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Optimal salt concentration of vehicle for plasmid DNA enhances gene transfer mediated by electroporation

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Abbreviations: ICP-AES, inductively coupled plasma-atomic emission spectrometer; IF, low frequency; RLU, relative luminescence unit

Abstract

***In vivo* electroporation has emerged as a leading technology for developing nonviral gene therapies, and the various technical parameters governing electroporation efficiency have been optimized by both theoretical and experimental analysis. However, most electroporation parameters focused on the electric conditions and the preferred vehicle for plasmid DNA injections has been normal saline. We hypothesized that salts in vehicle for plasmid DNA must affect the efficiency of DNA transfer because cations would alter ionic atmosphere, ionic strength, and conductivity of their medium. Here, we show that half saline (71 mM) is an optimal vehicle for *in vivo* electroporation of naked DNA in skeletal muscle. With various salt concentrations, two reporter genes, luciferase and β-galactosidase were injected intramuscularly under our optimal electric condition (125 V/cm, 4 pulses x 2 times, 50 ms, 1 Hz). Exact salt concentrations of DNA vehicle were measured by the inductively coupled plasma-atomic emission spectrometer (ICP-AES) and the conductivity change in the tissue induced by the salt in the medium was measured by Low-Frequency (LF) Impedance Analyzer. Luciferase expression increased as cation concentration of vehicle decreased and this result can be visualized by X-Gal staining. However, at lower salt concentration, transfection efficiency was diminished because the hypoosmotic stress and electrical injury by low conductivity induced myofiber damage. At optimal salt concentration (71 mM), we observed a 3-fold average increase in luciferase expression in comparison with the normal saline condition ($p < 0.01$). These results provide a valuable experimental parameter for *in vivo* gene therapy mediated by electroporation.**

Keywords: electroporation, gene therapy, drug delivery systems, sodium chloride; electric conductivity

Introduction

Although nonviral gene therapy has several advantages over viral gene transfer (John *et al*., 2001; Kay *et al*., 2001; Sun and Frederick, 2001), its transfection efficiency is still low (Templeton and Lasic, 1999; Han *et al*., 2000; Li and Huang, 2000). Recently, several approaches have been developed to enhance the efficiency of gene transfer and *in vivo* electroporation has emerged as a leading technology for developing nonviral gene therapies. Its advantages, compared with other nonviral gene delivery systems, are that gene expression is drastically increased (2- to 4-log fold), long-lasting (months), and very specific and localized (Mir *et al*., 1999). This technique has also been successful in various tissues such as skeletal muscle (Muramatsu *et al*., 1998; Aihara and Miyazaki, 1999; Gehl and Mir, 1999; Imai and Isaka, 1999; Mathiesen, 1999; Rizzuto *et al*., 1999; Bettan *et al*., 2000; Lemieux *et al*., 2000; Maruyama *et al*., 2000; Vicat *et al*., 2000; Lucas and Heller, 2001; Muramatsu *et al*., 2001), liver (Suzuki *et al*., 1998; Heller *et al*., 2000), testis (Muramatsu *et al*., 1997; Yamazaki *et al*., 1998; Yamazaki *et al*., 2000), skin (Johnson *et al*., 1998; Vanbever and Preat, 1999; Glasspool-Malone *et al*., 2000), cornea (Oshima *et al*., 1998; Sakamoto *et al*., 1999), cardiaovascular (Harrison *et al*., 1998; Martin *et al*., 2000) and mammary tumor tissue (Goto *et al*., 2000; Wells *et al*., 2000; Lohr *et al*., 2001). In particular, gene delivery to skeletal muscle is a promising strategy for the systemic secretion of therapeutic proteins.

The general mechanism of electroporation starts with an increase in membrane permeability following treatment with electrical pulses and is then followed by influx of DNA through the permeabilized membrane defect (Somiari *et al*., 2000). Under the influence of an electrical field, the distribution of ions adjacent to the inner surface of cell membranes are believed to be altered,

resulting in a series of membrane alterations that predispose to pore formation (Neumann *et al*., 1999). In the transport process, not only passive diffusion but electrophoretic and electroosmotic transport under the influence of electric fields may also facilitate transport of charged molecules and ions across the membranes (Golzio *et al*., 1998).

Recent reports have demonstrated optimized electroporation conditions including voltage, pulse duration, number of pulse, frequency, DNA concentration, and the amount of injected reporter gene in the tibialis anterior muscle (Golzio *et al*., 1998; Muramatsu *et al*., 1998; Gehl and Mir, 1999; Hofmann *et al*., 1999; Mir *et al*., 1999; Mekid and Mir, 2000; Miklavcic *et al*., 2000; Yoshizato *et al*., 2000; Lucas and Heller, 2001). It was also shown that square-wave electric pulses improve pulsed DNA delivery to living tissues (Mir *et al*., 1999). In addition, the low voltage-long duration approach maximizes gene delivery and minimizes tissue damage (Muramatsu *et al*., 1998). Nevertheless hitherto, while most electroporation stu-dies were focused on the electric conditions and electrotransferred DNA, none of these reports have ever compared the effects of vehicles for DNA with electroporation.

We hypothesized that salts in vehicle for plasmid DNA must affect the efficiency of DNA transfer because i) cations in vehicle could diminish electrophoretic and electroosmotic transport of plasmid in an electric field by interaction between cations in vehicle and negatively charged plasmid DNA, and ii) low conductivity at low ionic strength may cause membrane disruption, which leads to cell damage. In this study, we observed the variation of reporter gene expression at various cation concentrations of vehicle for plasmid DNA with our optimal electric parameters. Exact salt concentrations in DNA vehicles were measured by Inductively Coupled Plasma-Atomic Emission Spectrometer (ICP-AES) and the conductivity change in the tissue induced by the salt in the medium was measured by Low-Frequency (LF) Impedance Analyzer. In addition, traditional histological analysis was carried out to determine the transfection efficiency and the degree of cell damage depending on electroporation conditions. Here, we report that half saline is the optimal salt concentration for maximum transfection efficiency and minimum cell damage on *in vivo* electroporation.

Materials and Methods

Plasmid DNA preparation

The firefly luciferase gene and β-galactosidase gene were used as reporter genes to monitor the gene transfer results. The luciferase expression plasmid (pCN -luciferase) and β-galactosidase expression plasmid (pCN-lacZ) were constructed by subcloning *Photinus pyralis* luciferase or *E. coli* β-galactosidase cDNA to pCN, respectively. pCN vector contains the 600 bp HCMV IE promoter and its entire 5-untranslated region consisting of 122 bp exon 1, 827 bp intron 1, and 16 bp exon 2. pCN vector was known to express higher than the vector with only CMV promoter (Lee *et al*., 2000). Plasmid DNA was transformed into *Escherichia coli* TOP10 competent cells and plasmid DNA was isolated by plasmid purification Mega kits from Qiagen (Valencia, CA, USA) according to the manufacturers instructions. Plasmids were precipitated in isopropanol. For the first batch of DNA, the above plasmids were resuspended in water and for the second batch of DNA, plasmids were further washed twice with 70% ethanol and resuspended in water. DNA was stored at -20°C until use.

Measurement of salt concentration by ICP-AES

Emission measurements of salt concentrations were made with an ICP-AES, Model ICPS-10001V (Shimadzu, Japan).

Intramuscular DNA injection and electroporation *in vivo*

All animal experiments were carried out according to the Guideline for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Five-week-old female BALB/c-AnNCrj mouse was anesthetized by intraperi-toneal injection of 100 μl solution consisting of 2.215 mg ketamine (Ketalar 50 mg/ml, Yuhan Co, Korea) mixed with 0.175 mg xylazine (Rompun 23.32 mg/ml, Bayer, Korea). Fifty microgram DNA in 30 μl solution at each salt concentration was injected into the tibialis anterior muscle of the mouse with a 30-gauge insulin syringe (Becton Dickinson, Franklin Lakes, NJ). The syringe needle was fitted with a P10 tube adjusted to settle the depth of needle-tip penetration of a tibialis anterior muscle to 3.0 mm. Thirty seconds after DNA injection, transcutaneous electric pulses using ECM 830 (BTX Division of Genetronics, San Diego, CA) were applied through two electrodes (Tweezertrodes, BTX Division of Genetronics) placed on the surface of the injection site. Electric pulses at 125 V/cm, 50 ms duration and 1 Hz were applied four times and a further four times in the reverse direction.

Assay for luciferase activity *in vivo*

Unless otherwise stated, mice were humanely killed 7 days after DNA transfer. Tibialis anterior muscles were removed and homogenized in 2 ml of Reporter lysis reagent (Promega, Madison, WI). After cen-trifugation at 12,000 rev./min for 10 min, luciferase activity was assessed on 10 μl of supernatant with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). This assessment was made by measuring the in-tegration of

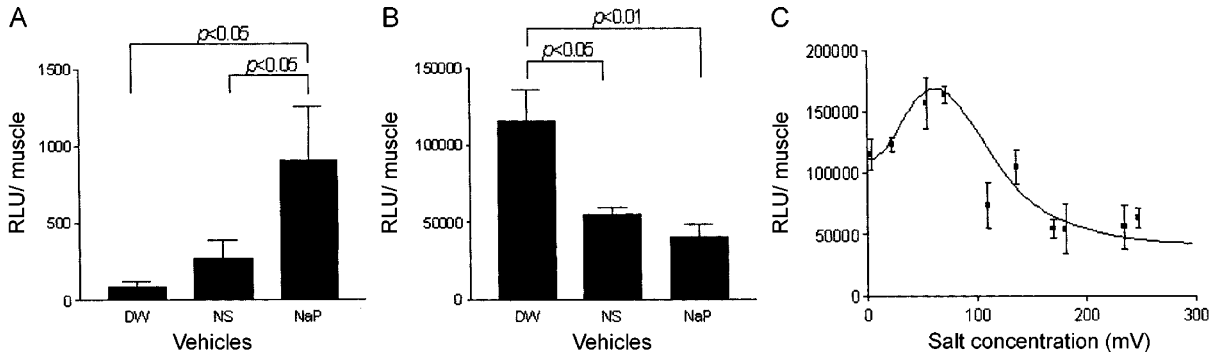

Figure 1. Dependence of luciferase expression on vehicles and on salt concentration in saline. Fifty microgram of pCN-luciferase DNA diluted in 30 ml distilled water, normal saline (150 mM Nacl), and sodium phosphate (150 mM) was injected into mouse tibialis anterior muscle in the absence (A) or presence (B) of electric pulses. Additionally, pCN-luciferase cDNA (50 μg) diluted in sterile saline at various concentrations was injected into mouse tibialis anterior muscle with the application of electric pulses (C). Electric pulses were applied 30 s after DNA injection with our optimal electroporation conditions (125 V/cm, 4 pulses x 2 times, 50 ms, 1 Hz). The luciferase activity was measured at 7 days after intramuscular injection and exact salt concentrations of DNA vehicle were measured by ICP-AES according to the protocol described in Materials and Methods. Gene expression of distilled water (DW) vehicle was significantly higher than those of NS (normal saline) and NaP (sodium phosphate) by Bonferroni's multiple comparison test ($P < 0.01$) when electric pulses were applied (B). When no electric pulse was applied, these results were radically altered. In this case, NaP vehicle showed the highest transfection efficiency (A, $P < 0.05$) and luciferase activity with distilled water vehicle was about 3 times lower than with normal saline ($P > 0.05$) Moreover, at optimal concentration (71 mM) we observed an average 3-fold increase in luciferase expression in comparison with normal saline ($P < 0.01$) (C). All data are means ± SEM of individual values ($n = 5$ per each).

the light produced during a period of 30 s, starting 3 s after the addition of 100 μl of luciferase assay substrate (Promega, Madison, WI) to the muscle-fiber lysate.

Histochemical staining for β-galactosidase activity and analysis of muscle cell damage

A 5-week-old female BALB/c-AnNCrj mouse was intramuscularly injected with 50 mg of pCN-lacZ in 30 μl solution at each salt concentration. One week later, tibialis anterior muscle in the injected mouse was removed and fixed with 4% paraformaldehyde (Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS) for 20 min. This sample was developed in a substrate solution [5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 10% sodium deoxycholate, NP40 and 50 mg/ml X-gal in PBS] overnight at 37°C. To prepare stained tissue cross-sections, tibialis anterior muscle was embedded in OCT (VWR, McGrau Park, IL, USA) and 10 mm sections were cut using a cryotome (Cryotome AS620, Shandon, UK). Sections were collected on microscopic slides (ProbeOn Plus Microscope Slides, Fisher Scientific, Pittsburgh, PA, USA), brought to room temperature and stained for hematoxylin and eosin (H&E). A similar protocol, except for the development of b-galactosidase, was observed for quantitative measurement of cell damage on electroporation. In this study, three vehicle systems with and without electric pulses were tested: distilled water (0 mM NaCl), half saline (75 mM NaCl) and normal saline (150 mM NaCl). The percentage of damaged cells was determined by light microscopy in muscle cross-sections. Five representative fields were counted for each slide.

Measurement of impedance parameters in the muscle

Five-week-old female BALB/c-AnNCrj mice were humanely killed and their tibialis anterior muscles were removed. Two parallel stainless-steel probes holding constant distance (about 6 mm wide) were inserted into the retrieved muscle and measurements of impedance parameters were carried out by means of LF Impedance Analyzer (model HP 4192A, Hewlett-Packard, Palo Alto, CA, USA) at constant 10 kHz frequency. Ten microliter of normal saline (150 mM NaCl) or distilled water (0 mM NaCl) was injected by 30-gauge insulin syringe into the muscle and after 30 s the changes of the conductivity and resistance were measured.

Statistical analysis of results

Each condition was tested on at least three different muscles. Results were expressed as the mean (RLU of luciferase per muscle)± SEM. Differences were tested by ANOVA for multiple-sample comparison with Bonferronis posthoc analysis. The level of significance was set at $P < 0.05$.

Results and Discussion

Determination of optimal electroporation parameters

In vivo electroporation protocols still lack standardized electrical parameters. Furthermore, the optimum conditions for *in vivo* electroporation may vary according to tissue, organs, and animal species (Suzuki *et al.*, 1998).

In order to determine our optimal conditions for *in vivo* electroporation, we made experiments by varying electrical parameters (data not shown). Maximum transfection efficiency occurred with the following electric conditions: a voltage to distance ratio of 125 V/cm, a pulse quantity of 8, a pulse duration of 50 ms, and frequency of 1 Hz. These optimal parameters are supported by similar results in other experiments (Muramatsu *et al*., 1998; Gehl and Mir, 1999; Mir *et al*., 1999; Lucas and Heller, 2001).

Comparison of the effect of the three vehicles

Firstly, we investigated the effects of three vehicles for plasmid DNA in the absence (Figure 1A) or presence (Figure 1B) of electric pulses. The preferred vehicle for naked DNA injections has been normal saline or PBS because these are isotonic, stable, and nontoxic. Recently it was reported that sodium phosphate enhances plasmid DNA expression *in vivo* possibly by inhibiting DNA degradation (Hartikka *et al*., 2000). When an electric pulse was not applied (Figure 1A), luciferase activity with distilled water vehicle was about 3 times lower than with normal saline [87 ± 36 versus 269 ± 115 relative luminescence unit (RLU) per muscle, $P > 0.05$] and about 10 times lower than with sodium phosphate (87 ± 36 versus 904 ± 351 RLU per muscle, $P < 0.05$). However, this behavior was radically altered in the presence of electric pulses (Figure 1B).

When electric pulses were applied (all experiments have been performed using our optimal electroporation condition; 4 pulses x 2 times, 50 ms, 8 pulses, 1 Hz), gene expression with distilled water was most efficient when compared with normal saline or sodium phos-phate. With distilled water vehicle, luciferase activity was about 2.1-fold higher than with normal saline (115,200 ± 20,180 versus 54,540 ± 4,746 RLU per muscle, $P < 0.01$) and about 2.9-fold higher than with sodium phosphate (115,200 ± 20,180 versus 40,190 ± 8,607 RLU per muscle, $P < 0.001$).

All vehicles showed an enhancement in transfection efficiency when electric pulses were applied, compared with that in the absence of electric pulses; with distilled water 1,317-fold (115,200 ± 20,180 versus 87 ± 36 RLU per muscle), with normal saline 203-fold (54,540 ± 4,746 versus 269 ± 115 RLU per muscle), and with sodium phosphate 44-fold (40,190 ± 8,607 versus 904 ± 351 RLU per muscle). In the case when distilled water vehicle was used, the enhancement of gene transfer by electroporation was the highest.

The mechanism for a reversed pattern of gene expression among different vehicles between the presence and absence of electroporation is not known. We hypothesized that salts in transferred vehicles for plasmid DNA could affect electrophoretic transport of plasmid DNA in an electric field by interaction between cations in vehicle and negatively charged plasmid DNA. This effect was examined in more detail.

Effect of sodium concentration on electroporation

In order to test our hypothesis, we investigated luciferase gene expression with varying degrees of ionic strength of NaCl solution. Figure 1C shows the effect on luciferase activities when various sodium concentrations were used. Exact salt concentrations in saline vehicles were measured by ICP-AES. Column-eluted DNA solution prepared without washing with 70% ethanol already possessed 23 mM sodium cation. When DNA was washed, practically no sodium cation was detected (< 3 mM) and other cations such as potassium were also not detected. Both kinds of DNA were used for different salt concentrations.

Consistent with our hypothesis, luciferase expression increased as cation concentration of vehicle decreased. However, when the ionic strength of NaCl solution was further decreased below that of half saline, the transfection efficiency was diminished (Figure 1C). At optimal concentration (71 mM) in the presence of electric pulses, we observed an average 3-fold increase of luciferase expression in comparison to normal saline condition (164,100 ± 6,550 versus 54,540 ± 4,746 RLU per muscle, $P < 0.01$) and 1.4fold to distilled water (164,100 ± 6,550 versus 115,200 ± 20,180 RLU per muscle, $P > 0.05$).

The decreased gene expression by electroporation at higher salt concentration could be explained by the fact that salt in the DNA vehicle influences gene transfer by altering ionic atmosphere and ionic strength. The basic mechanism of electroporation starts when a direct current electric field of uniform intensity is applied to an organ injected with naked DNA suspended in an aqueous medium. Electroporation causes DNA movement, distorts electric double layer surrounding the DNA, and induces electrical changes at the interfaces (Neumann *et al*., 1999; Somiari *et al*., 2000). The presence of ionic atmosphere around the DNA results in electrophoretic mobility slower than those expected when it is in the absence of ionic conditions. Such difference are caused by two effects. First, the potential at the surface of the DNA which dictates the rate of electromigration, is lowered by decreasing the effective electrostatic charge. Second, the electrical field also acts upon the ions surrounding the DNA. As the charge of the ionic cloud is unlike that of the DNA, the cloud will be moved in an opposite to that of the DNA, thus retarding the migration of the latter.

Electrophoretic mobility also decreases with ionic strength. This can be explained by specific binding of certain ions to the DNA by electrostatic interaction. The potential, which determines the rate of electromigration, is the potential at the boundary of the fixed and free liquid. This charge inside this surface determines the

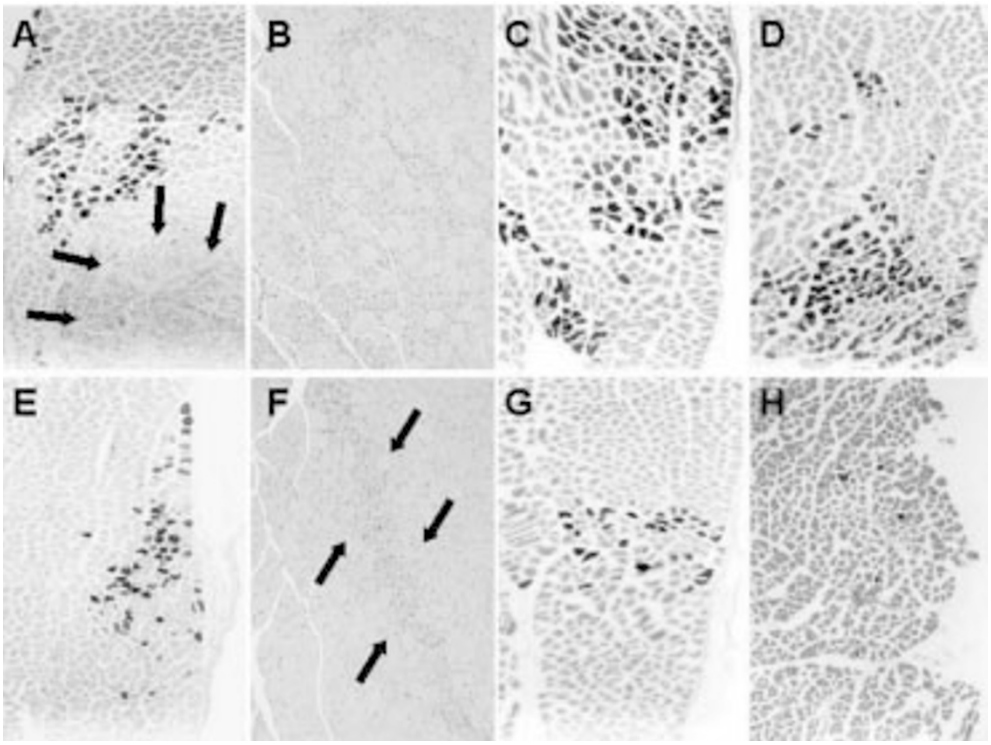

Figure 2. Histochemical staining for β-galactosidase expression. Fifty microgram of pCN-lacZ DNA diluted in sterile saline at various concentrations was injected into mouse tibialis anterior muscle in the presence (A-G: A, B with distilled water; C, with 50 mM NaCl; D, with 100 mM NaCl; E, F with 150 mM NaCl, G, with 200 mM NaCl) or absence (H, with 150 mM NaCl) of electroporation. After 7 days, X-gal staining was performed as described in Materials and Methods. The pattern of β-galactosidase expression had very high correlation with the results of luciferase expression (Figure 1C). The arrows in (A) indicate the damaged region. In (B), hematoxylin and eosin stain shows a large area of damage manifested as muscle degeneration, necrosis, infiltration of inflammatory cells or central nucleation. However, in others a minimal area of damaged cells along the track of the needle was observed (arrows in F). Original magnification, x 25 (A, C, D, E, G, H), x 200 (B, F).

zeta potential.

However, as the electric strength lowers below certain threshold (*i.e.*, 71 nM of NaCl in our data), gene expression level decreased unexpectedly. We surmised that this phenomenon was partly due to myocardial damage caused by hypoosmotic vehicle of plasmid DNA.

Histological analysis of β-galactosidase gene expression and muscle cell damage

Next, we used the β-galactosidase plasmid system to see the relationship between cell damage by electroporation and the salt concentration of DNA vehicles. Results are shown in Figure 2. Counting of β-galactosidase-positive stained fibers showed maximum transfection efficiency in the 50-100 mM concentration range (Figure 2C and D). Only a little staining was observed in the control with no electric pulse (Figure 2H). The pattern of β-galactosidase expression had a very high correlation with the results of luciferase expression (Figure 1C). Gene expression was limited only in the area where plasmid DNA was injected and it could potentially be a major benefit for electroporation that gene expression is focused on the site where electric pulses are applied. In the distilled water vehicle system, a large area of damage such as muscle degeneration, necrosis, infiltration of inflammatory cells or central nucleation was observed in most mice (Figure 2A and B). However, in other vehicles, a minimal area of damaged cells along the track of the needle was observed (Figure 2F).

Table 1. Comparison of cell damage depending on DNA vehicle and electric pulse. The percentage of necrotic cells was determined by light microscopy in muscle cross-sections stained by hematoxylin and eosin. With distilled water in the presence of electroporation, the proportion of damaged cells exceeded 32%, which was significantly higher than those of half saline and normal saline by Bonferroni's multiple comparison test ($P < 0.001$). In half saline with electric pulses, the proportion was around 7%, showing no significant difference from that of normal saline. Data are mean ± SEM.

DNA vehicle	With electric pulse (%)	Without electric pulse (%)
Distilled water	32.4 ± 2.6	< 3.0
Half saline	6.9 ± 1.8	< 3.0
Normal saline	4.7 ± 1.4	< 3.0

In Table 1, when quantitative evaluation of the myofiber damage was carried out in H&E stained sections of distilled water (0 mM NaCl), half saline (75 mM NaCl) and normal saline (150 mM NaCl) in the presence or

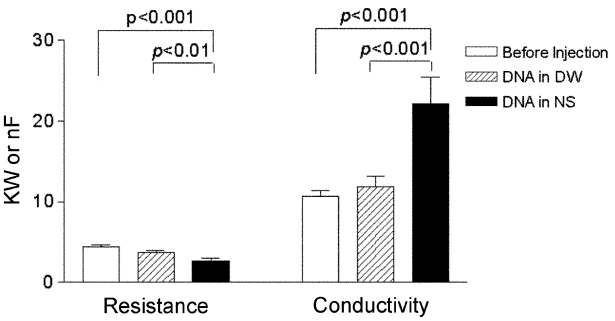

Figure 3. Change of electric parameters in muscle depending on the salt in the medium. Impedance parameters were measured using LF impedance analyzer. Two parallel stainless-steel probes holding constant distance were inserted (about 6 mm deep) into the retrieved tibialis anterior muscle of BALB/c-AnNCrj mice and measurements were carried out at constant 10 kHz frequency. Ten microliter of normal saline (150 mM NaCl) or distilled water (0 mM NaCl) was injected by 30-gauge insulin syringe and after 30 s the change of the conductivity (before injection 10.67 ± 0.80 nF; distilled water 11.90 ± 1.25 nF; normal saline 22.20 ± 3.25 nF) and resistance (before injection 4.45 ± 0.18 Ω; distilled water 3.71 ± 0.30 Ω; normal saline 2.73 ± 0.29 Ω) was measured. Figure shows that resistance with distilled water was higher than with normal saline ($P < 0.001$). On the other hand, conductivity with distilled water was lower than with normal saline ($P < 0.001$).

absence of electroporation, no significant damage was detected for any DNA vehicle in the absence of electroporation. On the other hand, when distilled water was used for DNA vehicle in elec-troporation condition, the average percentage of cell damage exceeded 32%. With normal saline, it never exceeded 5% of the total. And in the case of half saline, there was a similar degree of cell damage to normal saline.

Since muscle damage has been closely associated with reduced gene expression on electroporation (Vicat *et al.*, 2000), it is highly speculative that low ionic strength of vehicle alters its conductivity, increases electrical injury, and then decreases gene expression. Therefore, we have analyzed the change of conductivity depending on different vehicles injected.

Effect of salts in the DNA vehicle on the electric parameters

Normal saline (150 mM NaCl) or distilled water (0 mM NaCl) was injected into the retrieved tibialis anterior muscle, and then electrical resistance and conductivity were measured using low frequency (LF) impedance analyzer. Our results show that resistance with distilled water was higher than with normal saline (3.71 ± 0.30 versus 2.73 ± 0.29 Ω, $P < 0.001$. On the other hand, conductivity with distilled water was lower than with normal saline (11.90 ± 1.25 versus 22.20 ± 3.25 nF, $P < 0.001$) (Figure 3). Under low-conductivity conditions, it has been reported that the electric force contributes significantly to the enlargement of "electroleaks" in the plasma mem-brane generated by electric field (Sukhorukov *et al.*, 1998). This effect presumably results from the transient, conductivity-dependent deformation forces (elongation or compression) on the cell caused by Maxwell stress (Winterhalter and Helfrich, 1998). As ionic strength decreases until certain threshold (*i.e.*, 71 nM of NaCl in our data), this electropermeabilization increases gene transfer into the cell. However, below this point, the cell excessively distorted by low conductivity of medium may cause membrane disruption, which leads to cell death. These results indicate that salt in the medium is an important parameter for cell damage after electroporation.

In conclusion, we showed that half saline is an optimal vehicle for *in vivo* electroporation of naked DNA in skeletal muscle. The average enhancement by this vehicle was about three-fold in comparison to normal saline. Our results support the notion that not only the electromotive forces (*i.e.*, electrophoresis and electroosmosis) but also conductivity of DNA during the electric pulse might be another major factor for electrogenetransfer (Neumann *et al.*, 1999; Somiari *et al.*, 2000) and could provide a valuable experimental parameter for *in vivo* gene therapy mediated by electroporation. Further study of this mechanism may lead to a better understanding of the electrogenetransfer process.

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