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ARTICLE

Gene electrotransfer enhanced by nanosecond pulsed electric fields

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The impact of nanosecond pulsed electric fields (nsPEFs) on gene electrotransfer has not been clearly demonstrated in previous studies. This study was conducted to evaluate the influence of nsPEFs on the delivery of plasmids encoding luciferase or green fluorescent protein and subsequent expression in HACAT keratinocyte cells. Delivery was performed using millisecond electric pulses (msEPs) with or without nsPEFs. In contrast to reports in the literature, we discovered that gene expression was significantly increased up to 40-fold by applying nsPEFs to cells first followed by one msEP but not in the opposite order. We demonstrated that the effect of nsPEFs on gene transfection was time restricted. The enhancement of gene expression occurred by applying one msEP immediately after nsPEFs and reached the maximum at posttreatment 5 minutes, slightly decreased at 15 minutes and had a residual effect at 1 hour. It appears that nsPEFs play a role as an amplifier without changing the trend of gene expression kinetics due to msEPs. The effect of nsPEFs on cell viability is also dependent on the specific pulse parameters. We also determined that both calcium independent and dependent mechanisms are involved in nsPEF effects on gene electrotransfer.

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INTRODUCTION

Gene electrotransfer (GET) or gene transfer mediated by electric fields is a simple, direct, versatile physical method and low cost gene delivery approach. It has been broadly utilized for *in vitro* and *in vivo* gene delivery.[1–6] GET has been tested in preclinical and clinical trials to treat cancer[7] and several systemic disorders,[8,9] deliver DNA vaccines,[10,11] improve wound healing[12,13] as well as several other applications. While many clinical trials have been initiated and completed, one particular concern is its efficacy and adverse effects. The enhancement of GET efficiency will make it more attractive to clinical translation and broaden the potential applications. Although many efforts have been made to improve the efficiency of GET,[14–18] one big issue is the decrease of cell or tissue viability.

The transition of the use of high power pulse technology to the biomedical field is dependent on the evaluation of the effect nanosecond pulsed electric fields (nsPEFs)[19] on biological systems. Modeling studies showed that nsPEFs has a greater impact on intracellular organelles than microsecond pulses.[20,21] The biological effects of nsPEFs vary from cell function modulation to cell death. Low doses of nsPEFs have been shown to permeabilize the plasma membrane at a comparatively low energy dose[22] compared to typical electroporetic parameters. Previous research has demonstrated that both plasma and organelle membranes become permeabilized to small ions following exposure to nsPEFs.[23,24] The intracellular effects of nsPEFs include disruption of intracellular vesicles,[24,25] release of calcium from endoplasmic reticulum,[26,27] and immediate and prolonged loss of mitochondrial membrane potential.[28] Previous studies on the application of nsPEFs have focused on delivering lethal doses, or nsPEF ablation. High doses of nsPEFs induce apoptosis in mammalian cells and tissues.[29,30] Both hematologic and solid tumor cell lines have demonstrated decreased viability following high dose nsPEF treatment.[31] Localized nsPEF delivery has demonstrated complete local remission without recurrence in an *in vivo* melanoma mouse model.[32] A recent study reported delivery of a siRNA molecule with nsPEFs;[33] however, to date there has not been success in delivering plasmids. Since nsPEFs can influence intracellular events including disruption of nuclear envelope and cytoskeleton it may affect the efficiency of DNA delivery to nucleus. Two groups of investigators[34,35] have evaluated this issue by following a similar protocol but lead to controversial conclusions. Beebe *et al.*[34,36] showed that GFP expression could be increased about four times the levels achieved with classical millisecond electric pulse (msEP) when cells were exposed to 1 msEP then 1 nsPEF 30 minutes later. In contrast, Chopinet *et al.*[35] concluded that nsPEFs had no effect on GET by following the same order but with more sets of msEP and nsPEF parameters.

In this study, we first assessed the effect of nsPEFs on GET with classical msEPs. After we discovered that pretreatment with nsPEFs could synergize with GET to dramatically increase gene expression, we then characterized the properties of nsPEF effect, such as its' influence on kinetic gene expression, cell viability and the impact of interval between two pulses. We also explored the potential role of calcium on the effect of nsPEFs as a possible mechanism.

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RESULTS

Effects of nsPEFs on the cell viability of electrotransfer

One important issue we wanted to address was whether cells pretreated with nsPEFs could result in additional cell death. Cell viability was decreased proportionally after applied electric field was above 70 V for 1 msEP alone (Figure 1). Cells pretreated with nsPEFs amplified this influence. However, no additional cell death would be induced if no cell death occurred with 1 msEP alone with applied electric field below 70 V (Figure 1). This result was associated with particular parameters of nsPEFs, 24 KV/cm of applied electric field, 60 ns of pulse duration, 1 Hz of frequency, and 23 pulses. If the applied electric field was increased to 32 KV/cm without change of other parameters of nsPEFs, cell death would be observed even combined with 1 msEP at 50 V of applied electric field. Obviously, both parameters of msEPs and nsPEFs were critical to determine cell viability. Cell death could occur or increase if pulse number and/or electric field strength of either electric pulse(s) reached certain threshold or above. To minimize cell death and maximize gene expression, both parameters of msEPs and nsPEFs should be optimized.

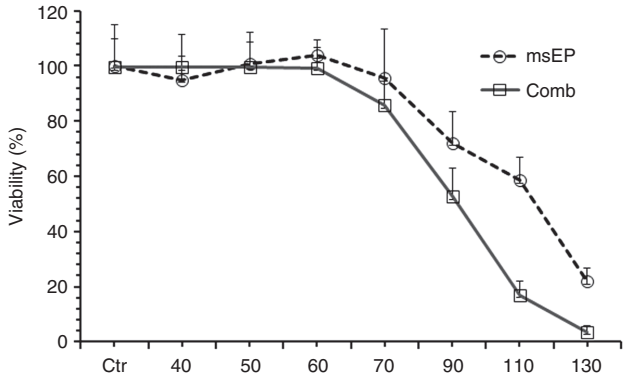

Figure 1 Viability of HACAT cells after gene electrotransfer (GET). msEP: 1 msEP with applied electric field 40–130 V. Comb: treated with nsPEFs then 5 minutes later with 1 msEP. nsPEF parameters: 60 ns, 24 KV/cm, 1 Hz, and 23 pulses; msEP parameters: 5 ms, 1 pulse, applied electric field as indicated. Error bars represent SD.

Enhanced electrotransfer efficiency by nsPEFs

Although it was reported that GFP expression was enhanced when nsPEFs was applied after msEPs in previous reports,[34,36] we have not observed any significant increase of luciferase expression of HACAT cells while similar parameters and the same order of two electroporations were adopted (Figure 2a). On the contrary, significant increase of gene expression was obtained while nsPEFs were applied prior to msEPs (Figures 2b and 3). The luciferase expression resulting from the combined electric pulses was enhanced two- to fourfold compared to 1 msEP alone dependent on the electric field strength of nsPEFs (Figure 2b, 12–24 KV/cm). The increase of gene expression was positively correlated with both electric field strength (Figures 2b and 3d) and pulse number (Figure 3c) of nsPEFs. Under the same parameters of nsPEFs, the percentage of GFP expression could be enhanced three- to tenfold dependent on the applied electric field strength of 1 msEP from 50 to 100 V (Figure 3). The fluorescence intensity of GFP was two- to threefold higher for the combined pulses than for one millisecond pulse alone. However, those results did not mean the total expression was increased to the same extent, because more cell death was observed along with higher electric field of millisecond pulses (Figure 1).

Kinetic gene expression with GET enhanced by nsPEFs

We further addressed if cells pretreated with nsPEFs could change the kinetics of gene expression by msEPs. Utilizing the IVIS Spectrum system, luciferase expression was evaluated at different time points with the same transfected cells. Two kinetic models of gene expression were found after GET with/without nsPEFs. Under parameters not causing cell death, in this case there were 5 ms of pulse duration and 50 V of applied electric field for 1 msEP with or without 60 ns of pulse duration, 24 KV/cm of electric field strength, 1 Hz of frequency, and 23 pulses for nsPEFs (Figure 4). Gene expression with 1 msEP alone or combined with nsPEFs was the highest at day 1 and rapidly dropped to the background level at day 4. Compared to 1 msEP alone, the nsPEFs enhanced gene expression about four times (Figure 4a). On the other hand, using parameters that cause death of majority of cells (Figure 1), applied electric field of 130 V for 1 msEP without change of other electrical parameters, gene expression reached the highest at day 1 and dropped in 8 days but

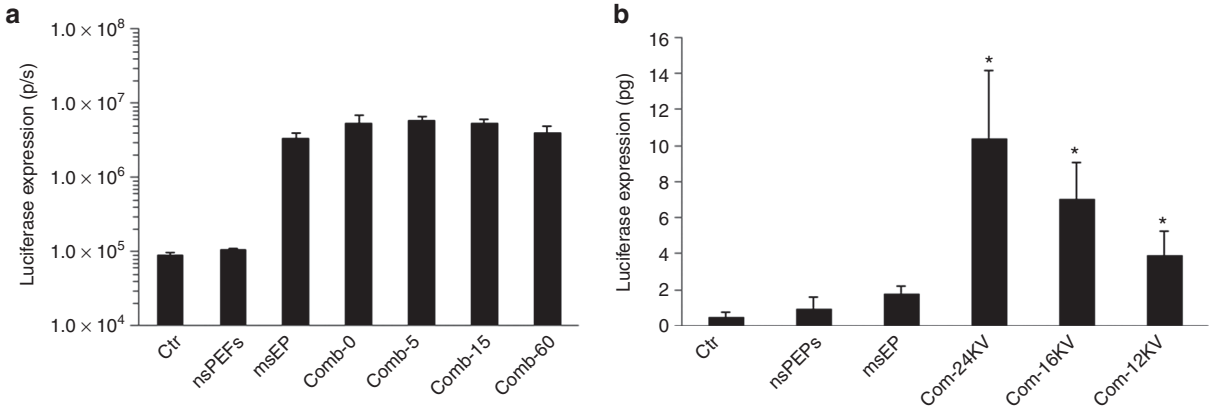

Figure 2 Luciferase expression after combination pulses. (**a**) luciferase expression of HACAT cells 1 day after gene electrotransfer (GET) with gWIZ-luc. Ctr: plasmid alone; nsPEFs: nsPEFs with pulse duration 60 ns, 24 KV/cm, 1 Hz, and 23 pulses; msEP: 1 msEP with pulse duration 5 ms and applied electric field 70 V; Comb-0, 5, 15, 60: treated with 1 msEP then followed by nsPEFs with time interval 0, 5, 15, and 60 minutes, respectively. (**b**) luciferase expression (pg/million cells) of HACAT cells 1 day after GET with gWIZ-luc. Ctr: plasmid alone; nsPEFs: nsPEFs with pulse duration 60 ns, 24 KV/cm, 1 Hz, and 23 pulses; msEP: 1 msEP with pulse duration 5 ms and applied electric field 50 V; Comb-12, 16, and 24 KV: pretreated with nsPEFs with electric field 12, 16, and 24 KV/cm, respectively then 5 minutes later followed by 1 msEP. Error bars represent SD. $n = 8–12$, $^*P < 0.001$ for Combination groups versus msEP.

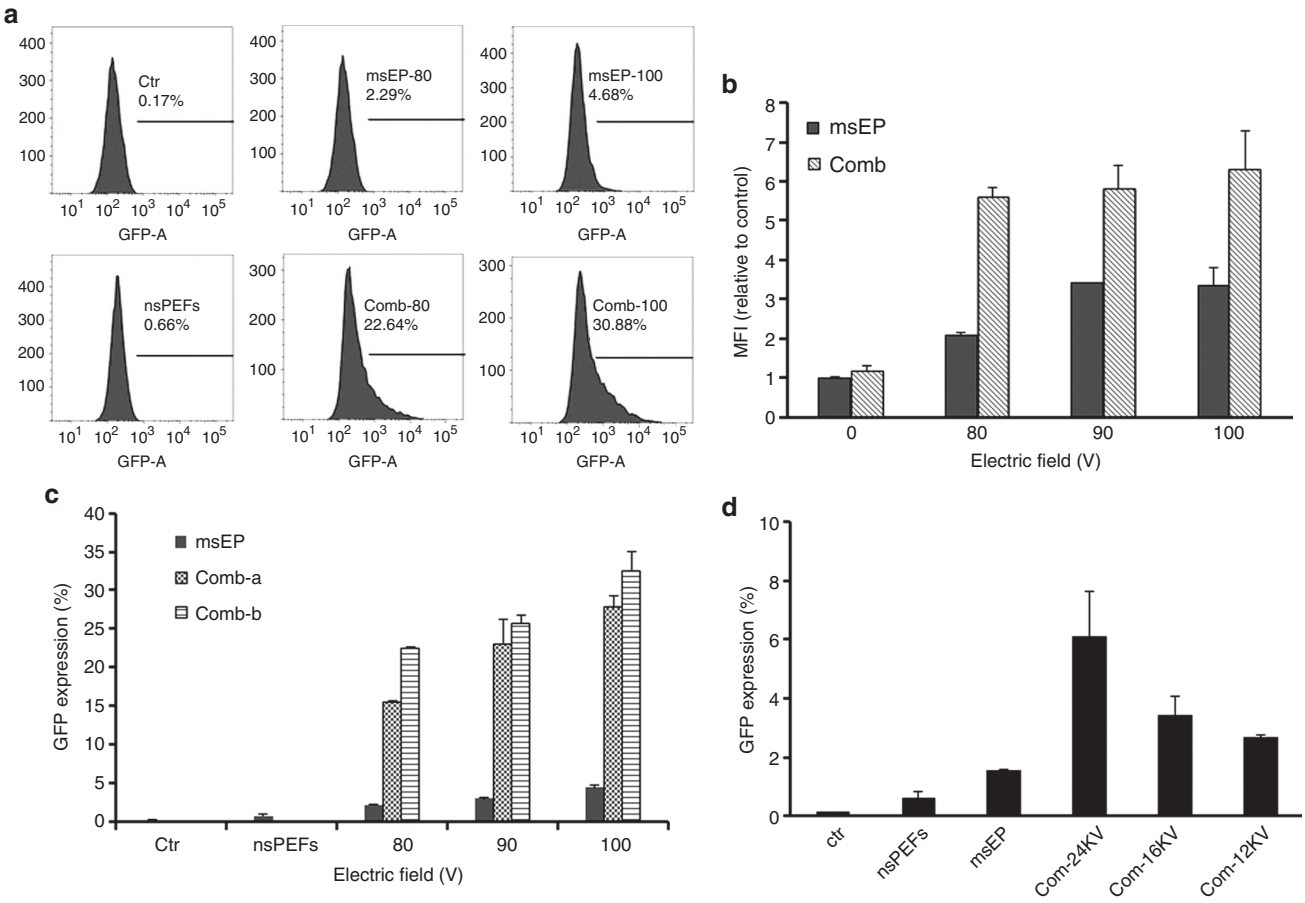

Figure 3 GFP expression after combination pulses. GFP expression of HACAT cells by flow cytometry after gene electrotransfer (GET) with comb-pulses and plasmid gWIZ-GFP. For A, B and C, Ctr: plasmid alone; nsPEFs: pulse duration 60 ns, 32 KV/cm, 1 Hz, and 23 pulses; msEP: pulse duration 5 ms, 1 pulse and applied electric field as indicated. Comb: pretreated with nsPEFs 5 minutes followed by 1 msEP. (**a**) Histogram of GFP expression. msEP –80 or 100: 1 msEP with applied electric field 80 V or 100 V. (**b**) Mean Flurorescence Intensity (MFI) of GFP expression. (**c**) Percentage of GFP positive cells. Comb-a: 16 pulses of nsPEFs; Comb-b: 23 pulses of nsPEFs. (**d**) Percentage of GFP transfected cells. Ctr: plasmid alone; nsPEFs: pulse duration 60 ns, 24 KV/cm, 1 Hz, and 23 pulses; msEP: pulse duration 5 ms, 1 pulse and applied electric field 50 V. Comb-12, 16, and 24 KV: pretreated with nsPEFs with electric field 12, 16, and 24 KV/cm, respectively, 5 minutes later followed by 1 msEP. Error bars represent SD.

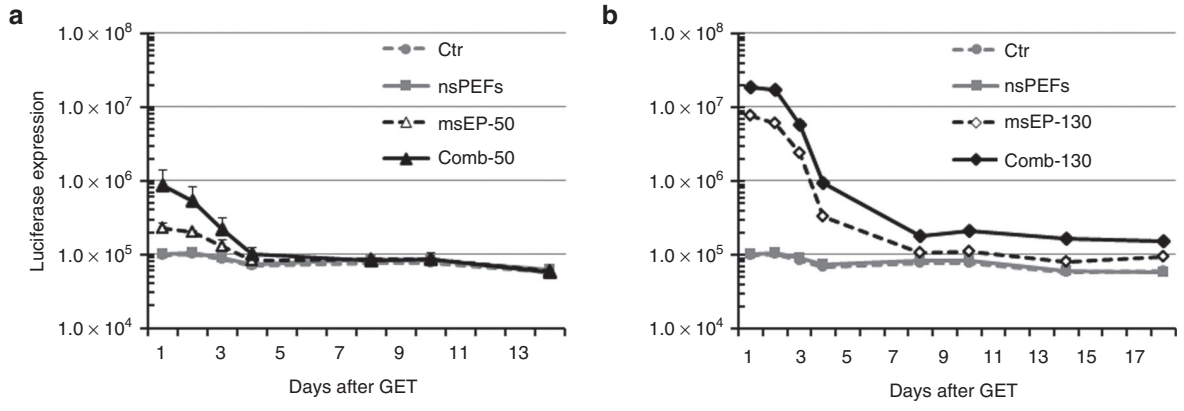

Figure 4 Kinetics of gene expression of HACAT cells after gene electrotransfer (GET). (**a**,**b**) Luciferase expression of HACAT cells after GET with gWIZ-Luc. Groups, Ctr: plasmid alone. nsPEFs: treated with nsPEFs alone. ms-50, 130: treated with 1 msEP applied electric field 50 V or 130 V. Comb-50, 130: treated with nsPEFs then 5 minutes later with 1 msEP applied electric field 50 V or 130 V. nsPEF Parameters: 60 ns, 24 KV/cm, 1 Hz, and 23 pulses; msEP parameters: 5 ms, 1 pulse, applied electric field as indicated. Error bars represent SD.

did not drop to background levels even after 18 days after transfection either with 1 msEP alone or with nsPEFs. In contrast to 1 msEP alone, the nsPEFs enhanced gene expression 1.7–2.8 times at different time points (Figure 4b). If we consider more cell death for the combined pulses than that for 1 msEP alone, the increase of expression on a per cell basis could reach 15-fold at day 1. For both kinetic models of gene expression, nsPEFs plays a role as an amplifier without changing the trend of gene expression due to msEPs.

By direct observation of GFP expression under fluorescence microscopy, GFP positive cells showed heterogeneous fluorescence intensity and diverse kinetics (Figure 5). HACAT cells with high fluorescence intensity could either rapidly drop GFP expression to background level in 4 days or maintain high expression longer than 4 days while cells were dividing. Meantime, cells with lower fluorescence intensity could maintain GFP expression at least 4 days. In contrast to results from live imaging of luciferase expression (Figure 4b), GFP expression lasted at least 9 days (Supplementary Figure S1). Although the half-life of GFP in HACAT cells was not determined precisely here, studies in human colon adenocarcinoma cells have shown a half-life of 28.3 hours.[37] A problem for the observation of long-term GFP expression is that cells were fused together and might go through aging or degradation.

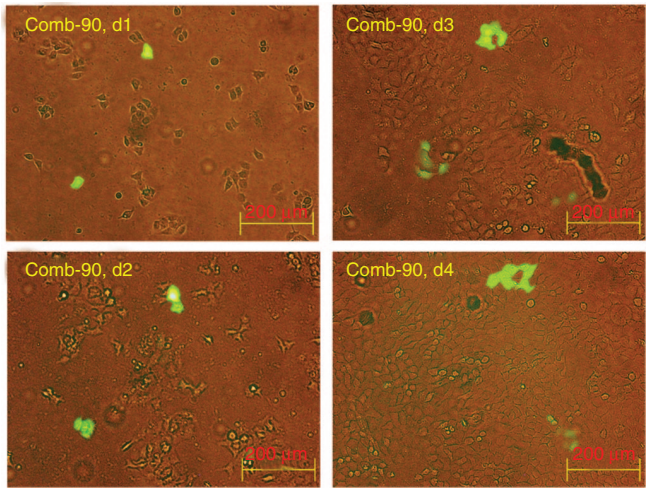

Figure 5 Diverse GFP expression and dynamic changes. Images of HACAT cells after gene electrotransfer (GET) with combination pulses. Comb-90: cells pretreated with nsPEFs 5 minutes followed by 1 msEP. nsPEF parameters: pulse duration 60 ns, 24 KV/cm, 1 Hz, and 23 pulses; msEP parameters: pulse duration 5 ms, applied electric field 90 V and 1 pulse. Day 1 (d1), d2, d3, and d4: images with the same area were taken after GET 1, 2, 3, and 4 days.

Time-restricted effects of nsPEFs on GET

Besides the order of millisecond and nanosecond electric pulses having a significant impact on gene expression efficiency (Figure 2), we also discovered that the effect of nsPEFs on gene transfection was time restricted. The enhancement of gene expression occurred immediately after nsPEFs and reached the maximum at posttreatment 5 minutes, slightly reduced at 15 minutes and had a residual effect at 1 hour (Figure 6a). Under the parameters we adopted in this experiment, in contrast to 1 msEP alone, luciferase expression with combined pulses was increased to 20.3-fold, 43.5-fold, 37.9-fold, and 9.5-fold (all $P < 0.001$), respectively for time gap 0 minute, 5 minutes, 15 minutes, and 1 hour. For this reason, we performed GET after cells pulsed with nsPEFs 5 minutes for all experiments except with these time-restricted assays. The extent of gene expression increase after cells pretreated with nsPEFs may vary depending on the parameters of nsPEFs and msEPs. For example, under another set of parameters, luciferase expression with combined pulses was enhanced to 3.4-fold, 6.5-fold, and, 6.1-fold, (all $P < 0.001$), respectively for the same time gaps as above (Figure 6b).

The role of calcium on nsPEFs' effect on gene expression

Calcium release induced by nsPEFs has been reported by several groups.[26,27] To address whether extracellular calcium plays a role on the effects of nsPEFs on GET, we performed experiments by replacing complete medium (DMEM with 10% FBS) with calcium free PBS (Dulbecco's Phosphate Buffered Saline). Surprisingly, gene expression from both 1 msEP alone and combination pulses was dramatically reduced (Figure 7a, b). However, the significant enhancement effects were still present by combination with nsPEFs. In contrast to 1 msEP alone, luciferase expression with combined pulses was increased to 2.6-fold, 3.5-fold, and 3.4-fold (all $P < 0.001$), respectively for time gap 0 minute, 5 minutes, and 15 minutes (Figure 7b). To assess the role of intracellular calcium on the nsPEF enhancement,

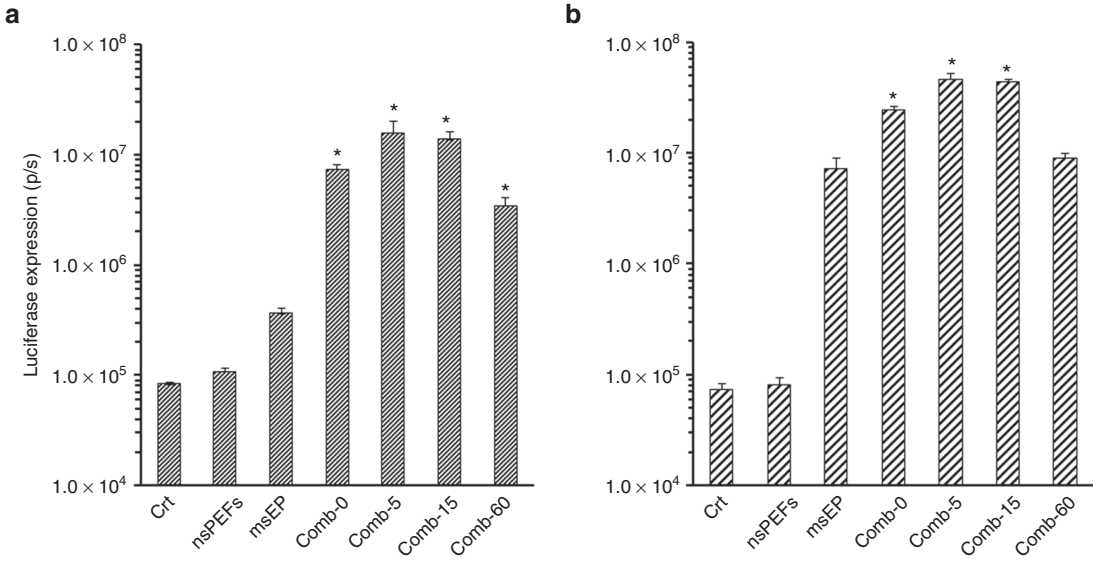

Figure 6 Time restricted effect of nsPEFs on gene electrotransfer (GET) with msEPs. Luciferase expression of HACAT cells after GET with comb-pulses and plasmid gWIZ-Luc. Ctr: plasmid alone; nsPEFs: pulse duration 60 ns, 24 KV/cm, 1 Hz, and 23 pulses; msEP: pulse duration 5 ms, applied electric field (**a**) 50 V or (**b**) 70 V, and 1 pulse; Comb-0, 5, 15, 60: pretreated with nsPEFs then followed by 1 msEP with time interval 0, 5, 15, and 60 minutes, respectively. $n = 3$ and error bar is represented as SD. *$P < 0.001$ for comb-groups versus msEP alone.

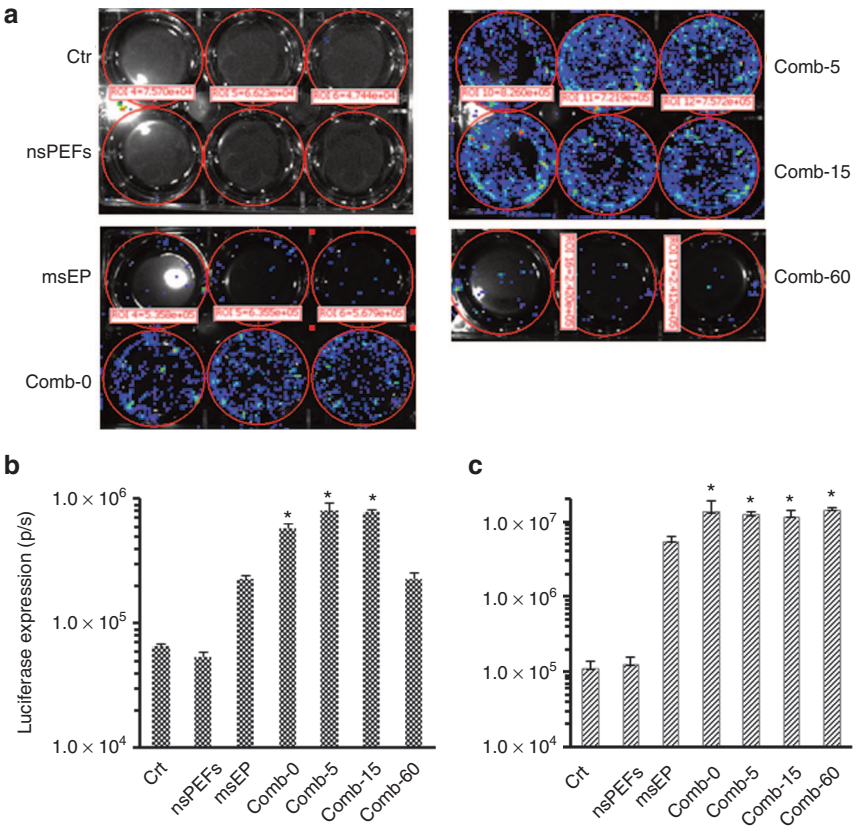

Figure 7 The effect of calcium on nsPEF enhancement for gene expression. (**a**,**b**) Luciferase expression with extracellular calcium depletion. (**a**) Live images of luciferase expression by IVIS spectrum at day 1. (**b**) Luciferase expression. (**c**) Luciferase expression with extra- and intracellular calcium depletion. Groups, Ctr: plasmid alone; nsPEFs: pulse duration 60 ns, 24 KV/cm, 1 Hz, and 23 pulses; msEP: pulse duration 5 ms, applied electric field 50 V for A and B, 70 V for C, and 1 pulse; Comb-0, 5, 15, 60: pretreated with nsPEFs then followed by 1 msEP with time interval 0, 5, 15, and 60 minutes, respectively. $n = 3$ and error bar is represented as SD. (**b**) $^*P < 0.001$ and (**c**) $^*P < 0.05$ for comb-groups versus msEP alone.

HACAT cells suspended in calcium free DMEM were pretreated with cyclopiazonic acid (CPA) at 20 µmol/l for 30 minutes then performed the experiment as above. Although immediate enhancement of nsPEFs was observed, the time-restrictive changes of nsPEFs within 1 hour were blocked by intracellular calcium depletion. Compared to 1 msEP alone, luciferase expression with combined pulses was increased to 2.4-fold, 2.2-fold, 2.1-fold, and 2.6-fold (all $P < 0.05$), respectively for time gap 0 minute, 5 minutes, 15 minutes, and 1 hour (Figure 7c).

DISCUSSION

In comparison to viral gene delivery system, one of the disadvantages for non-viral delivery is low efficiency. To enhance delivery a variety of approaches have been developed including physical methods. One method that has shown a great deal of potential is electro transfer. At least two strategies have been developed for the improvement of GET efficiency. One is the optimization of various influencing factors including DNA concentration, electrodes and pulse parameters (field strength, field direction, pulse number and frequency). Another is the enhancement of GET with other factors that do not deliver DNA but may synergize with GET.[15–17] Our present approach is one example of second strategy since nsPEFs alone do not sufficiently deliver plasmid DNA into cells. Our results clearly demonstrated that pretreating cells with nsPEFs could significantly enhance the efficiency of GET with both mild and intense msEP parameters. However, no major impact on gene expression, which is consistent with Chopinet's report,[35] was observed if cells were treated with msEPs first and nsPEFs later. This is against our initial hypothesis that nsPEFs would facilitate transport of the DNA from the cell membrane to the nucleus after classical electroporation. The mechanism why the order of two electric pulses is critical to influence gene expression is unclear. Noticeably, it appears that nsPEFs can amplify the effects of msEPs whether it's on gene expression or on cell damage. This property could be potentially utilized to enhance GET efficiency or improve the efficacy of irreversible electroporation (IRE).

Cell viability is critical for GET to treat metabolic diseases, inoculate vaccines, etc. A common issue for the enhancement of GET is concomitant additional cell damage or cell death. Additional cytotoxicity with 80% or 50% cell death[38] was present while antioxidant vitamin C (6–8 mmol/l) or tempol (8 mmol/l) was utilized to increase GET efficiency. Cemazar *et al.*[15] reported that pretreatment of tumors with a combination of hyaluronidase and collagenase can significantly enhance GET efficiency. However, the tumor growth with combination enzymes and GET was observed slower than control or GET alone group within 5 days.[15] Our viability study showed that the enhanced efficiency of GET without cause of cell death can be achieved by optimizing both pulse parameters. This feature indicates the possibility of our combination approach for many *in vivo* applications. As a matter of fact, total gene expression was still higher for our combination pulses than for msEPs alone at extreme electric field strength (1,040 V/cm) while the combination approach resulted in fourfold more cell death. It suggests the increase of GET efficiency from the combination approach overcomes the efficiency

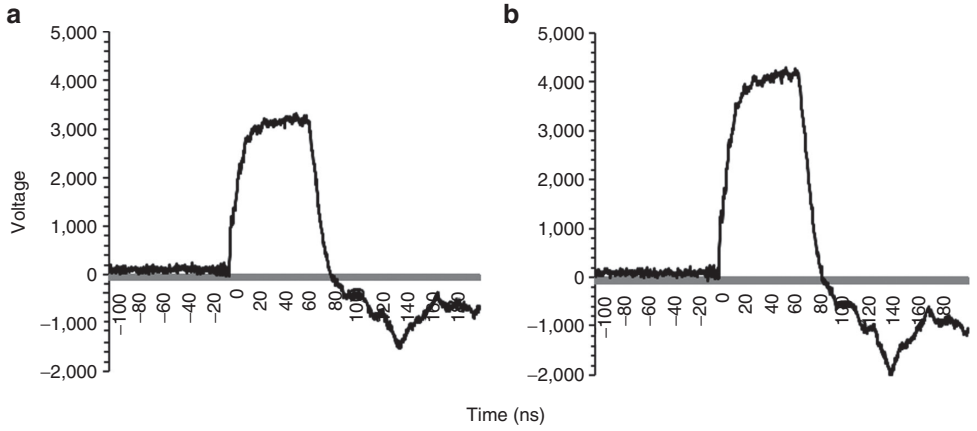

Figure 8 The Profiles of nanosecond electric pulses. (**a**) A representative 60 ns pulse with applied electric field 3 KV or 24 KV/cm. (**b**) A representative 60 ns pulse with applied electric field 4 KV or 32 KV/cm.

decrease caused by additional cell death under intense msEP parameters. This property makes our combination approach useful for cancer gene therapy when more gene expression is needed but cell viability is not critical.

Combined high- and low-voltage (HV+LV) electric pulses with pulse duration under microsecond to millisecond range have been demonstrated as an efficient GET protocol *in vitro* and *in vivo* by several groups.[14,39–41] It appears our combination pulses is quite distinctive approach in many aspects. First, both HV and LV pulses are capable of effectively delivering DNA into cells, and either single pulse protocol has been developed to achieve *in vitro* and *in vivo* GET. In contrast, the successful gene transfer by nsPEFs has not been proven. Second, HV pulse, which is applied first in the combination, can achieve effective gene expression while LV pulse may or may not be sufficient to obtain gene transfer depending on the parameters, then facilitates the gene transfer induced by the former HV pulse. However, in our combination strategy, it is the msEP, the second pulse that achieves effective gene transfer, while pretreatment with nsPEFs, which is unable to achieve any meaningful gene expression, increase the gene transfer efficiency. Third, the influence of the interval between two pulses on gene transfer is different. The enhancement effect of HV+LV pulses reaches the peak with a very short lag time microsecond to millisecond[42,43] then maintains the same level with a lag 5 seconds[43] to 50 minutes[42] depending on the parameters. Nevertheless, it takes 5 minutes for cells to obtain the maximal enhancement effect by nsPEF treatment. This enhancement is slowly diminished and can present with lag time longer than 1 hour. Fourth, above comparisons may also suggest that the mechanisms involved in these two combinations are different.

The mechanism behind our discovery is quite interesting. For the HV+LV combination, it was suggested that HV pulses permeabilized cell membrane for DNA access then LV pulses electrophoretically moves DNA into cells or nuclei.[43] It would be possible that nsPEFs act in similar way by causing membrane perturbation[23,26] to facilitate subsequent DNA transfer mediated by longer electric pulses. Moreover, a number of intracellular events occur after nsPEFs, including cell swelling,[44] calcium mobilization,[26,27] cytoskeleton (actin) disruption,[45] nuclear membrane damage,[46] etc. Several other hypothetical processes are potentially involved in the enhancement mechanism of nsPEFs for GET. First, cell swelling, which has been observed by Romeo *et al.*[44] and in our experiments (data not shown), induced by nsPEFs may increase permeabilized membrane area for consequent DNA access. Second, intracellular changes caused by nsPEFs, including actin disassembly,[45] calcium mobilization, organelle membrane modulation, may help intracellular DNA transport to the nucleus. Third, nuclear membrane disruption[46] may increase DNA diffusion but also decrease active DNA trafficking into nucleus. Fourth, changes in the nucleus including chromatin rearrangement[46,47] could also influence DNA stability. In this study, we were particularly interested in the role of intracellular calcium release or extracellular calcium mobilization from nsPEFs. It appears the enhancement effect is largely present after both intra- and extracellular calcium depletion. Nevertheless, intracellular calcium release likely amplifies this enhancement since the blockage of further increase with 5 minutes pulse interval was observed after intracellular calcium depletion. Our data also suggest that there are more than one mechanism involved. One of the major components is calcium independent enhancement occurring immediately after nsPEFs and slowly diminished. Another component is calcium dependent amplification, which is indirectly induced by nsPEFs, reaches the maximum 5 minutes following nsPEFs. Obviously, more studies will be needed to explore the detail mechanisms of our combination pulses.

In conclusion, we first discovered that pretreatment but not posttreatment with nsPEFs was critical to significantly influence the gene expression of msEPs. We demonstrated the efficiency of GET with msEPs could be greatly enhanced up to 40-fold by combination with nsPEFs. The kinetics of gene expression with GET was not changed by nsPEFs under both mild and intense msEP parameters. The enhancement effects occurred immediately after nsPEFs, reached the maximum 5 minutes later and slightly decreased at 15 minutes and could be seen after 1 hour. Cell viability was determined by both parameters of msEPs and nsPEFs. The enhancement effect of nsPEFs is largely calcium-independent and occurs immediately after nsPEFs. However, intracellular calcium release by nsPEFs is likely involved in time-restricted impact of nsPEFs on GET. Our novel combination pulse approach can be utilized for the improvement of GET efficiency while higher levels of gene expression are needed.

MATERIALS AND METHODS

Materials

The reporter plasmids encoded luciferase (gWiz-Luc) and green fluorescent protein (gWiz-GFP) were purchased from Aldevron (Fargo, ND). CPA, a reagent for intracellular calcium depletion, was obtained from Iurii Semennov (Old Dominion University). D-Luciferin, which was utilized for luciferase assay or

live cell imaging, was purchased from Goldbio Technology (St Louis, MO). WST-1 for cell viability assay was obtained from Roche Applied Science (Indianapolis, IN).

Cell culture

Human keratinocyte cell line (HACAT) was grown in DMEM (Cellgro, Mediatech, VA) media with 10% FBS and antibiotics penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 °C in an incubator under 5% CO_2. Cells were harvested for experiments while 90% confluency was reached.

GET protocol

Electrotransfer was performed by using the ECM 830 Square Wave Electroporation System (BTX, Harvard Apparatus, MA). Cells were trypsinized with trypsin-EDTA (0.05% trypsin/0.025% EDTA), washed with PBS and suspended in complete medium or calcium free medium at a concentration of 5×10^6/ml. After mixed with plasmid DNA (final concentration 10 µg/ml) 140 µl HACAT cell suspension was placed in a 1.25 mm gap electroporation cuvette (BioSmith, CA). Cells were then exposed to the pulsed electric fields with pulse duration 5 ms, frequency 1 Hz, pulse number from 1 to 16 and applied electric field from 40 to 130 V (equal to 320–1,040 V/cm) dependent on experiment designs. Cells were either pretreated or posttreated with nsPEFs with interval 0 minute to 1 hour relying on the purpose of experiments. Cells were then transferred to a six-well cell culture plate (Corning Incorporated Life Sciences, MA) and incubated in 2 ml complete medium per well for 1–18 days for analysis dependent on the experimental designs.

Nanosecond pulsed electric fields

NsPEFs were produced by a custom-designed nanosecond pulse generator that can generate fixed 60 ns PEFs with adjustable pulse frequency and applied electric field. The pulse profile was monitored and recorded by a digital phosphor oscilloscope (TDS3052B, Tektronix, OR) (Figure 8), and power was provided by a high voltage power supplier (EH60R1.5, Glassman High Voltage, NJ).

Prior to or after electrotransfer, 140 µl HACAT cells (5×10^6/ml) in a cuvette were pulsed with nsPEFs. The pulse parameters were pulse duration 60 ns, frequency 1 Hz, applied electric fields from 1.5 to 4 KV (equal to 12–32 KV/cm) and pulse number 16 or 23 dependent on experimental designs. If the interval between GET and nsPEFs was longer than 5 minutes, the cells were re-suspended by a pipette with a 200 µl tip immediately before the second time electric pulse delivery. Cells then were transferred, incubated and analyzed as mentioned above.

GFP expression

To observe the dynamic GFP expression, complete medium was replaced with prewarmed PBS. Live cells on the plate were then examined by Olympus IX71 inverted fluorescence microscopy (Olympus, Tokyo, Japan) at different time points after GET. Pictures were captured by DP Controller software (Version 3.1.1., Olympus).

To quantify GFP positive cells, transfected cells were harvested and resuspended in PBS with 2% FBS after gene delivery 2 days. Samples were analyzed on BD FACSAria flow cytometer (BD Biosciences, San Jose, CA). A minimum of 15,000 events was collected and the analysis of GFP expression cells was performed with FACSDiva software (BD Biosciences).

Living imaging of luciferase expression

Live cell bioluminescence imaging was performed with the IVIS Spectrum system (Caliper Life Sciences, Hopkinton, MA). At different time points culture medium was removed from six-well plate and replaced with 300 µl per well prewarmed complete medium with Luciferin (300 µg/ml, Goldbio Technology). Images were acquired 10 minutes after cells incubated with luciferin at 37 °C. Quantitation of luciferase activity was analyzed with the Living Image acquisition and analysis software (Caliper Life Sciences).

Luciferase assay

Luciferase activity was quantified as the following steps. Growth medium was removed from six-well plates 18 hours after GET. Cells were washed with 2 ml 1× PBS twice. 250 µl Cell Culture Lysis Reagent (CCLR) was dispersed into each well. Cells were scraped and transferred together with solution into a microcentrifuge tube. After brief centrifugation, the supernatant was transferred to a new tube. 30 µl cell lysate was mixed with 150 µl Luciferase Assay Buffer (25 mmol/l glycylglycine, pH 7.8, 15 mmol/l KPO_4, pH 7.8, 15 mmol/l $MgSO_4$, 4 mmol/l EGTA, 2 mmol/l ATP, 1 mmol/l DTT, and 100 µmol/l Luciferin). Luciferase activity was quantitated with a Dynex MLX microplate luminometer (Dynex Technologies, Chantilly, VA). Serial dilutions of recombinant luciferase (Promega, Madison, WI) were utilized to make a standard curve. Relative light unit (RLU) values were converted to luciferase activity (pg), which was reported as total pg luciferase per million cells.

WST-1 viability assay

After GET with or without nsPEFs, 10 µl of cell suspension was added into a clear-flat-bottom 96-well plate filled with 90 µl complete medium per well. Cells were incubated at 37 °C in an incubator under 5% CO_2. After 18 hour incubation, 10 µl of WST-1 reagent was added to each well of 96-well plate. Cells were incubated with WST-1 for 2 hours. The absorbance was measured by MultiSkan MCC/340 microplate reader (Fisher Scientific, Hampton, NH) with a test wavelength at 450 nm and a reference wavelength at 630 nm. The cell viability was calculated with an equation: Treated sample (OD450-OD630)/control (OD450-OD630) × 100%. 140 µl cells mixed with same amount of DNA in a cuvette was placed into the cuvette holder for the same duration as treated cells but no exposure to electric field as a control.

Statistical analysis

All values are reported as the mean ± SD. Analysis was completed by one-way ANOVA for many groups or two-tailed Student's *t*-test for two groups. Statistical significance was assumed at $P < 0.05$. All statistical analysis was completed using the SigmaPlot 11.0 (Aspire Software International, Ashburn, VA). For experiments with small size samples ($n = 3$), the statistical analysis was justified to obtain a $P < 0.05$ with a power of 0.9 (PS Power and Sample Size Calculations, Version 3.0).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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