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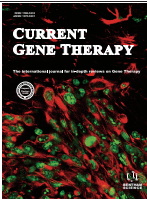
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Thermal Assisted *In Vivo* Gene Electrotransfer

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Abstract: Gene electrotransfer is an effective approach for delivering plasmid DNA to a variety of tissues. Delivery of molecules with electric pulses requires control of the electrical parameters to achieve effective delivery. Since discomfort or tissue damage may occur with high applied voltage, the reduction of the applied voltage while achieving the desired expression may be an important improvement. One possible approach is to combine electrotransfer with exogenously applied heat. Previous work performed *in vitro* demonstrated that increasing temperature before pulsing can enhance gene expression and made it possible to reduce electric fields while maintaining expression levels. In the study reported here, this combination was evaluated *in vivo* using a novel electrode device designed with an inserted laser for application of heat. The results obtained in this study demonstrated that increased temperature during electrotransfer increased expression or maintained expression with a reduction in applied voltage. With further optimization this approach may provide the basis for both a novel method and a novel instrument that may greatly enhance translation of gene electrotransfer.

Keywords: Gene electrotransfer, Skin, Gene therapy, Heat, Electroporation.

1. INTRODUCTION

A critical aspect for gene therapy is appropriate delivery. Non-viral delivery has a favorable safety profile; however, expression levels are typically low without the addition of physical, chemical or viral delivery methods. Physical approaches, including ultrasound, hydrodynamic pressure, and electrotransfer, enhance delivery to a variety of tissues and are increasingly evaluated for therapeutic applications [1-7]. Gene electrotransfer (GET; electroporation) uses electric pulses to transiently permeabilize cell membranes allowing entry of extracellular nucleic acids into the cell. The current challenge is to develop protocols that result in appropriate transfection efficiency without adverse effects [8, 9].

Skin is an excellent target for gene therapy [10-15] due to its easy accessibility for delivery and monitoring. GET of skin is a simple and direct method for gene therapy and can be accomplished in a minimally invasive way. Several approaches with potential therapeutic or prophylactic applications have been evaluated including wound healing [16-18], ischemia [19-21], infectious disease vaccines [22-25] and cancer vaccines and therapies [5, 8, 26]. To date, sixty-eight clinical trials are being enrolled, are ongoing, or are completed using GET (clinicaltrials.gov “electroporation”, “electrotransfer”). Most of these trials are designed to evaluate DNA vaccines against infectious agents or cancer or the effectiveness of cancer immunotherapy [27].

For optimal delivery GET electrodes must be designed based on the target tissue and/or the desired endpoint analysis, for example secreted protein, antibody production, or cell death. Therefore, important focus areas for GET research have been electrode development and the identification of “ideal” pulsing parameters. A variety of electrodes for *in vivo* use have been developed including needle, caliper, plate, and non-penetrating pin electrodes [23, 28, 29]. While each electrode type has its advantages and disadvantages, the surface electrodes are attractive because they allow for delivery of DNA non-invasively. A surface electrode design is well-suited for delivery to the skin. However, the skin is also well-adapted to serving as a barrier from intrusion. Our lab has developed two surface electrodes for gene delivery to the skin that provide high levels of transgene expression. The first was the 4 plate electrode (4PE) designed with 4 plates at 90° angles with a stopper to maintain a constant distance between electrodes [28].

This electrode worked well as it provided a fixed distance and allowed for directional control of the applied electric field in two perpendicular directions without the need for moving the electrodes. While an advantage of this design is the fixed distance, the treatment of larger areas also required an increase in the electrode gap. Since the applied voltage is based on the distance between the electrodes, higher voltages are required to achieve effective delivery. These voltages potentially cause cellular damage or discomfort [28, 30]. To minimize cellular damage or discomfort, a multi-electrode array (MEA) was designed. This applicator consisted of 16 flat pin electrodes 2mm apart in a 4X4 array [23, 29, 31]. The electrode covered the same tissue area as the 4PE but

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smaller electrode distances and an increased number of electrodes allowed for enhanced directional control and a reduction in applied voltage.

Despite many successes of GET and the development of an increasing number of GET-based therapies, further improvement is needed, for example by reducing the necessary applied voltage and by increasing the ability to control expression. While GET is generally a non-thermal process, the administration of low heat from an exogenous source could potentially enhance delivery. Changes in both skin and core body temperature have been demonstrated to affect vasodilation and constriction. Additionally, local warming of tissue increases vasodilation, reaching a maximum blood flow in the skin at 42°C. This effect is maintained for up to one hour after heating [32-34]. The use of heat to enhance delivery is based on the hypothesis that permeability of cell membranes can be affected by temperature [35]. For example, hyperthermia has been used to enhance delivery of chemotherapeutics [36-39]. Increased temperature can influence the efficiency of electrotransfer while decreasing the temperature to 4°C reduces delivery [35].

The goal of the current study was to determine if thermal assisted gene electrotransfer (TAGET) could enhance transgene expression and/or allow for a reduction of applied voltages *in vivo*. We hypothesized that moderate heating prior to GET *in vivo* would allow the use of lower applied voltages while maintaining gene expression without adverse effects on the tissue.

2. MATERIALS AND METHODS

2.1. Animals

Animals used for this study were 6-8 weeks old Female Hartley guinea pigs weighing approximately 200-250g. The animals were housed at the Old Dominion University animal facility and procedures approved by the Old Dominion Institutional Animal Care and Use Committee.

2.2. Plasmids

Endotoxin free preparations at 2mg/ml in physiological saline of gWizLuc, encoding firefly luciferase and gWizGFP green fluorescence protein (GFP) were purchased (Aldevron, Fargo, ND) in this study. Plasmids were prepared endotoxin free by Aldevron (proprietary fermentation technology and DNA purification) and suspended at 2mg/ml in physiological saline.

2.3. Heat Application

Animals were anesthetized with O₂ containing 2.5-3.0% isoflurane. Both left and right flanks were shaved using Wahl Shears and extraneous hair removed by cleansing with water. To determine the intradermal skin temperature from exogenous heating, an 18G needle was inserted intradermally into the left or right flank then replaced with a thermocouple temperature probe (Omega, Stamford, CT, USA). Baseline tissue temperature readings were recorded. Temperature measurements during heat application and GET were collected by positioning the electrode array on the skin above the temperature probe. Exogenous laser heat

was applied at 1, 2 or 3 amps and temperature measured in real time.

2.4. Electrode Design

Electric pulses were administered utilizing a specially constructed applicator. The electric pulse delivery system had an integrated infrared radiation emitter Fig. (1) to heat the tissue radiatively. The emitter consisted of an optical fiber centered between 4 pins with rounded ends spaced 5 mm apart that acted as electrodes. The optical fiber has advantages over other nonionizing radiation emitters: its small diameter (< 1 mm) and dielectric material can be easily integrated into an existing electrical pulse delivery system. The fiber was connected to an infrared semiconductor laser which provided up to 8W of radiative power at a wavelength of approximately 1µm. Adjusting the distance of the fiber to the tissue irradiated a well-defined circular area of the tissue.

2.5. Gene Delivery

Injection sites were marked to ensure accuracy of data collection. Gene delivery and TAGET were assessed after intradermal injections of 50 µl of plasmid to multiple sites on both left and right flanks. The electrode array was positioned over the injection area with or without exogenous heating. The heat was applied as determined in the first round of experiments at 1 amp for 30 seconds. The tissue remained at 43°C for approximately 10 seconds, allowing adequate time for pulsing at a range of voltages from 50-125V for luciferase experiments and 50-100V for GFP experiments. Based on our previously published results with a similar electrode, pulse number, time between pulses and pulse length were maintained at 8 pulses, 150ms, and 150ms, respectively [23, 29, 31].

During the course of these experiments we evaluated laser amperage, duration of thermal assistance and applied voltage. Gene expression levels were measured for all conditions by *in vivo* bioluminescent imaging or fluorescent imaging. Visual assessment of the skin was used to determine the extent of external tissue damage from the addition of moderate heat.

2.6. *In Vivo* Bioluminescent Imaging

On days 1, 2, 5, 7, 10, and 14, animals were anesthetized with O₂ containing 2.5-3.0% isoflurane followed by injections of 50 µl of 15 mg/ml D-luciferin (Gold Biotechnology, St. Louis, MO) at the plasmid injection sites. The animals were confined in an anesthesia chamber for two minutes then transferred to the IVIS[®] Spectrum (Perkin Elmer, Akron, OH), imaging chamber under constant anesthesia (O₂ containing 2.5% isoflurane). Regions of interest (ROI) were selected on the image to encompass the entirety of each injection site independently and compared to untreated control ROIs. After background correction bioluminescence results were represented as average total flux, photons/sec (p/s).

2.7. GFP Expression and Analysis

Forty-eight hours after plasmid delivery, the animals were euthanized and the treated areas were excised, fixed in 4% paraformaldehyde for 1-2 hours, equilibrated in optimum

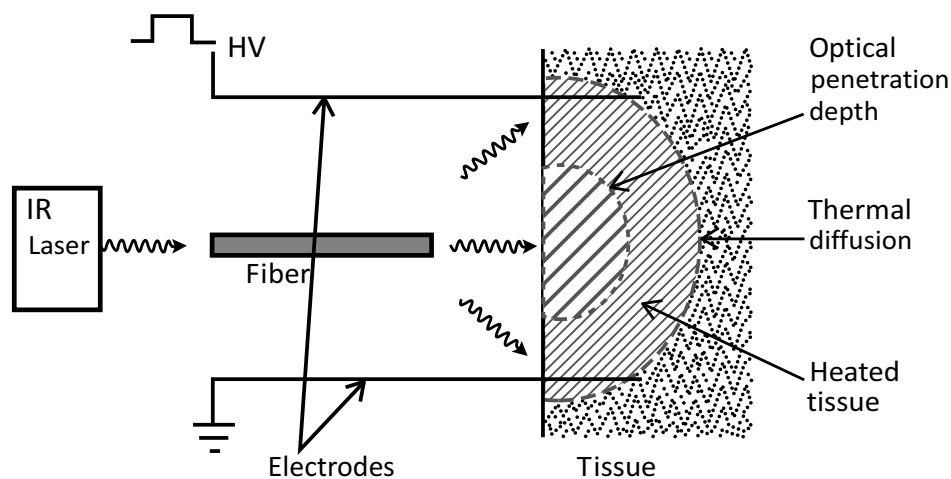


Fig. (1). TAGET Device. Schematic of an optical fiber that was connected to an infrared laser and inserted into the device. The fiber is situated centrally between 4 electrodes. The fiber is also placed 1 cm above a 3 mm opening. This gap and opening allows for an increased spot size of the light as it exits into the space between the opening and the tissue which is set at 5 mm.

cutting temperature (OCT) compound, snap frozen using dry ice and stored at -80°C . Samples were sectioned on a cryostat and mounted in Vectashield[®] mounting media with 4', 6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA) was used for nuclear staining. Slides were imaged on an Olympus BX51 fluorescence microscope. The analysis of the tissues was performed in a blinded fashion. Two members of the research team performed the evaluation independently without knowledge of the specific treatment protocol for the sample being evaluated.

2.8. Statistical Analysis

Temperature and luciferase measurements are represented as the mean \pm SD. Comparisons between specific voltage with or without the addition of thermal component were performed by Student T-Test. Significance was assumed at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Electrode Design, Development and Temperature Determination

An *in vivo* surface array was designed that incorporated features from both the 4PE and MEA [23, 28-31]. This new array consisted of four electrodes with 5mm length and 5mm gap. Centered within the array was an infrared laser which sat flush to the electrode handle and applied a focused beam of light to the skin (Fig. 1). The electric pulses were administered in 2 sets of four pulses orthogonally. As shown in the schematic (Fig. 2), the pulses were administered simultaneously from the electrodes for four pulses (*i.e.*, from 1 and 4 to 2 and 3) then using a manual switch the direction was rotated 90° and pulses administered for four additional pulses (1 and 2 to 4 and 3). Fig. (2) provides the full final protocol for administration of TAGET (not to scale). The total time required for 1 treatment was approximately 45 seconds.

In a previous *in vitro* study we evaluated the use of low and moderate heat conditions prior to application of GET to increase gene expression and determine if reduction in absolute voltage could achieve a reduction in cell mortality [40].

This study demonstrated that the application of moderate levels of heat could achieve increased gene expression, although utilizing maximal electric fields came at the expense of cell viability. Interestingly, we found that when moderate exogenous heat was applied prior to pulsing, similar gene expression levels could be achieved using about 30% lower electric fields. These increased gene expression levels were obtained with a significantly higher level of cell viability. An important observation from this work was that temperatures higher than 43°C resulted in increased cell death [40] while lower temperatures did not produce consistently higher gene expression. Therefore, 43°C was used for all *in vivo* experiments.

The first set of experiments was designed to determine the time it took to heat the tissue to 43°C . Using a temperature probe inserted laterally into the dermis of the guinea pig, the time to reach 43°C was measured from application of direct currents between 1 and 3 amps. Averages of 29 (+/-4), 23 (+/-7), and 17 (+/-2) seconds were measured for 1, 2, and 3 amps respectively (Fig. 3A). However, at both 2 and 3 amps the temperatures continued to rise quickly beyond 43°C . A consistent rise in heating was obtained by using 1 amp for 30 seconds. In addition, the temperature probe was left inserted in the skin after heating and application of TAGET to determine the increase in temperature at the end of delivery. Consistent with the previously published *in vitro* results and reports on the lack of heat generation from reversible electroporation, changes in temperature were less than 1°C . However, minimal visual damage that typically healed within 2-4 days was noted at the location of electrode contact (Fig. 3B, C).

3.2. Evaluation of In Vivo Luciferase Gene Expression After TAGET

Luciferase expression was measured to determine whether the addition of heat prior to GET would enhance expression *in vivo* over time. Injection of DNA only, thermally assisted injection of DNA, and GET were included as controls in these experiments. Guinea pigs received an intradermal injection of 50 μl gWizLuc on Day 0 immediately

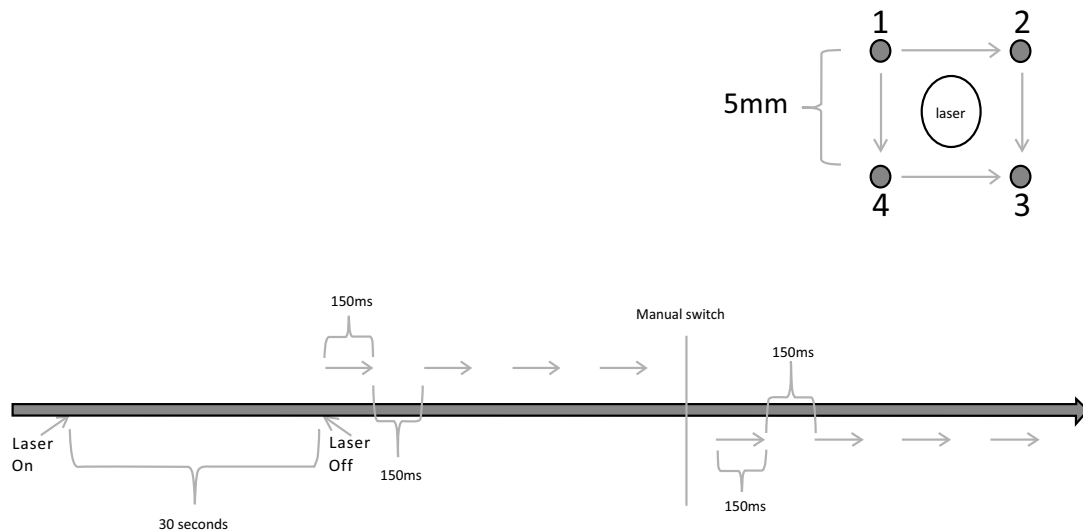


Fig. (2). Experimental Setup and Delivery Protocol. Upper panel: The pulse sequence is outlined. All 4 electrodes are active for each pulse, but are applied in two perpendicular directions in sequence. Four pulses in one direction followed by four in a perpendicular direction. Lower panel: schematic of the delivery protocol to the skin.

followed by TAGET consisting of 30 seconds of heating at 1 amp at an applied voltage of 125V, 8 pulses for 150ms. Luciferin was injected into the same location as treatment on Days 1, 2, 7, and 14 and photon emissions measured on the IVIS Spectrum. No differences between the application of DNA + heat as compared to DNA alone were observed and as expected both had lower expression than DNA + GET (Fig. 4). Interestingly the greatest differences between TAGET and GET were noted at Day 2 (a 12 fold increase). On Day 7, the greatest difference (6-8000 fold) observed was between TAGET and DNA injection. At Day 14 TAGET groups decreased below the levels of GET.

While the addition of heat alone did not result in enhanced gene expression as compared to injection of DNA alone, the combination of heat pretreatment and GET did result in higher gene expression at specific time points. Therefore, we sought to determine the most appropriate GET voltage condition for enhancing gene expression with thermal assistance. The previous heating conditions were maintained, but the applied voltage varied from 50 to 125V (Fig. 5A). The highest gene expression was achieved at an applied voltage of 100V with or without the addition of heat. However, the addition of heat at 50V resulted in significantly ($p < 0.05$) increased gene expression as compared to GET alone at Days 6 and 7 (Fig. 5B). Significant ($p < 0.05$) gene expression increases were also noted at the same time points for 100V. TAGET at lower voltages resulted in similar gene expression levels as higher voltage GET conditions. For example expression levels achieved with TAGET at 50V were similar to levels following 100V GET (Fig. 5B).

3.3. Location of Gene Expression by TAGET

A disadvantage of surface electrodes is their inability to penetrate deep into the tissue without using high potentially damaging electric fields. The addition of heat prior to GET might facilitate delivery and might produce gene expression deeper within the tissue. To analyze this, gWizGFP plasmid

was injected and the site treated with and without heat and with and without GET at 50 and 100V. After 48 hours, increased GFP protein expression was observed in TAGET groups (Fig. 6); however, deeper penetration into the dermis was not observed. As with GET, the use of TAGET appears to localize expression within the epidermis.

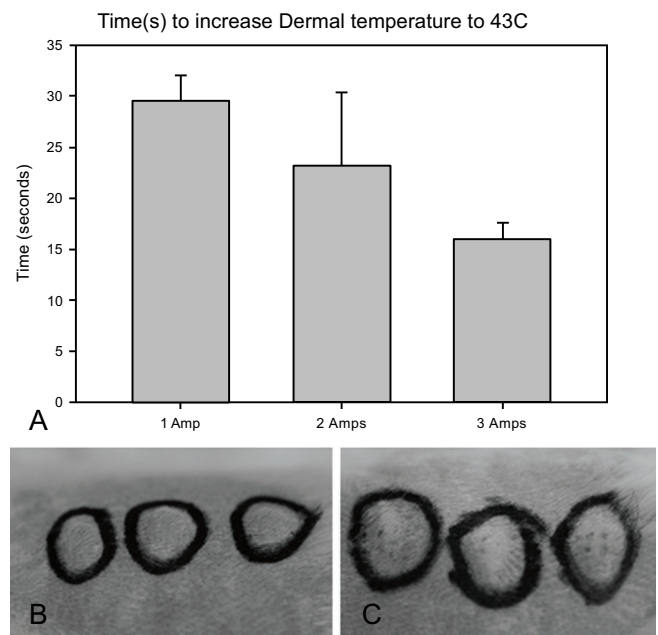


Fig. (3). Thermal Application Timing and Effect on Skin. (A) Comparison of various laser amperages and the time necessary to increase intradermal skin temperature to 43°C. Error is represented as standard deviation (SD). (B) Evaluation of tissue damage by visual analysis after application of heat and prior to pulsing. (C) Evaluation of tissue damage by visual analysis after application of heat and after pulsing.

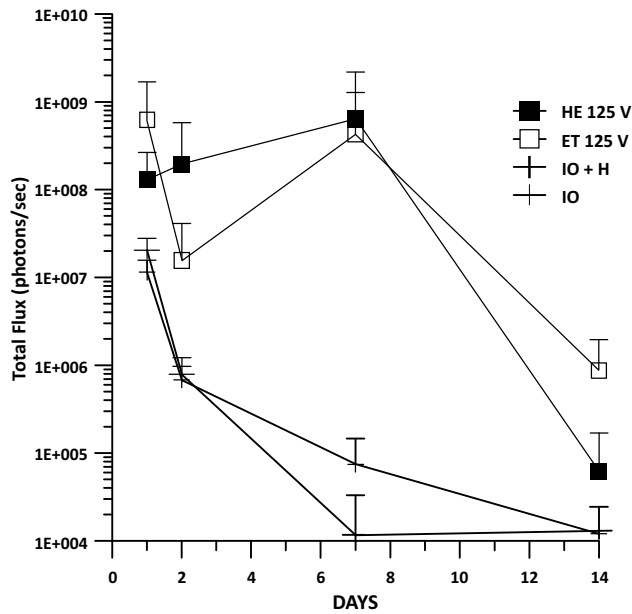


Fig. (4). Gene Expression Resulting from TAGET. Luciferase gene expression was evaluated during a 2 week time course following delivery with or without heat pretreatment and GET. 8 pulses of 150ms were administered with an applied voltage of 125 with heat (HE) or without heat (ET). This was compared to injection of plasmid DNA with heat (IO + H) or without heat (IO). Error is represented as SD.

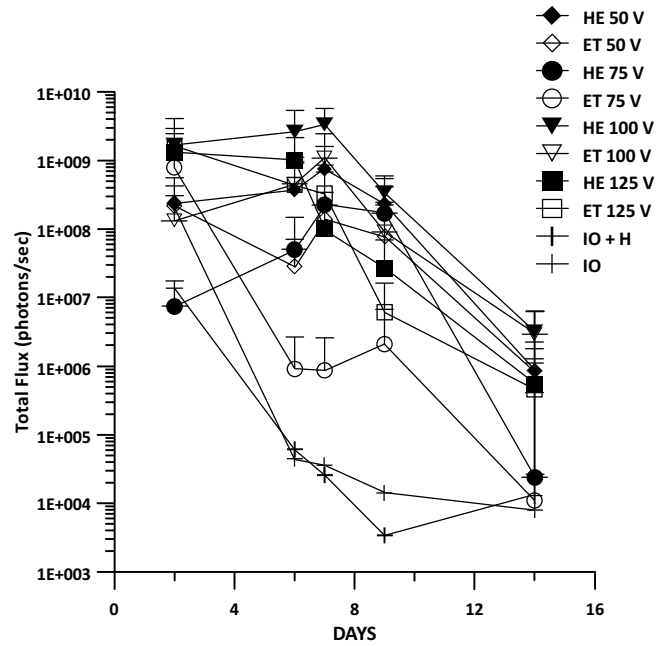
CONCLUSION

In this study, we demonstrated a novel method of *in vivo* GET employing exogenous heat. The intradermal skin temperature was increased to 43°C using an infrared laser and electrical fields applied *via* electrodes. We previously demonstrated that the addition of exogenous heat to GET *in vitro* resulted in maximal gene expression with a reduction of applied voltage by 30% and an increase in cell viability [40]. This current study demonstrated that *in vivo* TAGET maintained gene expression levels while utilizing 50% lower applied voltage with only short-term adverse effects to the tissue. In addition to gene expression, our current study also demonstrated that TAGET did not result in temperature increases above those induced by the exogenous heating source.

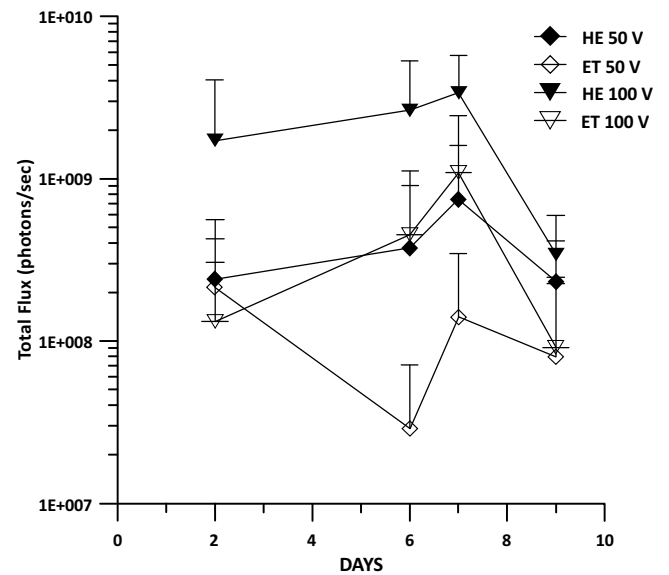
We did not demonstrate an increased depth of penetration for gene delivery. However, there were several parameters which were not evaluated. Throughout the current experimental design the heat level and duration at 43°C and pulse number, length, and interval were maintained. In the future, these parameters could be adjusted to assess the effect of their changes. GET parameters can be designed to increase penetration by changing electrode distance or electric field [28, 29]. The current electrode design could be adjusted to evaluate the combination of these factors by reducing the electrode distance or increasing the field.

While this method still requires optimization, the initial results suggest that TAGET could prove to be a valuable tool for obtaining better control over delivery and subsequent protein expression. In addition, to the overall reduction of

applied voltage, targeted application of heat could aid in localization of plasmid expression. Future studies will evaluate alternate electrode designs, heat application and electrical parameters with the goal of optimizing the applications of *in vivo* TAGET.



(A)



(B)

Fig. (5). Comparison of Various Applied Voltages with or without Heat Pretreatment. Luciferase gene expression was evaluated with GET administered at various applied voltages. Baseline values of IO and HIO were also determined. (A) Applied voltages were administered from 50-125 with (HE) or without (ET) heat pretreatment. (B) Enlarged graph highlighting differences at 50 and 100 volts with and without heat pretreatment. All errors are represented as SD.

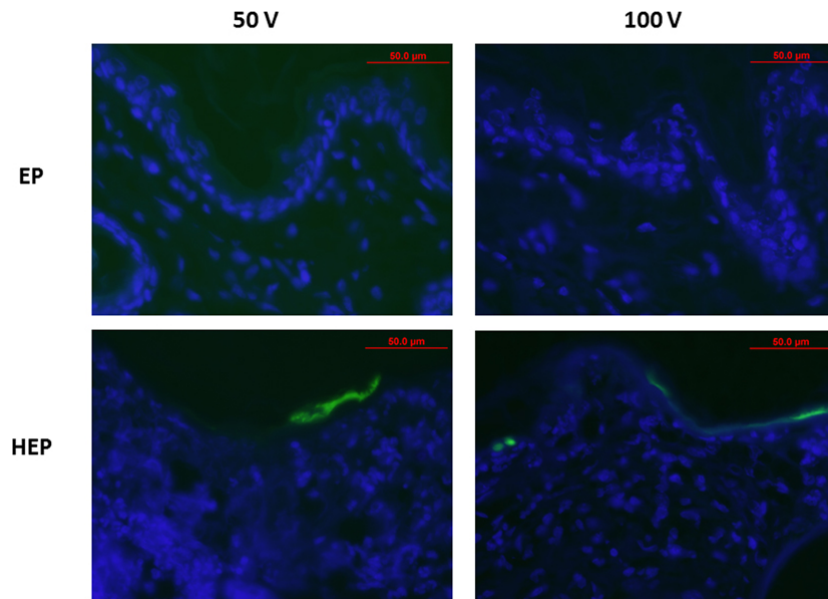


Fig. (6). Evaluation of Distribution Following TAGET Delivery of gWizGFP. Plasmid encoding GFP was delivered by GET with or without heat pretreatment at applied voltage of 50 or 100. Tissue was harvested at 48 hours, sectioned, counter stained with Dapi, and fluorescent image analyzed.

CONFLICT OF INTEREST

Drs. R. Heller and K. Schoenbach are inventors on patents and patent applications which cover the technology that was reported in this manuscript. This research was supported in part by a research grant from the National Institutes of Health R01 EB018956. The funders had no role in study design, data collection, analysis, decision to publish or preparation of the manuscript.

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