Department of Basic Veterinary Medical Science, Jordan University of Science and Technology, Irbid, Jordan and [3]Department of Animal Sciences, Purdue University, W. Lafayette IN 47907, USA

Email: Jordan Taylor - jat4@cec.wustl.edu; Charlie F Babbs - Babbs@purdue.edu; Mohammed Borhan Alzghoul - alzghoul@just.edu.jo; Aaron Olsen - aleroyo2002@cableone.net; Mickey Latour - Latour@purdue.edu; Amber L Pond - pond@purdue.edu; Kevin Hannon* - Hannonk@purdue.edu

* Corresponding author

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Abstract

Background: Electroporation (EP) is a widely used non-viral gene transfer method. We have attempted to develop an exact protocol to maximize DNA expression while minimizing tissue damage following EP of skeletal muscle *in vivo*. Specifically, we investigated the effects of varying injection techniques, electrode surface geometry, and plasmid mediums.

Results: We found that as the amount of damage increased in skeletal muscle in response to EP, the level of β-galactosidase (β-gal) expression drastically decreased and that there was no evidence of β-gal expression in damaged fibers. Two specific types of electrodes yielded the greatest amount of expression. We also discovered that DNA uptake in skeletal muscle following intra-arterial injection of DNA was significantly enhanced by EP. Finally, we found that DMSO and LipoFECTAMINE™, common enhancers of DNA electroporation *in vitro*, had no positive effect on DNA electroporation *in vivo*.

Conclusions: When injecting DNA intramuscularly, a flat plate electrode without any plasmid enhancers is the best method to achieve high levels of gene expression.

Background

The field of gene therapy has great potential in terms of therapeutic applications. The goal of this science is to develop a method by which genes can be delivered to tissue *in vivo* and produce high localized expression without severe damaging effects. Recently, a novel gene therapy technique has emerged that involves injection of naked DNA coupled with electroporation (EP) [1,2].

During EP, a large voltage gradient is produced across the muscle for a fraction of a second. This gradient produces pores in the membranes of muscle cells, allowing extracellular plasmids to enter the cell. The exact mechanisms of this phenomenon are currently unknown. The amount of gene expression is highly variable, depending upon electrical parameters, plasmids, and injection methods. Additionally, EP can cause damage to the tissue resulting in mineralized and hylanized necrosis. The amount of damage the tissue experiences may be a function of the exact method of EP. In the present research, we attempted to develop an exact protocol to maximize gene expression while minimizing expression variability and tissue

damage. Specifically, this work investigated the effects of injection techniques, electrode surface geometry, and plasmid mediums.

Methods

Standardized electrical field parameters

A 2100 isolated pulse stimulator (AM-systems, Carlsborg, WA) gating a GRASS S44 Solid State Wave Generator stimulator (GRASS Instruments, Quincy, MA) connected to a caliper electrode (BTX, San Diego, CA) was used to perform the EP. The thickness of the mouse's muscle was measured by using the BTX caliper electrode and the electrical parameters were adjusted accordingly to achieve a voltage of 200 V/cm for 20 ms at 1 Hertz, as determined previously by Mir *et al* to be an effective waveform for naked DNA expression in mice undergoing EP [1]. The mice were anesthetized with ketamine/xylazine (0.1 cc per 10 g body weight) before the DNA injection and EP. All experimental mice were NIH Swiss Mice (Harlan, Indianapolis, IN) and received the same treatment unless otherwise noted.

Whole tissue β-Gal assay

The muscle was prepared by a homogenizer (Brinkman, Pittsburgh, PA) in potassium phosphate buffer with PMSF and centrifuged at 3000 gs for 15 minutes. β-galactosidase (β-gal) enzyme levels in the supernatant were assayed with the Galacto-Star (Tropix, Foster City, CA) kit using a Lumat LB 9507 luminometer (Berthhold Technologies, Oak Ridge, TN). β-Gal activity was, therefore, reported in relative light units. A standard curve for the luminescent reaction was developed with known amounts of β-Gal enzyme (Sigma, St. Louis, MO) and an equation relating relative light units to β-Gal protein amount was determined and used to convert sample luminescence into mg β-Gal protein by linear regression. The total amount of protein in each muscle sample was also determined by a DC Protein Assay Kit (Bio-Rad, Hercules, CA) and divided into the β-Gal amounts to normalize the data and correct for the different masses of the muscles. All the data are reported as mean % β-Gal expression (average value of [β-Gal activity (mg)/total protein (mg)]. All results were then analyzed by Statistical Analysis Software (SAS, Cary, NC) for continuous variables.

Damage assessment

Hemotaxlyin and Eosin (H&E) staining of light microscopic sections was used to quantify the amount of damage the muscle experienced due to EP. The gastrocnemius of the mouse was embedded in O.C.T. compound (Tissue-Tek, Torrance, CA) and frozen in liquid nitrogen. The tissue was cross-sectioned using a cryostat (ThermoShandon, Pittsburg, PA) at -28°C. The slides were allowed to dry and were then stained using H&E. The slides were coverslipped by using Immunomount (ThermoShandon, Pittsburgh, PA) and were photographed using a Leaf microlumina mounted to a microscope (Olympus, Melville, NY). After H&E staining, necrotic fibers appear dark purple and are distinct from surrounding uninjured fibers. By using Adobe Photoshop® (San Jose, CA) the area of necrosis for each section was highlighted and the number of pixels in this location was recorded. The total area of each section was highlighted and the total number of pixels was recorded. The area of necrosis was divided by the total area of skeletal muscle tissue in the section. To normalize the data, the amount of damage of each section for each mouse was averaged to take in account the expression variation between the proximal and distal portion of the muscle.

Electrode geometry

After a 50 μl aliquot of saline containing 50 μg CMVLacZ plasmid was injected into the right and left gastrocnemius of the mice using a Hamilton syringe (Hamilton, Reno, NV), four different electrodes were designed with varying degrees of perforation in the plates (Figure 1). Both legs were electroporated using one of the four different electrodes (3 mice per electrode, Groups 4, 5, 6 and 7 in Table 1).

Histochemical staining

The mice were euthanized after 7 days and both gastrocnemius muscles were harvested. The left muscles were prepared and assayed as described. The right gastrocnemius of each mouse was embedded in O.C.T. compound and frozen in liquid nitrogen. The tissue was cross-sectioned using a cryostat at -28°. The slides were allowed to dry and were then stained for LacZ activity using X-gal. The slides were coverslipped by using Immunomount and photographed. By using Adobe Photoshop® the area of expression for each section was highlighted and the number of pixels in this location was recorded. The total muscle area of each section was highlighted and the total number of pixels was recorded. The area of expression was divided by the total muscle area of the section.

Intra-arterial and intramuscular injection

Fifty μl of sterile saline containing 50 ug of CMVLacZ construct was injected into the right and left femoral arteries of 4 NIH Swiss Mice (Harlan, Indianapolis, IN) using a Hamilton syringe. The right limb was electroporated with the BTX caliper flate plate electrode, while the left leg was not (Table 1, Groups 1 and 2). The mice were euthanized 7 days later and the gastrocnemius muscle of each leg was harvested. The muscles were prepared and the β-Galactosidase enzyme content was determined.

To compare intra-arterial injection (IA) and intramuscular injection (IM), the same concentration of the same plasmid was injected into the right and left gastrocnemius of

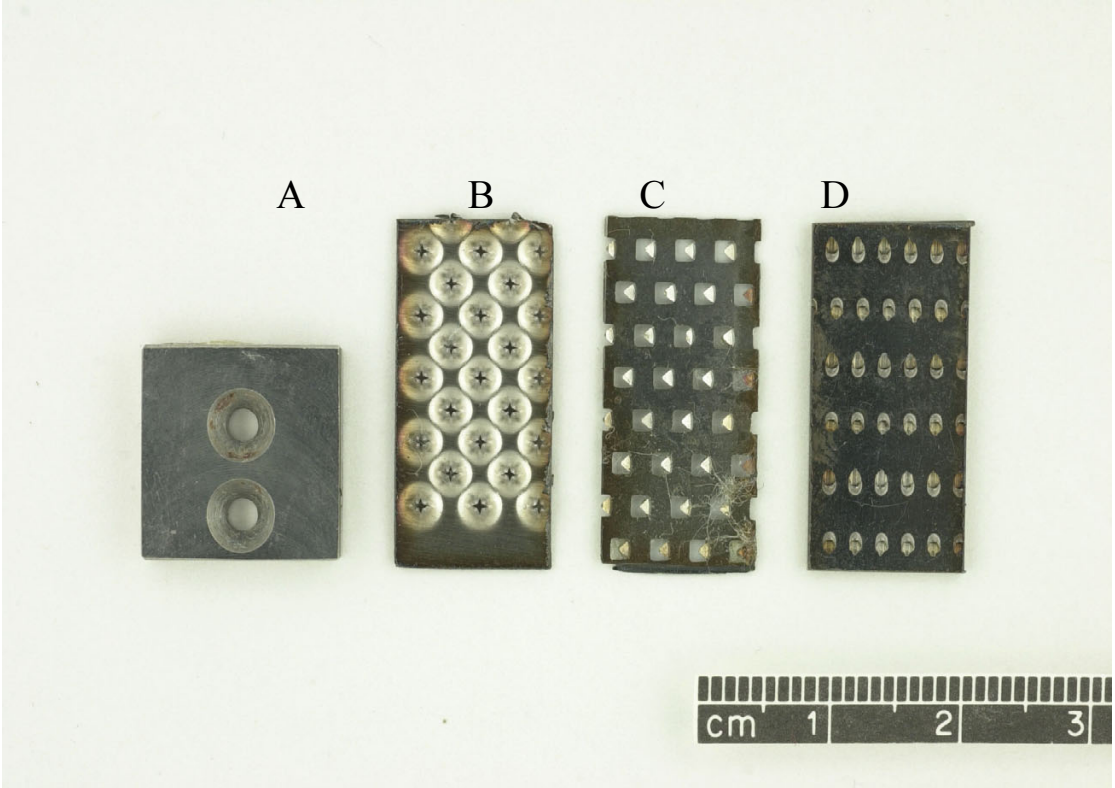

Figure 1
Electrode Surface Geometry. Four electrodes, labeled above, were tested on mice to determine the effect of surface geometry of DNA expression following electroporation into skeletal muscle.

an additional 4 mice. The right limb was electroporated, while the left leg was not (Table 1, Groups 3 and 4). The mice were euthanized 7 days later and the gastrocnemius muscle of each leg was harvested. The muscles were prepared and the β-Galactosidase enzyme content was determined.

LipoFECTAMINE and DMSO

The LipoFECTAMINE™ and the CMVLacZ plasmid were combined at a 1:5 ratio and allowed to react for 6 hours for optimal expression [3]. The plasmid/LipoFECTAMINE™ solution was injected into the right and left gastrocnemius of the mice using a Hamilton syringe. The right limb was electroporated with the BTX caliper flat plate electrode, while the left limb was not (Table 1, Groups 9 and 10). The mice were euthanized after 7 days and both gastrocnemius muscles were harvested. The muscles were assayed.

A 50 μg sample of CMVLacZ plasmid was solubilized in a sterile normal saline solution containing 1.25% DMSO. This solution was injected into the right and left gastrocnemius of 7 Harlan mice using a Hamilton syringe. Only the right limb was electroporated (Table 1, Group 8). The mice were euthanized after 7 days and both gastrocnemius muscles were harvested. The muscles were prepared and assayed.

Table 1: Comparison of the different injection and electroporation techniques. Intramuscularly injected mice are represented as IM and intra-arterially injected mice are represented as IA. Mice that were electroporated are identified with a + sign and mice that were not electroporated are identified with a - sign. The four different electrode types are identified with letters that correspond to the letters in figure 3. [a] mean % β-Gal expression = average value of [β-Gal activity (mg)/total protein (mg)]. [b,c,d,e,f,g] statistical significance (p < .05) between groups with differing superscripts. n = 7 for each treatment group.

Group #	Limb	Injection	EP	Electrode Type	Mean % β-Gal expression[a] (+/- SD)	Mean % Area of Muscle Damage (+/- SD)
1	Left	IA CMVLacZ in 0.9% Saline	No EP	No EP	0.0475[b] ± 0.0377	
2	Right	IA CMVLacZ in 0.9% Saline	+	A	0.4900[h] ± 0.0200	
3	Left	IM CMVLacZ in 0.9% Saline	No EP	No EP	0.0200[b] ± 0.0082	
4	Right	IM CMVLacZ in 0.9% Saline	+	A	7.790[c,e] ± 4.5254	5.6 ± 4.9
5	Right	IM CMVLacZ in 0.9% Saline	+	B	5.04[c,d,f] ± 2.4683	8.5 ± 4.9
6	Right	IM CMVLacZ in 0.9% Saline	+	C	3.6167[d] ± 2.2234	21.3 ± 6.1
7	Right	IM CMVLacZ in 0.9% Saline	+	D	11.650[e,g] ± 3.0645	3.4 ± 4.9
8	Right	IM CMVLacZ in 1.25% DMSO	+	A	8.190[e,f,g] ± 1.9986	
9	Left	IM CMVLacZ in LipoFECTAMINE	No EP	No EP	0.0214[b] ± 0.0107	
10	Right	IM CMVLacZ in LipoFECTAMINE	+	A	0.0414[b] ± 0.1912	

Results

Necrosis

We found that as the amount of muscles damage (reported as percent necrosis) increased, the level of β-Gal expression significantly decreased (Figure 2). A second order polynomial was fit to the data with a correlation coefficient of 0.826 ($p < .01$). Furthermore, there was no evidence of β-Gal expression in damaged fibers (Figure 3).

Electrode geometry

The difference between electrode types is reported in Table 1. With constant 200 V/cm voltage gradient shocks, electrode A (group 4) and D (group 7) delivered the highest levels of B-gal activity, but were not significantly different from one another ($p < 0.17$). Electrode A and electrode D resulted in greater levels of expression, ($p < 0.02$) and ($p < 0.01$) respectively, than electrode C (group 6). Electrode D delivered higher levels of β-Gal activity ($p < 0.04$) than electrode B (group 5). There was no statistical difference between electrodes B and C. By using the H&E staining, the level of damage was analyzed. When the muscles were analyzed for damage, there was a trend for greater damage with electrode D. However, there were no statistically significant differences between the different electrodes (Table 1).

Intra-arterial and intramuscular injection

There was no statistically significant difference in the β-Gal expression levels of the muscles receiving IA and IM injections (Table 1, group 1 and 3, respectively). Both IA and IM injection followed by EP (groups 2 and 4, respectively) produced significantly higher ($p < 0.01$) β-Gal expression than the injection of naked DNA alone without EP by either injection route (groups 1 and 3). When expression levels of the mice that received IA injection followed by EP (group 2) were compared to the expression levels of mice that received IM injection followed by EP (group 4), it was found that IM injection produced higher levels of B-gal activity ($p < 0.05$). These results demonstrate that EP indeed improved β-Gal expression levels regardless of injection route, but IM injection of DNA followed by EP is the most efficient method in producing high levels of DNA expression.

LipoFECTAMINE and DMSO

Mice that received injection of CMVLacZ in LipoFECTAMINE with EP (group 10) and mice that received injection of CMVLacZ and LipoFECTAMINE without EP (group 9) did not express β-Gal levels significantly higher than background. In addition, there was no statistically significant difference between the two groups (Table 1).

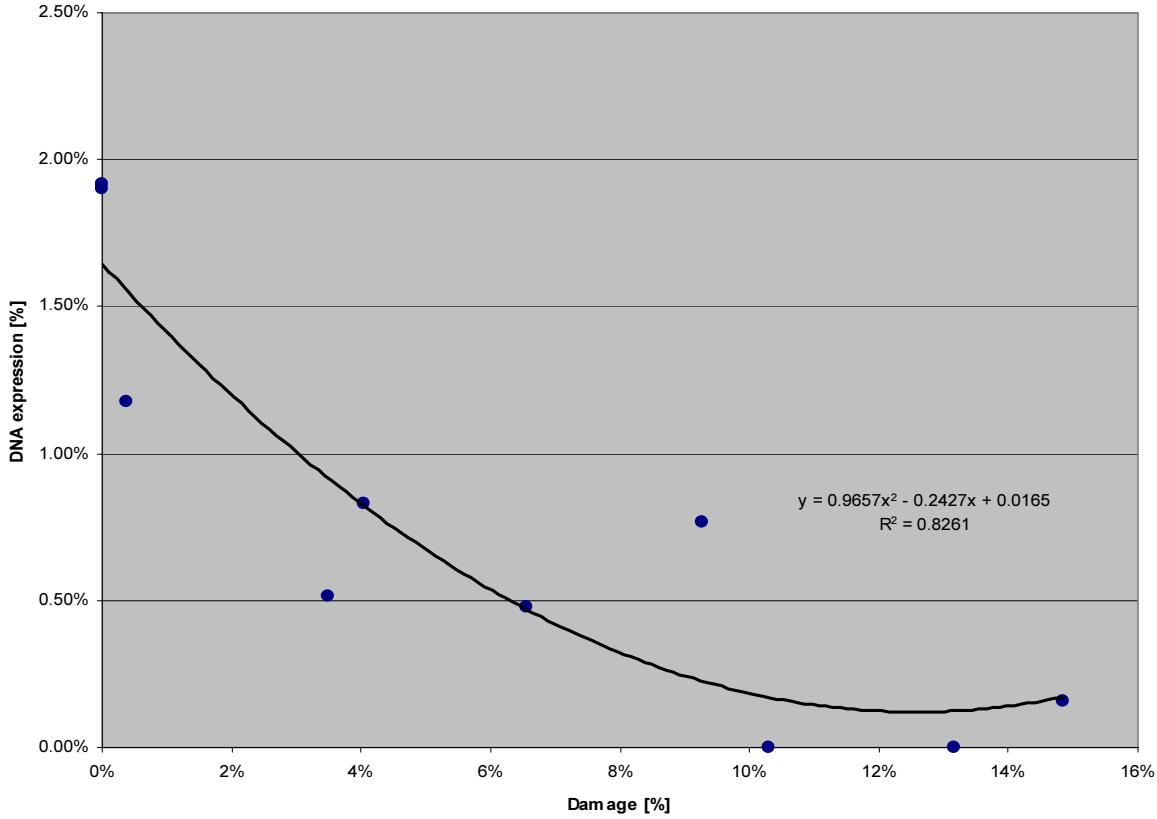

Figure 2
Ectopic DNA expression decreases with increasing tissue damage following electroporation. LacZ expressing cDNA was electroporated into the gastrocnemius/soleus skeletal muscle. DNA expression was quantitated by measuring the amount of b-gal staining per area total muscle, and is reported as a percent of the gastrocnemius/soleus muscles. Tissue damage was measured and is reported as a percent of damage per total gastrocnemius/soleus muscle area. A second order polynomial was fitted to the data and it was found that there was a correlation coefficient of 0.826, which corresponds to a statistical significance of $p < 0.01$. This polynomial demonstrates that with increasing skeletal muscle damage, the amount of ectopic DNA expression decreases.

Comparing the muscles that were injected with CMVLacZ in 1.25% DMSO (group 8) and the muscles injected with CMVLacZ in normal saline (group 3) demonstrated that no significant difference existed between the groups (Table 1).

Discussion

Tissue damage

We found that there was a decrease in β-Gal expression with increasing muscle damage (Figure 2). Areas of the tissue that experience damage and necrosis do not express CMVLacZ because the fibers are dead (Figure 3). The damage in Figure 3 is apparent because necrotic, mineralized fibers appear opaque on phase-contrast microscopy and gene expressing fibers appear blue due to the X-gal staining. Further investigation is required to find the optimum voltage for maximal expression and minimal damage.

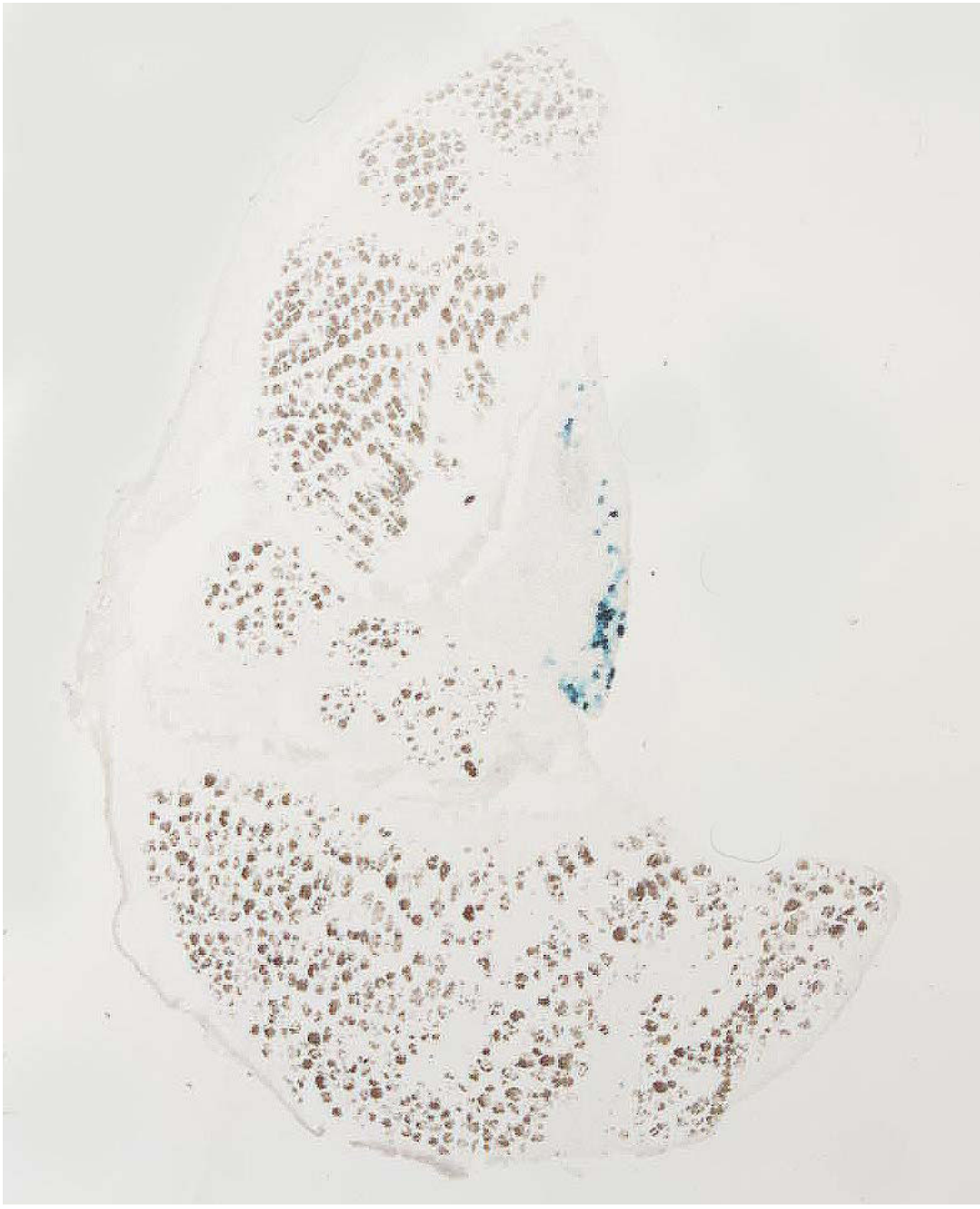

Figure 3
Ectopic DNA expression following electroporation does not occur in damaged muscle fibers. Micrograph above represents an example of a seriously injured muscle following electroporation. Blue fibers are cells that are expressing ectopic LacZ. Mineralized necrosis is apparent in the opaque, brown fibers. Ectopic DNA expression and muscle damage are mutually exclusive.

Electrode geometry

For most EP, plate electrodes are used because these are less invasive, easier to use and contact a larger area resulting in a greater distribution of expression. For our study, plate electrodes were designed to decrease the actual surface area contact by adding perforations of varying number, size, spacing and geometry. The perforations within a plate electrode will decrease actual surface area contact and may decrease the total area of damage while maintaining current flow over a larger area than a needle point electrode. By choosing electrodes of this geometry, it is intended that the area of tissue damage resulting from electrode contact will be reduced while maintaining a large area of current flow. By varying the surface geometry of the electrodes, the current concentration geometry may be changed in the muscle. We hypothesized that the different current patterns might cause increased expression of β-Gal because DNA uptake is proportional to current concentration. Additionally, Liu and Guang, reported that tissue damage could be curtailed by decreasing the contact points of the electrodes with the tissue [4]. In their study, a syringe electrode was used to deliver the DNA and perform the EP [4]. A syringe electrode has only one contact point with the tissue and this situation decreases the area of the current flow, henceforth increasing current density to this smaller area. Therefore, this electrode reduced the amount of damage resulting from the procedure. However, the area of expression was also reduced. Our results did not demonstrate a significant difference in the amount of damage between plain or perforated electrodes. However, our data demonstrated that the flat plate electrode A (group 4) and electrode D (group 7) had the highest mean level of expression. Therefore, either of these two electrodes would be favorable for EP.

Intra-arterial and intramuscular injection

Zhang *et al* showed previously that IA injection of CMVLacZ into the femoral arteries of rats and monkeys increased β-Gal activity in skeletal muscle [5]. It is suggested that the artery transported the injected DNA throughout the leg and into the cells. Zhang also suggested that the increase in pressure from the injection device causes the cell to porate [5]. We hypothesized that combining EP with IA injection would greatly increase expression levels of the injected DNA and better distribute the expressed protein. Although IA injection with EP increases the expression of β-Gal, it does not produce more expression than IM injection. IA injection may be promising in full dispersion of the CMVLacZ since the blood will transport the DNA to most areas of the hindlimb, but more volume of DNA and greater coverage of EP would be needed. The advantage to using IA injection and EP is that DNA can be delivered to a greater area, while IM injection is localized to the injection site. Another advantage to using the IA injection is that the procedure allows for two mechanisms for extracellular DNA uptake. Recent studies suggest that the blood transports the pDNA (~10 nm) to the cells and any molecule under 20–30 nm in size will be consumed by the muscle endothelium [6]. The mechanism for capillary delivery of macromolecules is unclear, but it is proposed that it occurs through transcytosis [7]. Our data demonstrated that IA injection of DNA followed by EP increased expression in comparison to IA injection alone. However, IM injection of DNA with EP produced expression over 15 times that of IA injection and EP. Further investigation and refinement of the IA injection technique will be needed to fully develop its potential.

LipoFECTAMINE and DMSO

DMSO is an amphillic compound that stabilizes the cell membrane and it has been shown to increase transfection efficiency in cultured cells [8]. We coupled DMSO with the plasmid before injection into the muscle to determine if it would increase DNA expression when EP followed the injection. In our model, the addition of DMSO did not increase expression *in vivo* as it does in vitro.

LipoFECTAMINE™ is a lipid that binds with DNA to make it more lipophilic and, therefore, more likely to gain entry into the cell [3]. It has been shown that combining LipoFECTAMINE™ with CMVLacZ plasmid increases transfection in cell lines [3]. When LipoFECTAMINE was combined with CMVLacZ and coupled with EP, it prohibited β-Gal expression. The exact cause of it is currently unknown. Recent studies have shown that LipoFECTAMINE only works in a serum-free environment [3]. DOSPER, which is another lipophilic medium, has proven to work in serum environments with comparable expression as LipoFECTAMINE in the transfection of cell lines [3]. Another cause of LipoFECTAMINE stopping DNA transfer during electroporation is because the reaction causes the CMVLacZ to become more lipophilic, which in turn reduces the charge on the DNA. This lack of charge may prohibit EP because EP may rely on the extended portion of the 20 ms square wave to actually drive the DNA into the cell. If the DNA were lacking any charge, then the electroporation efficiency would be greatly reduced, as seen by Table 1.

Conclusions

Our objective was to develop and refine a protocol to maximize electroporated DNA expression in skeletal muscle while minimizing tissue damage. Our data suggests that when injecting DNA intramuscularly, a flat plate electrode without any plasmid enhancers is the best method yet tested for high levels of gene expression. In addition, we found that ectopic expression of DNA within skeletal muscle following IA injection is significantly enhanced by EP. Finally, we observed that ectopic DNA expression and

muscle damage are mutually exclusive. Understanding mechanisms of, and prevention of, muscle damage following EP could significantly enhance the efficiency of DNA electroporation into skeletal muscle *in vivo*.

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