Gene Ther Mol Biol Vol 9, 329-338, 2005

Innovative electroporation for therapeutic and vaccination applications

Review Article

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Key words: electroporation, plasmid, gene transfer, skeletal muscle

Abbreviations: Electroporation, (EP); short interfering RNAs, (siRNA)

Received: 4 October 2005; Accepted: 17 October 2005; electronically published: November 2005

Summary

Electroporation (EP) has been used for years as a tool to increase macromolecule uptake in tissues, including nucleic acids for gene therapeutic applications. Skeletal muscle is a preferable target tissue for a number of reasons including long-term secretion of therapeutic proteins for systemic distribution and promotion of strong humoral and cellular immune responses post-vaccination. All of these DNA-mediated applications are significantly improved by *in vivo* EP. We have demonstrated previously that constant-current EP is effective for intramuscular plasmid delivery in mammals and does not cause permanent damage to cells. Numerous other factors impact plasmid uptake and expression after intramuscular injection followed by EP, such as plasmid size, formulation, concentration, injection volume, intensity of the electric field, target muscle, and species and age of the treated subject. These improvements in the conditions of EP can increase the efficacy of plasmid transfer and lower the total amount of plasmid and DNA vaccines required to generate targeted levels of biologically active proteins or antibodies.

I. Introduction

Recent developments in gene transfer technology are promising and could either supplement or potentially replace the classic recombinant protein or vaccination regimens, eventually leading to the development of effective and efficient therapeutic or preventive strategies (Herweijer and Wolff, 2003). Gene delivery allows the transgene product to be synthesized within target cells using host expression machinery taking advantage of host secretory or antigen presentation mechanisms. An ideal delivery system for *in vivo* gene transfer should have well-characterized pharmaceutical properties; have few, if any, safety issues; provide prolonged expression of transgenes at therapeutic levels if delivered into tissues with low turnover time, with the ability to be re-dosed; avoid auto-antibody responses following re-administration (Jiao et al, 1992); and last, but not least, be easy to manufacture at a reasonable cost (Hebel et al, 2005). Plasmids can fulfill all these requirements. Furthermore, plasmids can be naturally taken up into undamaged organs and tissues (Wolff and Budker 2005). Nevertheless, when compared to viral or liposomal vectors, there are disadvantages such as extracellular degradation and binding and poor nuclear uptake (Wolff et al, 1997; Hebert, 2003). For instance, less than 0.01% of plasmid directly injected into the skeletal muscle will be taken up and possibly expressed (Wolff et al, 1990, 1992b).

While the target organ vastly depends on the particular gene therapeutic application, the skeletal muscle is a preferable target tissue for numerous reasons: there is convenient access to multiple muscles with direct intramuscular injection resulting in a localized expression site (Jiao et al, 1992); skeletal muscle can act as an effective platform for the long-term secretion of therapeutic proteins for systemic distribution similar to an endocrine organ (Goldspink, 2003); the transgene expression product may have a better pharmacological profile compared to repeated injection of recombinant proteins; a pressing need exists to develop effective therapies for muscular dystrophies (Braun, 2004); and introduction of DNA vaccines into skeletal muscle promotes strong humoral and cellular immune responses (Shedlock and Weiner, 2000). The innovative EP technique overcomes a major disadvantage of plasmid administration: insufficient uptake in the undamaged muscle. Using this technology, transgene products can approach levels previously achieved only with viral

vectors for intracellular and secreted products (Draghia-Akli and Fiorotto 2004; Fattori et al, 2004; Molnar et al, 2004). Numerous factors can impact plasmid uptake and expression after intramuscular injection followed by EP, such as plasmid size, formulation, concentration, injection volume, intensity of the electric field, pulse length, lag time between injection and EP, target muscle, species and age of the treated subject. This review will focus on recent studies analyzing these and other factors that can increase the efficacy of plasmid transfer and lower the total amount of plasmid and DNA vaccines required to generate targeted levels of biologically active proteins or antibodies.

II. Factors relating to target species and muscle

A. Species

Electroporation has been extensively used in mice (Lucas et al, 2001; Vilquin et al, 2001; Lesbordes et al, 2002), rats (Terada et al, 2001; Yasui et al, 2001), dogs (Fewell et al, 2001), pigs (Draghia-Akli et al, 1999, 2002b), cattle (Tollefsen et al, 2003; Brown et al, 2004), and non-human primates (Fattori et al, 2004) to deliver therapeutic genes that encode for a variety of hormones, cytokines, enzymes or antigens. Nevertheless, optimal conditions of electroporation varied in different animal models as well as in the same animal model depending upon physiological or pathological state of the treated animal. Theoretical and practical data also suggest that the cell size in the region perpendicular to the electric field plays a crucial role in determining the permeabilization parameters (Somiari et al, 2000). The larger the "functional" size of the cell, the lower is the field strength necessary (Neumann et al, 1999). For example, tissues containing cells that communicate through tight gap junctions amplify transmembrane potential changes. Thus, the skeletal muscle as compared to other tissues can be electroporated at lower field intensities (Fear and Stuchly, 1998), resulting in decreased tissue damage. Rodent muscles have been electroporated using current intensities as low as 50-100 mA (Zampaglione et al, 2005), and as high as approximately 1 Amp, and electric field intensities of 100-200 V/cm (Mir et al, 1999; Bettan et al, 2000), while in larger mammals, such as pigs or cattle, lower electric field intensities, 0.4 to 0.6 Amp and voltages, 80 to 120 V/cm, are required to avoid tissue damage (Khan et al, 2003; Brown et al, 2004). In some disease models, such as in mdx mice, higher voltages of approximately 200 V/cm are needed (Wong et al, 2005), while milder conditions have been described for diabetic mice than for normal mice (Wang et al, 2005).

B. Target muscle

Plasmids have been delivered to most surface muscles in different animal species, with the transgene product directly measured in the target muscle or in the systemic circulation. The results are difficult to compare as an entire spectrum of conditions can directly impact the results: fiber type composition of the muscle, which has been substantially studied *in vitro*, but not *in vivo*, is an important factor in rodents, where muscle can have predominantly type I or type II fibers (Cardoso et al, 2004); eventual fibrosis (Vilquin et al, 2001), collagen or fat content, or atrophy (Alzghoul et al, 2004); the choice of promoter driving transgene expression (most muscle-specific promoters have a fiber type preference) (Bertrand et al, 2003); vascularization, etc. In large animal species, where muscles have essentially mixed fiber composition and are well vascularized, plasmid injection followed by electroporation of different large surface muscles results in similar transgene expression levels (Draghia-Akli et al, 2002b, 2003; Khan et al, 2003).

C. Age

Recent studies have described a significant difference between transgene product levels in normal adult and young animals. The pattern of expression seems to fit a Gaussian curve, with normal younger and very old animals showing less expression than adult ones. Intramuscular injection and EP into muscles of young mice resulted in lower number of transduced fibers than in adults (Molnar et al, 2004), and a more rapid loss of expression. Lower expression level was described in old rodents (Wang et al, 2005). On the other hand, the slower rate of turnover in adult animals may contribute to the longer duration of expression. Similar results have been described in pigs, with young animals having higher muscle resistance, and requiring increased electric field intensities for optimum expression when compared to older pigs (Khan et al, 2005). Muscle properties are affected by the age of the animal and these factors may affect muscle resistance during the course of the EP procedure. It seems that the uptake and activity of smaller nucleic acid molecules, such as short interfering RNAs (siRNA), are less impacted by particular variable (Golzio et al, 2005). Age differences are also less pronounced in disease models of muscle dystrophy, such as mdx mice (Gollins et al, 2003; Wong et al, 2005).

III. Factors relating to plasmids and its formulation

A. Plasmid size

A general consensus exists on this important factor: the smaller, the better. Numerous studies found that plasmids of smaller size entered cells more efficiently than large plasmids (Bloquel et al, 2004; Molnar et al, 2004; Wang et al, 2005). Also, small nucleic acid fragments, such as siRNA seem to be uptaken very efficiently by this method (Kishida et al, 2004; Golzio et al, 2005).

B. Plasmid formulation

Under some conditions, EP procedures can inflict fatal stress on some skeletal muscle cells (Fewell et al, 2001; McMahon et al, 2001) or degrade the plasmid (Hartikka et al, 2001). Polymers such as poly-vinylpyrrolidone (PVP), poly-(L)-glutamate (PLG) at high or low concentrations, or mild surfactants in low concentration such as poloxamer 188 have been used to demonstrate increased plasmid uptake and reduced tissue damage. Poloxamer 188 may induce sealing of

permeabilized lipid bilayers to rescue cells that were not extensively heat-damaged, and consequently, cause an increase in plasmid expression levels (Lee et al, 1999). Following EP of the skeletal muscle of mice, rats, dogs or pigs (Mumper et al, 1998; Fewell et al, 2001; Nicol et al, 2002; Draghia-Akli et al, 2002a), plasmid formulated with PLG or PVP has been observed to increase gene expression up to 10 fold compared to non-formulated plasmid. In mice, pre-injection of the electroporated muscle with hyaluronidase, an enzyme that hydrolyzes hyaluronic acid, a ubiquitous component of the extracellular matrix, increases gene expression up to 5 fold with minimal tissue damage (Mennuni et al, 2002; Molnar et al, 2004).

Further experiments in our laboratory have elucidated that the formulation of plasmid can enhance expression *in vivo*. Our strategy uses low concentrations of the transfection-facilitating anionic polymer sodium PLG of low molecular weight. Previous studies have demonstrated that mice receiving SEAP construct coated with PLG at a concentration of 0.01 mg/ml had the least inflammation associated with the delivery procedure at 3 days post-injection (Draghia-Akli and Smith, 2003). We determined that a mol/mol ratio of DNA phosphate groups to PLG carboxyl groups yields the optimum concentration for gene therapeutic applications to the skeletal muscle and results in increased expression of the transgene with no damage to the target tissue in numerous animal species. The minimum number of repeat units, as well as lot-to-lot (even when the compound is purchased from the same vendor) variability of PLG was also analyzed in mice (**Figure 1**) and pigs (**Figure 2**) and found to be an important factor in the larger animal species, but not in rodents. Other experimental excipients, such as $MgCl_2$ which promotes sealing of membrane pores, was also be analyzed, and was determine to favorably impact expression levels in rodents.

C. Plasmid concentration and dose

Studies have shown that plasmid concentration directly correlates with expression in rodents and other species (Bettan et al, 2000; Khan et al, 2003; Wang et al, 2005) up to an optimum concentration. Mechanistic studies related to cellular entry of plasmids after EP show that the level of transfection remains unchanged whether electric pulses are delivered at various periods of time after injection and at plasmid doses that vary by a factor of 100 (Bureau et al, 2004). Thus, it is possible that at low plasmid concentrations the relative proportion of plasmid that will be rapidly cleared will be greater when compared to the plasmid protected from DNAses which would be available for cell entry following EP (Wolff et al, 1992a). We have shown that increasing the number of injection sites by dividing a certain plasmid dose does not result in increased transgene expression (**Figure 3**).

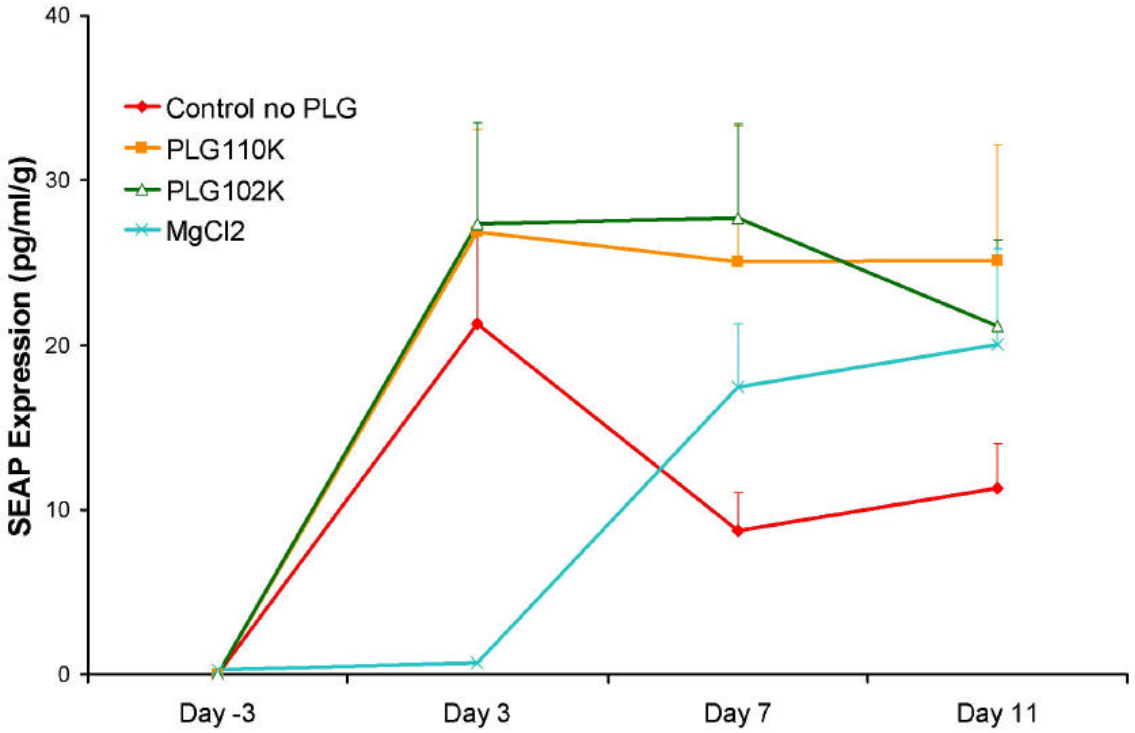

Figure 1. The biological comparability of poly-L-glutamic acid (PLG) sodium salt (Sigma, St. Louis, MO) is examined in this experiment by measuring SEAP expression in C57/Bl6 mice (n=6/group). SEAP plasmid is formulated with different lots of PLG denoted as PLG110K and PLG102K and SEAP levels are measured following electroporation. A group received 3 mM $MgCl_2$, while the control group received SEAP plasmid without PLG. Animals receiving plasmid with PLG lot 110 exhibited the highest expression levels. Values depicted are mean ± SEM.

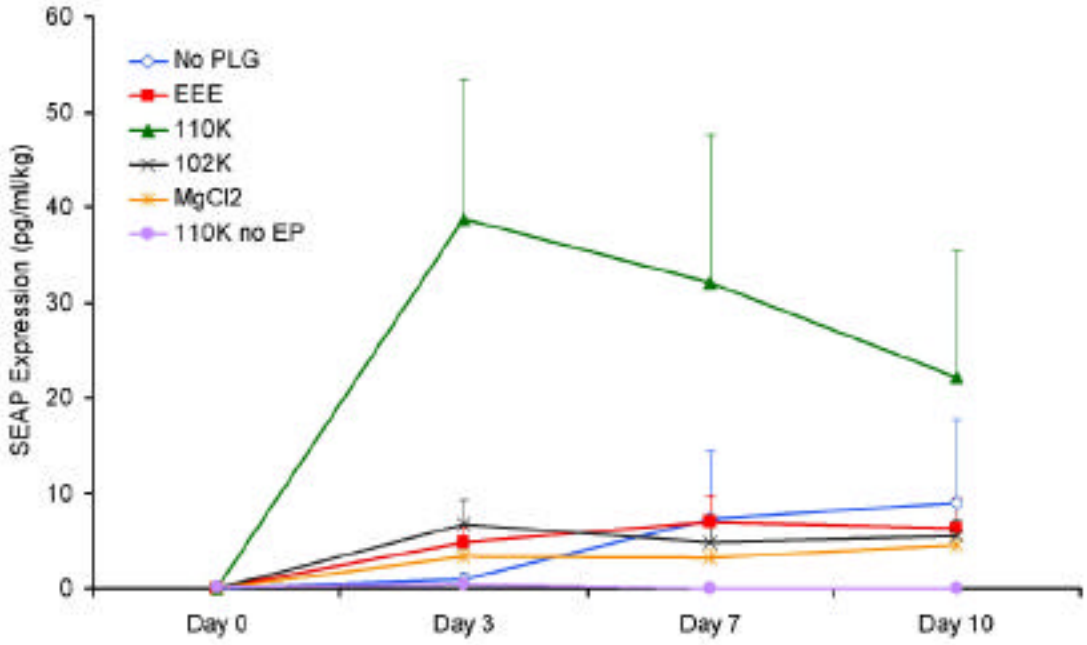

Figure 2. Different plasmid formulations were tested in male pigs using SEAP expression as the endpoint. Four week-old barrows (n = 4 to 6/group) weighed 10 kg on average. SEAP plasmid (0.5 mg in 2 mL injection volume) was formulated containing 0.01% (w/w) peptide (PLG lot 102, PLG lot 110, or a synthetic tripeptide, EEE) and injected intramuscular at 1 site in the sternocranialis muscle, followed by electroporation. 3 mM $MgCl_2$, which promotes sealing of membrane pores, was also be tested. Control groups included no PLG and PLG110K without electroporation. The highest expression from day 3 until the end of the study was elicited in the group of pigs injected with plasmid containing PLG Lot 110K ($p = 0.06$, 110K vs. control). Values depicted are mean ± SEM.

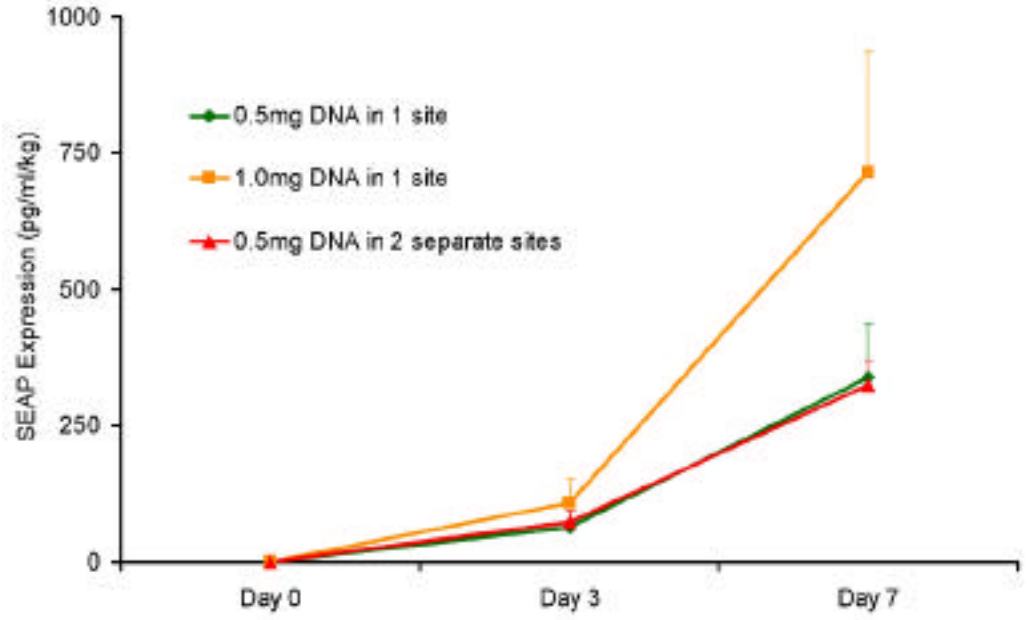

Figure 3. This study examined whether SEAP plasmid expression increases in a dose-response fashion when administered in one versus two injection sites in young, male pigs. The doses included 0.5 mg in one site, 1.0 mg in one site, and 0.5 mg in two different sites. All plasmid was administered via injection in the right semimembranosus muscle followed by electroporation. At study day 7, the group administered 0.5 mg SEAP plasmid in two different sites exhibits similar expression to the group administered 0.5 mg SEAP plasmid in one site. Values depicted are mean ± SEM.

Despite the success of DNA technologies in rodents, the clinical translation to larger animals and humans may be hampered by the large quantities of plasmid required to attain therapeutic levels of the desired transgene product (Fewell et al, 2001). Since high doses of plasmid can saturate a single injection site, multiple injections may be required in larger animals to achieve optimal gene transfer (Fattori et al, 2005). Various attempts have been made to increase the effectiveness of plasmid-based expression systems, including significant improvements to plasmid

design. We have shown in our experiments that under the proper EP conditions as little as 0.1 mg of a plasmid encoding for a *therapeutic protein* delivered can accomplish these requirements in pigs (Draghia-Akli et al, 2003) or 2.5 mg in dairy cattle (Brown et al, 2004). This plasmid quantity is 100-fold lower than a hypothetical dose obtained by direct extrapolation of the dose used in rodents (an average of 1 mg/kg). Therapeutic and vaccine applications differ by the amount of protein production that is necessary, as vaccine applications usually require lower amounts of protein production. A recent study of influenza vaccine demonstrated that a single injection of 0.03 mg plasmid encoding neuraminidase from influenza virus, followed by EP, was able to provide long-term protection from influenza challenge in mice (Chen et al, 2005).

IV. Factors relating to electroporation conditions

Existing EP technologies that deliver square waves targeted to the skeletal muscle are based upon either constant-voltage or constant-current concepts. Due to variations in tissue resistance during the EP process, a predetermined voltage pulse may cause an unregulated variation in the current flowing through the tissue during each pulse. The result is a loss of the perfect square-wave function (current intensity versus time), tissue damage and reduced plasmid uptake and expression (Draghia-Akli and Smith, 2003). By contrast, constant-current EP maintains a square wave function in the target tissue irrespective of changes in tissue resistance (Khan et al, 2005). Electroporation parameters such as pulse pattern, tissue resistance, momentary electric field intensity, and voltage can be studied only in software-driven systems that are capable of instant feed-back and recording of both current and volts during pulses (Hebel et al, 2005) as well as adapting the output to the changing conditions in the tissue. Thus, software-driven devices that can adapt the current intensity output and prevent high variations in voltage and prevent tissue damage could be better adapted to animal and human applications of this technology.

A. Pulse pattern

Pulse patterns can impact expression levels, with lower number of pulses and no reverse orientation during EP with needle-electrodes resulting in a higher transgene expression (Khan et al, 2005). These results are in agreement with previously published studies showing that optimization of cumulated pulse duration and current intensity dramatically reduced gene electrotransfer-associated muscle damage (Durieux et al, 2004).

B. Electric field intensity (current, voltage, resistance)

Electroporation settings as low as 0.1 Amp are able to successfully transfect the mouse tibialis anterior muscle and ensure high levels of SEAP expression when using a constant-current device (Khan et al, 2005). Electroporation at amperage settings of 0.2, 0.3, and 0.4 may induce higher voltage settings and cause damage to the surrounding muscle tissue. Analysis of the data recorded during EP of mice and young and old pigs revealed that muscle resistance was significantly higher in mice than in pigs, with a lower resistance in the older animals compared to younger animals. For constant-voltage devices (**Figure 4**) electric field intensities can approach 1 Amp and may result in tissue damage and pain (Rizzuto et al, 1999; Gronevik et al, 2005; Tjelle et al, 2005). Furthermore, tissue damage may induce cellular repair processes that could either replace damaged myocytes that were successfully transfected or stimulate plasmid degradation (Hartikka et al, 2001; Cappelletti et al, 2003).

C. Pulse length

From the first comprehensive studies of Mir et al, (1999) and Aihara and Miyazaki (1998), it has been determined that very efficient plasmid DNA transfer in muscle fibers by using square-wave electric pulses requires low field strength (less than 300 V/cm) and long duration (more than 1 ms). Most studies describe EP to the muscle for the different applications referenced above use pulse length from 10-400 microseconds to 20-60 milliseconds.

D. Lag time

In previous studies, we and others have used time lags between plasmid injection and EP of approximately 120 seconds (Aihara and Miyazaki, 1998; Bettan et al, 2000) and subsequently 80 sec (Khan et al, 2003). Very recent studies performed in different muscle groups in mice describe procedures that allow for a minimal (20 seconds or less) lag time (Khan et al, 2005; Wang et al, 2005). This finding indicates that gene expression is dependent on the time needed for the plasmid solution to disperse into surrounding tissue, but it is not as critical in smaller murine muscles (Bureau et al, 2004). Future studies analyzing the impact of plasmid concentration, formulation, target muscle and species on lag time are needed for larger animal species and humans.

E. Electrode configuration and orientation

The influence of electrode configuration on the electric field distribution has been shown by measuring ^{51}Cr-EDTA uptake *in vivo* (Gehl and Mir 1999; Gehl et al, 1999). Both plate electrodes (Hartikka et al, 2001; Draghia-Akli et al, 2002b), a pair of wire electrodes (Mathiesen 1999) or two-needle devices (Tjelle et al, 2005) have been shown to be effective. The calculated electric field distribution is more homogenous with larger electrode diameter (Miklavcic et al, 2000; Davalos et al, 2003). In order to maintain constant-current parameters, a multiple needle electrode array is preferred, as reviewed in (Draghia-Akli and Smith 2003). Recent studies from our laboratory have shown that electrode orientation can impact animal-to-animal variability, with randomly oriented electrodes resulting in higher animal-to-animal variability in plasmid uptake and expression (**Figure 5**). Also, recent studies suggested that precise delivery of the plasmid formulation in the area delineated by the

electrodes is essential for optimum expression in the large animal and potentially humans (Khan et al, 2003).

V. Conclusions

Non-viral gene delivery using plasmids followed by EP is widely being used. The conditions of EP as well as the characteristics and formulation of the plasmid should be optimized to ensure high levels of expression. This review demonstrates the need to analyze all elements involved in this delivery process, because minor alterations can dramatically affect plasmid expression levels. We predict that improvements to the EP methodology in large animal models constitute a step forward to deliver hormones, enzymes or cytokines, or vaccines to humans.

Acknowledgments

The authors would like to particularly thank Dr. Louis C. Smith for his significant contribution to these studies and Ms. Catherine Tone for her editorial correction of this manuscript. We acknowledge support for this study from ADViSYS, Inc. (The Woodlands, Texas).

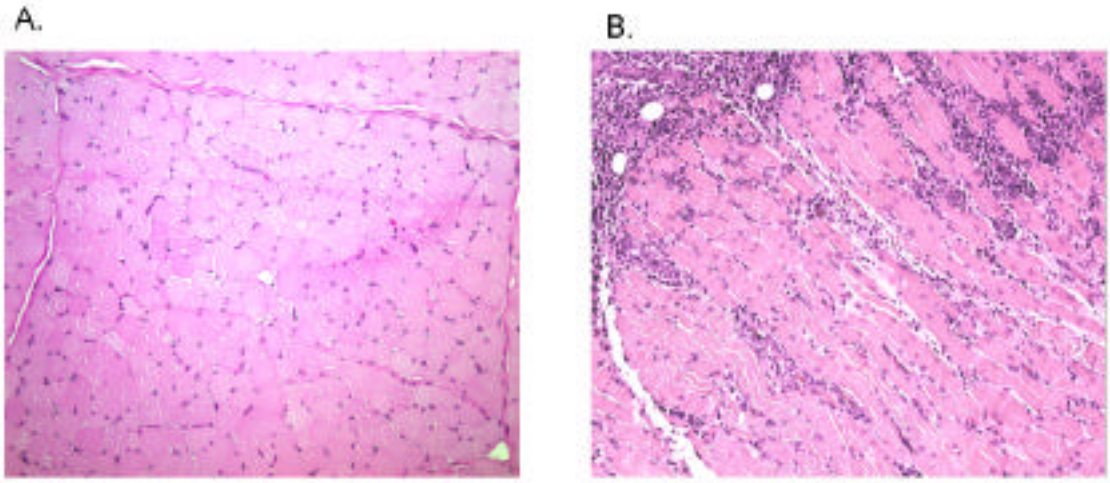

Figure 4. Histological analysis (hematoxylin/eosin staining) of muscle samples in pigs injected and electroporated with **A.** a constant current electroporator, or **B.** a constant voltage electroporator. In both cases the electroporation was performed using internal needle electrodes.

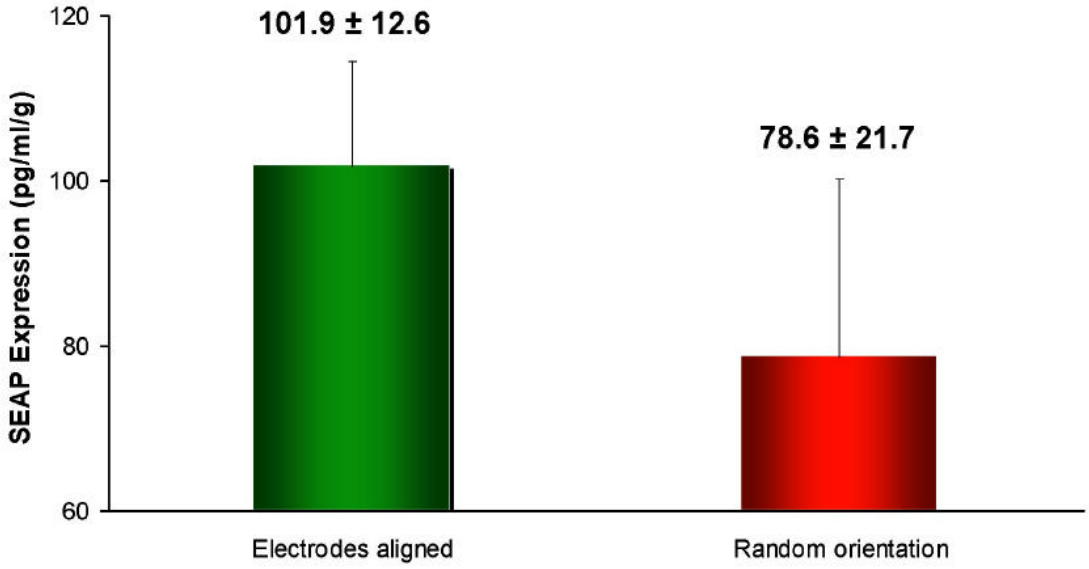

Figure 5. The orientation of the custom-designed reverse trochar point of the 3- needle electrodes is tested in C57/Bl6 male mice to determine if this orientation is essential for optimal plasmid expression. Mice were injected with SEAP plasmid (10 μg) in the right tibialis muscle in a volume of 25 μl. After 2 minutes, groups of animals were electroporated (0.4 Amp, 2 pulses, 52 msec pulse duration, 1 sec between pulses) using constant current EKD electroporator and an electroporator array with oriented electrodes, versus electrodes in random orientation (oriented electrode arrays have all needles with the 120°, large trochar side, facing inward, with a ± 2 degree of variation; the random oriented electrode arrays have the needle trochar side facing randomly). Serum SEAP levels were measured. As

shown, SEAP levels were 30% lower and the SEM was increased approximately 2 fold, when non-oriented needles were used. Values depicted are mean ± SEM.

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