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Moderate Heat-Assisted Gene Electrotransfer for Cutaneous Delivery of a DNA Vaccine Against Hepatitis B Virus

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An estimated 350 million people are living with chronic Hepatitis B virus (HBV) worldwide. Preventative HBV vaccination in infants has reduced the disease burden; however, insufficient immunization programs and access obstacles leave vulnerable populations at risk for infection in endemic regions. Gene electrotransfer (GET) using a noninvasive multielectrode array (MEA) provides an alternative platform for DNA vaccination in the skin. DNA vaccines are nonlive and nonreplicating and temperature stable unlike their counterparts. In addition, their simple engineering allows them to be manufactured quickly at a low cost. In the current work, we present the combination of GET and moderate heating for delivery of a DNA vaccine against HBV. Our laboratory has previously shown the synergy between moderate tissue preheating at 43°C and GET with the MEA as a means to reduce both the applied voltage and pulse number to achieve similar if not higher gene expression than GET alone. In this study, we expand upon this work, by optimizing the plasmid dose to achieve the highest level of expression. Using the reporter gene luciferase, we found that an intradermal injection of 100 μL at 1 mg/mL induced the highest expression levels across all tested GET conditions. We then evaluated our moderate heat-assisted GET platform for the intradermal delivery of a plasmid encoding Hepatitis B surface antigen (pHBsAg) via a prime and prime plus boost vaccination protocol. At 18 weeks, following the prime plus boost protocol, we observed that a high-voltage low-pulse GET condition with moderate heating (45 V 36 p+heat) generated antibodies against Hepatitis B surface antigen (HBsAb) at peak measuring 230-fold over injection of plasmid DNA alone with moderate heating. HBsAbs remained robust over the 30-week observation period. These data suggest that moderate heat-assisted GET has the potential to induce strong immune responses, an attractive feature for development of an alternative vaccine delivery platform.

Keywords: electrotransfer, DNA vaccine, gene delivery, electroporation, Hepatitis B, multielectrode array

INTRODUCTION

Hepatitis B virus (HBV) is a leading global cause of liver disease.[1–4] HBV infection is endemic in Asia, the Pacific Islands, Eastern Europe, and sub-Saharan Africa where most of the population are carriers.[5–7] The virus is transmitted via blood or sexual contact and is quite resilient, able to survive outside the body for up to 7 days. Each year there are ~20,000 new HBV cases in the US.[8] The symptoms of HBV infection are nonspecific, and infection is generally confirmed through blood laboratory analysis once latent indications appear. Preventative HBV vaccination has reduced the disease burden; however, an estimated 350 million people worldwide are living with chronic HBV.[9]

Vaccination schedule compliance is critical for HBV prevention. Current guidelines recommend beginning vaccine dosing within 24 h of birth, followed by two to three additional doses within the first year of life.[10,11] Global vaccination estimates of children are unclear on the extent to which vaccines are administered completely and on time. In practice, vaccines tend to be given later rather than earlier. When HBV vaccination is delayed, children fail to receive adequate protection when they are most vulnerable. Late vaccinations raise the risk of HBV infection by lengthening the period of susceptibility. This can have important implications in countries where HBV infection is endemic, increasing the probability of transmission. In this situation, catch-up vaccination of older children has relatively little impact for prophylaxis because infection may already have been acquired by the time they present for vaccination. Vaccine compliance

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in endemic regions is related to access barriers. Limited facilities, inadequately funded vaccination programs, means and distance of travel to clinics, storage logistics, and capacity hinder immunization coverage. The current recommended HBV vaccine is recombinant protein consisting of the viral envelope protein Hepatitis B surface antigen (HBsAb) and requires strict cold storage to maintain its stability.[3,8–10]

DNA vaccines are an attractive alternative to standard vaccine types, given their innocuous make-up and simplicity of production requirements; however, there are several pitfalls. Previous DNA vaccines in the clinical pipeline have demonstrated poor cellular uptake and immunogenicity rendering them inconsistent or ineffective for conferring protection. These shortcomings can be mediated through enhanced delivery protocols beyond the standard needle-fitted syringe administration. Delivery of plasmid encoding viral antigen through gene electrotransfer (GET) has potential as a DNA vaccination platform.[12–17] A recent clinical study delivering a DNA vaccine via GET showed HBV-specific T cell responses in humans.[18]

In this work, we present a novel GET vaccine delivery platform utilizing a combination of moderate heating and noninvasive electrotransfer. We have previously shown the advantages of combining electrotransfer with moderate heating where both pulse number and applied voltage could be significantly reduced resulting in a shorter and less painful method.[19,20] Futhermore, using the multielectrode array (MEA) for intradermal delivery has been shown to be both effective and tolerable.[20–23] Minimizing discomfort is an important consideration for translation to nonlife-threatening applications such as vaccination, in which schedule compliance is critical to achieve immunization coverage.

Here, we present moderate heat-assisted GET for the delivery of a DNA vaccine against HBV. To evaluate this method, we utilized a guinea pig model for delivery of a DNA vaccine encoding HBsAb. The objective of this work was to determine the appropriate dosing and moderate heat-assisted GET parameters for achieving elevated Hepatitis B surface antibodies in circulation with the overarching goal of conferring humoral immunity against HBV.

MATERIALS AND METHODS

Infrared laser system for moderate heating

Moderate heating was applied to maintain a surface temperature of the treatment site at 43°C. The moderate heating and GET delivery is incased in a single device. The heating system consists of a 980 nm wavelength infrared laser (Lasermate Group, Inc., Walnut, CA), an optical fiber that delivers the infrared laser light to the skin, and an emission splitter to distribute infrared laser light through nine optical fibers, positioned between 16 conductive spring-loaded electrodes (Fig. 1).

For moderate heat application, the electrode was placed with the spring-loaded GET electrode pins in contact with the skin, leaving a fixed distance of 8 mm between the optical fibers and the vaccine target. The laser was applied for a total of 30 s following injection of plasmid DNA. This duration allowed for 43°C to be maintained for 30 s after removal of the moderate heating source (Edelblute *et al.*, Moderate heat-assisted GET as a potential delivery approach for protein replacement therapy in the skin, submitted; under review, 2021). The GET protocol was ~20 s in duration, enabling the tissue to remain heated during its application. Moderate heating was always applied before and never concurrently with GET. Safety precautions such as eyewear and barriers were in place during all laser operations.

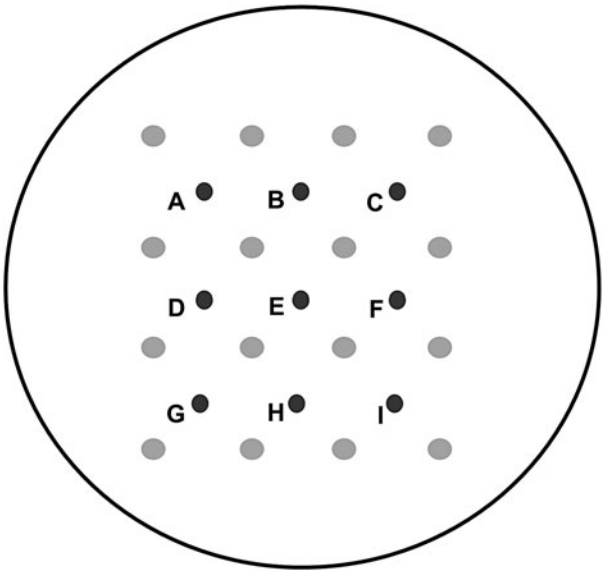

Figure 1. Moderate heat-assisted GET device. Moderate heating is performed via an infrared laser split into nine equally spaced fibers A–I positioned in rows between the conductive MEA pins (*black circles*). GET is applied from 16 spring-loaded gold-plated round-tipped pins placed in contact with target (*gray circles*). GET, gene electrotransfer; MEA, multielectrode array.

GET technology platform

The moderate heating and GET delivery was incased in a single device for ease of use. The design of the MEA GET delivery device has been previously described.[22–24] In brief, the electrode has 16 pins arranged in a 4×4 pattern at 2-mm-apart. Each pair of electrodes is programmed to administer either a train of 4 pulses with total 72 pulses or 2 pulses for a total of 36 pulses depending on pulsing conditions. The applied voltage was either 35 or 45 V between two conductive pins, each with a pulse duration of 150 ms and a 150 ms delay between pulses. Electrotransfer was performed using the UltraVolt Model: Rack-2-500-00230 (Ultravolt, Inc., Ronkonkoma, NY).

Plasmids

For the initial dosing experiment, endotoxin-free plasmid encoding the reporter gene firefly luciferase (gWizLuc) was

commercially prepared at 2 mg/mL in 0.9% sterile injectable saline (Aldevron, Fargo, ND). For the HBV vaccination study, endotoxin-free plasmid encoding Hepatitis B surface antigen (gWizHBsAg) was professionally prepared and suspended to 2 mg/mL in 0.9% sterile injectable saline (Aldevron). Both plasmids have a gWiz backbone and a human cytomegalovirus (pCMV) promoter.

Animals

Female guinea pigs aged 8-10 weeks weighing ~350 g were used for this study. Animals were housed at the ODU AAALAC accredited animal facility and all procedures were approved by the ODU IACUC protocol #17-022. Animals were quarantined for 7 days before conducting any procedure.

Moderate heat-assisted GET delivery and plasmid dosing

Volume and concentration of plasmid DNA was first assessed to determine the appropriate dose to achieve the highest expression level with minimum adverse tissue effects. For this experiment, we used a plasmid encoding the reporter gene luciferase. Anesthesia was performed by placing the animals in an induction chamber infused with 3–4% isoflurane and 96–97% oxygen gas.

After animals were sufficiently anesthetized, they were fitted with a standard rodent mask supplied with 3% isoflurane and oxygen to maintain a surgical plane of anesthesia. Animals were placed on a heating pad to ensure thermoregulation during the entire procedure. Animals were shaven and washed with mild soap (Dial) and water in an outward circular motion to remove any loose hair or an overabundance of oil. Injection sites were marked to ensure accuracy of data collection. gWizLuc plasmid doses included volumes of 50 or 100 μL at 0.5, 1, or 2 mg/mL.

The electrode array was immediately positioned over the injection area. When applicable, moderate heating was always applied after injection, but before GET. The four experimental groups included injection only (IO), injection of pDNA followed by 72 pulses at 45 V (45 V 72 p), injection of pDNA followed by 72 pulses at 35 V with moderate heat (35 V 72 p+heat), and injection of pDNA followed by 36 pulses at 45 V with moderate heat (45 V 36 p+heat). Each experimental group was tested after injection of the respective plasmid concentration and volume. Gene expression levels were measured 2, 5, 7, 9, and 14 days after gene delivery via *in vivo* bioluminescence imaging.

In vivo bioluminescent imaging and kinetic expression analysis

On days 2, 5, 7, 9, and 14, animals were anesthetized with O_2 containing 2.5–3.0% isoflurane followed by a single subcutaneous injection of D-luciferin (Gold Biotechnology, St. Louis, MO) at 150 mg/kg administered at the neck scruff. The animals were confined in an anesthesia chamber for 8 min then transferred to the IVIS® Spectrum (Perkin Elmer, Akron, OH), imaging chamber under constant anesthesia. Regions of interest (ROI) were selected on the resulting image to encompass the entirety of each injection site independently. These results were compared to untreated control ROIs. After background correction, bioluminescence results were represented as average total flux in photons/sec (p/s).

Moderate heat-assisted GET delivery of plasmid encoding Hepatitis B surface antigen

Female guinea pigs weighing ~350 g were used for this study as they represent a model for human skin. Anesthesia was performed as previously described. After animals were sufficiently anesthetized, they were fitted with a standard rodent mask supplied with 3% isoflurane and 97% oxygen to maintain a surgical plane of anesthesia. The right flank was shaved and washed in the same aforementioned manner, ensuring the removal of sebaceous oils before GET.

Moderate heat-assisted GET was applied after the preoperative cleansing procedure. The five tested groups included IO+heat (injection of plasmid without GET and with moderate heating), plasmid encoding Hepatitis B surface antigen (pHBsAg) +45 V 36 p (plasmid with high-voltage low-pulse GET), pHBsAg +45 V 36 p+heat (plasmid with moderate heating and high-voltage low-pulse GET), pHBsAg +35 V 72 p (plasmid with moderate heating and low-voltage high-pulse GET), and pHBsAg +45 V 72 p (plasmid with high-voltage high-pulse GET). pHBsAg was injected at a single site intradermally in a 100 μL volume at a concentration of 1 mg/mL (100 μg total) immediately before moderate heating via infrared laser or GET application.

Operating parameters for both moderate heating and GET were delivered as previously described. After primary vaccination, each animal was monitored continuously until they recovered from anesthesia, as indicated by their ability to maintain sternal recumbency and exhibit purposeful movement. Boost vaccinations were administered 14 days after the prime protocol in appropriate subjects in the same manner, except on a different location on the animal's flank.

Total HBsAg antibody production

An enzyme-linked immunosorbent assay (ELISA) kit specific for guinea pig anti-HBsAg (KA0286; Novus Biologicals) was used to detect anti-HBs in all animal subjects. Sera was collected via a jugular vein puncture and isolated in serum separator tubes, which were then assayed per the manufacturers' instructions. Serum was collected 6, 11, 18, 24, and 30 weeks after immunization. Sera collected before vaccination was used to determine baseline levels of circulating anti-HBs. Two standard deviations from the mean of this baseline served a

positive result. Serial dilutions were performed on the serum samples to accommodate these criteria. The results are reported as HBsAb geometric mean titer.

Statistical analysis

Statistical significance between groups for the reporter gene dosing experiment was determined using a two-way analysis of variance (ANOVA) with a Tukey-Kramer multiple comparisons posttest (GraphPad Prism Software, La Jolla, CA). Results are expressed as the mean of 4–8 replicates per group (±standard error of the mean [SEM]). Significant results were determined with respect to animals receiving injection of plasmid DNA alone unless otherwise noted. A p-value <0.05 was considered significant.

Statistical significance between the groups for HBV DNA vaccination was determined by two-way ANOVA with a Tukey-Kramer multiple comparisons posttest (GraphPad Prism Software). Results are expressed as the mean of 5 individuals per group (±SEM). A p-value <0.05 was considered significant.

RESULTS

Increasing the volume of plasmid DNA, not the concentration, yielded highest expression levels

Plasmid DNA dosing was optimized by varying both the concentration and intradermal injection volume at each delivery site. For this work, the reporter gene

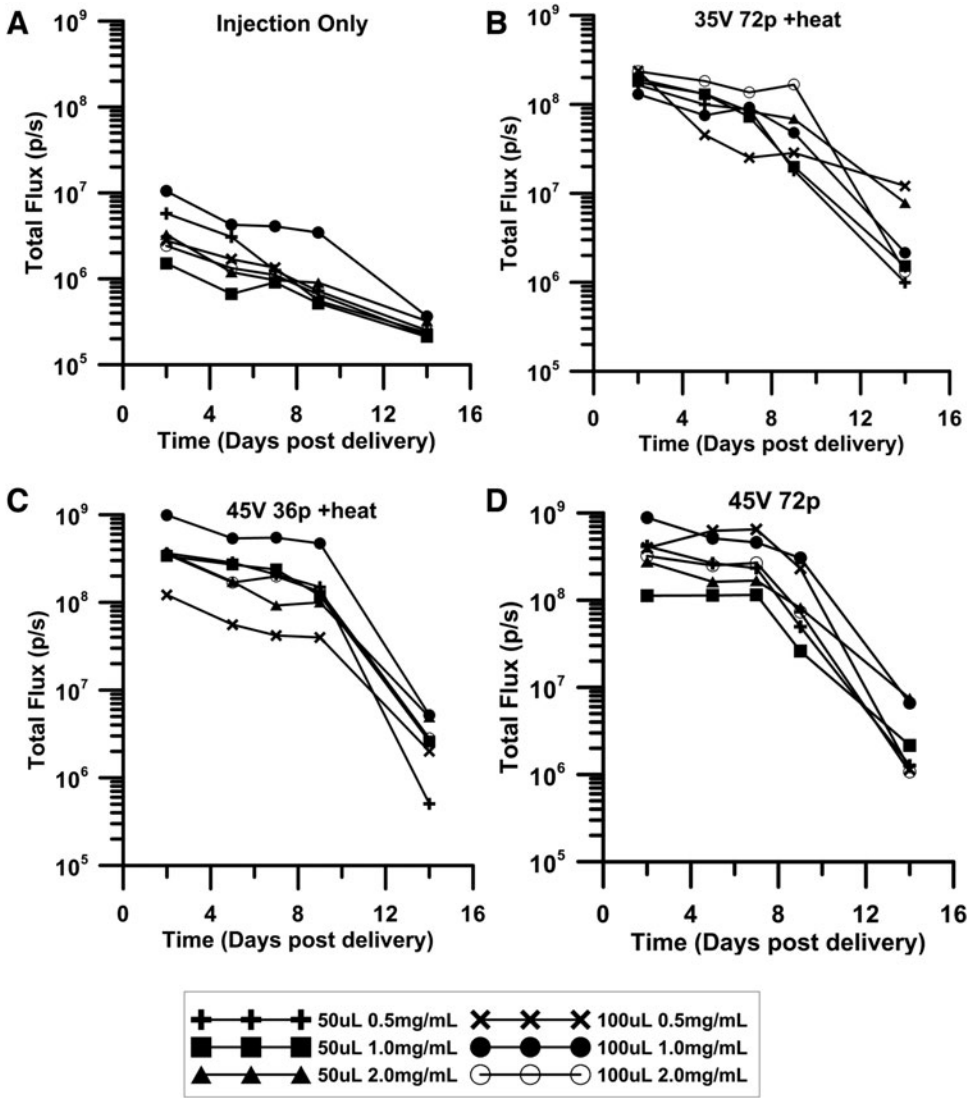

Figure 2. Optimization of plasmid DNA dosing for moderate heat-assisted GET to the skin. Experimental groups included **(A)** injection pDNA only (IO), **(B)** 72 pulses at 35 V with moderate heat (35 V 72 p+heat), injection of pDNA followed by GET with **(C)** 36 pulses at 45 V with moderate heating (45 V 36 p+heat), or **(D)** 72 pulses at 45 V without moderate heating (45 V 72 p). Plasmid DNA doses included volumes of 50 or 100 μL at 0.5, 1, or 2 mg/mL. Luciferase expression levels reported as average total flux (photons/second) ± SEM, n = 5–8 individual sites per group. IO, injection only; SEM, standard error of the mean.

luciferase was used to determine the kinetics of luciferase expression following the application of various pulsing parameters with and without the addition of moderate heat. Two volumes, 50 and 100 μL, and three concentrations, 0.5, 1, and 2 mg/mL, were evaluated across four experimental conditions, including IO, moderately heated GET conditions of 36 pulses at 45 V and 72 pulses at 35 V, and ambient GET conditions of 72 pulses at 45 V.

Peak luciferase expression *in vivo* was observed 48 h after exposure in all tested parameters and decreased gradually over the 2-week period of observation (Fig. 2). A 100 μL injection at 1 mg/mL yielded the highest overall level of luciferase expression in all tested parameters compared to all other concentrations and volumes. The highest expression was shown in those animals receiving 36 pulses at 45 V with a total flux of $9.9\times10^8\pm1.8\times10^8$ photons/s after just 2 days (Fig. 2C). These results were significant compared to injection of plasmid DNA alone ($p<0.001$) (Fig. 2A). Similarly, 72 pulses at 45 V delivered at ambient temperature following a 100 μL injection at 1 mg/mL resulted in a total flux of $8.84\times10^8\pm5.66\times10^8$ photons/s (Fig. 2D). However, this condition served as a positive control, in that although expression levels were high, the superficial damage attributed to these GET parameters were unfavorable and negated its potential use for translation.

In the high-pulse, low-voltage moderately heated GET condition (35 V 72 p+heat), the highest expression level was also observed at day 2 with a total flux of $2.38\times10^8\pm2.82\times10^7$ photons/s after a 100 μL injection at 1 mg/mL (Fig. 2B). Although at a peak level lower than that of the other tested GET conditions, these results were significant with respect to those animals receiving injection of plasmid DNA alone of the same volume and concentration ($p<0.01$). Although not significant, on observation days 7 and 9, animals receiving the higher plasmid DNA concentration of 2 mg/mL in an injection volume of 100 μL followed by 35 V 72 pulses plus moderate heating, displayed a trend of elevated expression levels compared to the other five dosing parameters at the same time point.

Moderate heat-assisted and ambient GET induces the production of antibodies against HBV surface antigen following intradermal injection of pHBsAg

Circulating antibodies specific to HBV surface antigen have been shown to be efficacious for conferring HBV humoral immunity. Surveillance of HBsAb titer is used clinically to determine if a follow-up boost vaccination is warranted, as a drop in levels of this antibody suggests a

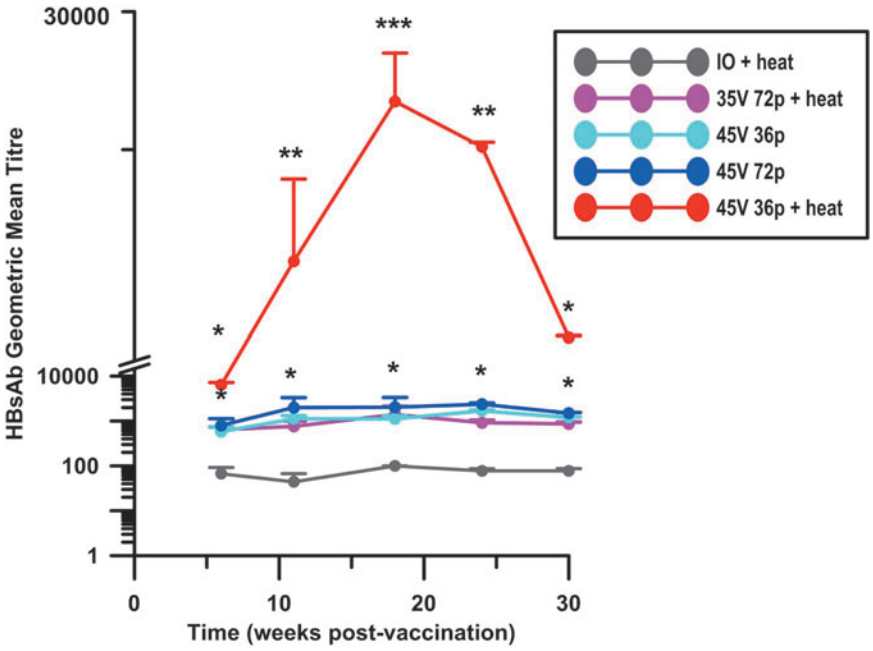

Figure 3. HBsAb production induced by moderate heat-assisted GET after a prime-boost DNA vaccination protocol against HBV. Prime and boost vaccinations were separated by 2 weeks. All animals received an intradermal injection of a pHBsAg in a 100 μL volume at 1 mg/mL. Experimental groups included injection pDNA only plus moderate heating (IO+heat), injection of pDNA followed by GET with 72 pulses at 45 V (45 V 72 p), 72 pulses at 35 V with moderate heat (35 V 72 p+heat), and 36 pulses at 45 V with (45 V 36 p+heat) or without moderate heating (45 V 36 p). Serum was collected via jugular vein puncture 6, 11, 18, 24, and 30 weeks after immunization. Serum collected before vaccination was used to determine baseline levels of circulating HBsAbs. HBsAb titer in serum was measured by ELISA. Two standard deviations from the mean of this baseline served as a positive result. Serial dilutions were performed on the serum samples to accommodate these criteria. The results are reported as HBsAb geometric mean titer ± SEM, $n=5$ individuals per group. ***$p<0.001$, **$p<0.01$, *$p<0.05$. ELISA, enzyme-linked immunosorbent assay; HBsAb, Hepatitis B surface antigen; HBV, Hepatitis B virus; pHBsAg, plasmid encoding Hepatitis B surface antigen.

drop in immunization protection. The ability of moderate heat-assisted GET to induce the production of HBsAbs after injection of a plasmid encoding HBV surface antigen was tested by comparing the levels of antibody titers in the serum to unheated GET conditions. All GET parameters had been previously tested for gene expression in the dosing optimization experiment. Based on results from the dosing experiment, an intradermal injection of 100 μL at 1 mg/mL was used for this work.

GET parameters for DNA vaccination against HBV included IO plus moderate heating, 45 V 36 pulses with and without moderate heating, 35 V 72 pulses plus moderate heating, and 45 V 72 pulses without moderate heating. Guinea pigs were injected with pHBsAg followed by appropriate GET and/or moderate heating conditions. A boost vaccination was given in the same manner after 2 weeks at a different site than the prime. Serum collected via the jugular vein over a 30-week observation period was assayed using an ELISA.

Overall, HBsAb titers induced by DNA vaccination with pHBsAg via all GET parameters, moderately heated or ambient, were significantly higher than an IO control with moderate heating. All GET groups displayed a steady increase in antibody production, peaking at week 18 and waning gradually to the end of the observation period. Notably, 45 V 36 pulses with moderate heating yielded significantly higher HBsAbs titers compared to all other tested conditions at all observation time points ($p < 0.001$) (Fig. 3). By week 18, HBsAb titers in this condition (45 V 36 p+heat) peaked and were 230-fold higher than those in an IO control with moderate heating (IO+heat) ($p < 0.001$). Furthermore, antibody production at week 18 in the experimental condition 45 V 36 pulses with moderate heating were 20-fold over the HBsAb titer achieved by its ambient counterpart (45 V 36 p) ($p < 0.01$).

Although our dosing experiment carried out with the luciferase reporter gene indicated that expression levels following GET with 45 V 72 pulses were similar to 45 V 36 pulses plus moderate heating, we did not observe such similarities in HBsAb antibody production between these two treatments. Specifically at peak antibody production, 45 V 36 pulses with moderate heating was still nearly 10-fold higher than the levels achieved with 45 V 72 pulses delivered at ambient temperature ($p < 0.01$).

Moderate heat-assisted GET elicits significantly higher HBsAbs after both prime and boost vaccination schedules

HBV DNA vaccination was evaluated after a single primary dose to determine HBsAg antibody production at a reduced schedule. For this work, we assessed efficacy of the four previously tested GET groups: 35 V 72 pulses with moderate heating, 45 V 72 pulses, and 45 V 36 pulses

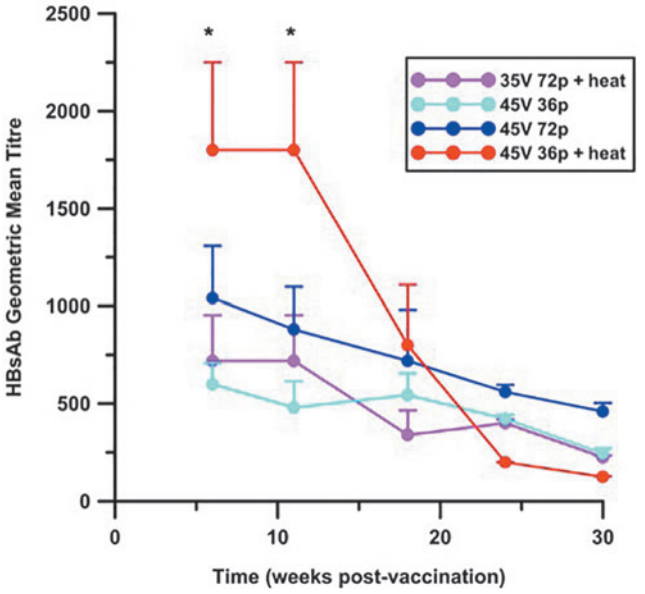

Figure 4. HBsAb production induced by moderate heat-assisted GET after a prime only DNA vaccination protocol against HBV. No boost vaccination was administered in this experiment. All animals received an intradermal injection of a pHBsAg in a 100 μL volume at 1 mg/mL. Experimental injection of pDNA followed by GET with 72 pulses at 45 V (45 V 72 p), 72 pulses at 35 V with moderate heat (35 V 72 p+heat), and 36 pulses at 45 V with (45 V 36 p+heat) or without moderate heating (45 V 36 p). Serum was collected via jugular vein puncture 6, 11, 18, 24, and 30 weeks after immunization. Serum collected before vaccination was used to determine baseline levels of circulating HBsAbs. HBsAb titer in serum was measured by ELISA. Two standard deviations from the mean of this baseline served as a positive result. Serial dilutions were performed on the serum samples to accommodate these criteria. The results are reported as HBsAb geometric mean titer ± SEM, $n = 5$ individuals per group. *$p < 0.05$.

with and without moderate heating. After the prime vaccination protocol, peak antibody production was observed at week 6 across all tested GET parameters (Fig. 4). Just as we observed after the boost vaccination, HBsAb titers in those animals receiving pHBsAg followed by the high-voltage low-pulse moderately heated condition (45 V 36 p+heat) were significantly higher than all other GET conditions. At week 6, HBsAb titers were 3-fold higher in the experimental condition 45 V 36 pulses with moderate heating compared to its ambient counterpart (45 V 36 p) ($p < 0.05$). Similarly, this condition was 1.73- and 2.5-fold higher than 45 V 72 pulses without moderate heating and 35 V 72 pulses plus moderate heating, respectively.

Overall, antibody production from the prime protocol was far lower than those levels achieved from two doses: a prime and boost given 2 weeks apart. However, although a lower level of antibody production was observed, the same trends between the tested GET parameters were replicated in this experiment where 45 V 36 pulses plus moderate heat induced the highest overall level of HBsAb production.

DISCUSSION

Whole-pathogen and subunit vaccines predominate current vaccine schedules.[25] Whole-pathogen vaccines contain either killed or live-attenuated or weakened strains that cannot cause disease, but are able to elicit an immune response. Subunit vaccines comprised only the essential antigens of a particular pathogen along with adjuvants to achieve long-term immunity, as antigen alone is often not enough to confer protection. Although effective, these formulations require strict cold storage to maintain stability, are costly to manufacture, require multiple scheduled doses to achieve life-long protection, and can have serious side effects in some recipients, such as those with allergies or compromised immune systems, and in rare cases can lead to reversion to a disease-causing state.[25–27]

DNA vaccines consist of a plasmid that carries genes encoding proteins from the pathogen of interest.[15,16] When delivered, the plasmid enters host cells and serves as a genetic template for the translation of its antigen. DNA vaccines are nonlive and nonreplicating, leaving little risk for secondary infection from vaccination. Due to well-established production protocols, DNA plasmid vaccines can be made rapidly, shortening the time between an outbreak and the public health response. This streamlined approach is advantageous with the emergence of new pathogens. DNA vaccines are an ideal global vaccination candidate due to their robust temperature stability, specificity to target antigen, and ease of large-scale manufacturing. These advantages make DNA vaccines an inexpensive option and therefore more feasible in low-income regions where access to the general population is a barrier.[15]

Reversible electroporation, where the cells survive, is largely considered a nonthermal process. GET falls under this category where a high-voltage pulse is applied creating transient permeations in the cell membrane that are resealed once the pulse is removed. Physical methods of GET[28,29] are used both *in vitro* and *in vivo* and are well tolerated in living tissues.[18,30] By adding moderate heating to this approach, there is an increase in membrane fluidity allowing for the widening of the transient permeations already caused by GET.[31,32] Thus, the interaction of moderate heating and GET affords both a reduction in the necessary pulse number and applied voltage to achieve a similar or enhanced result.[19,20] In practice, moderate heat-assisted GET provides a faster and less painful delivery platform with all the same benefits as ambient GET.

In the current work, we evaluated the use of moderate heat-assisted GET for the delivery of a DNA vaccine against HBV. Our initial testing to confirm plasmid dosing allowed for more prudence in determining the proper volume and concentration of plasmid to deliver in our vaccination study. We found that a 100 μL injection at 1 mg/mL achieved the highest level of luciferase expression when followed by 45 V 36 pulses with moderate heating. This is likely attributed to better coverage of the plasmid DNA injection by the applied electric field and exogenous moderate heating source, given that a 100 μL injection measures $\sim$8 mm, while a 50 μL spans $\sim$5 mm. Previous studies using reporter genes have determined that a total concentration of 100 μg achieved the highest expression levels.[19,20,33] Our results are in line with these data.

In all of our experiments, we chose to use 45 V 72 pulses as a positive control, which in our experience is a MEA condition that reliably induces high expression levels.[16,17,19,20,22,24,34] However, we hypothesized that this high-voltage high-pulse condition would not be suitable for translation due to observable skin damage caused from its application (Edelblute *et al.*, Moderate heat-assisted GET as a potential delivery approach for protein replacement therapy in the skin, submitted; under review, 2021). On this basis, we sought to reduce either the pulse number and/or the applied voltage with the addition of moderate heat to compensate for expression loses all the while maintaining tissue health.

Tissue health is critical for reversible electroporation approaches such as GET, where live cells are needed to achieve gene expression in the host. Furthermore, in nonlife threatening applications such as vaccination visible scarring and pain at the delivery site are unappealing side effects. These could be overlooked in some cases, where the benefit outweighs the detriment of the side effect, but it is not ideal. We found that with the addition of moderate heat, we were able to reduce the applied voltage by 23% and the pulse number by 50% all the while achieving a similar luciferase expression level and eliminating the tissue damaging side effects.

For the delivery of a DNA vaccine against HBV, we evaluated both a prime and a prime plus boost vaccination protocol. We found that GET parameters of 45 V 36 pulses with the addition of moderate heat far exceeded the serum levels of HBsAbs produced by any of the other GET conditions we tested in both the prime and prime plus boost vaccination protocols. These levels were especially elevated in those animals receiving the prime plus boost vaccination schedule and remained stable during the entire 30-week observation period. This suggests that the effects of moderate heat-assisted GET with respect to antibody production are long lasting. Vaccine longevity is just as critical as compliance, so these results are encouraging.

Furthermore, all of the GET conditions we evaluated induced significant HBsAb production compared to injection of pHBsAg alone. Interestingly, although the high-voltage high-pulse condition (45 V 72 p) showed similar expression levels in our reporter gene experiment, this did not translate to high antibody production in our vaccination study. This could imply that the tissue damage induced by these delivery parameters is too severe to achieve a robust antibody response, where the inflammation at the

target site is being addressed at a greater magnitude than HBsAg uptake and antigen presentation to mediate the generation of HBsAg antibodies. While in the moderately heated low-voltage, high-pulse condition (45 V 36 p+heat), no gross skin damage was observed, and total antibody production was thus higher.

The results of the prime vaccination protocol followed the same trend as the prime plus boost protocol, just at a far lower magnitude. We still observed that GET parameters of 45 V 36 pulses with moderate heating induced the highest level of HBsAb production compared to the other tested GET conditions. These results suggest that prime vaccination alone can prompt enduring antibody production in this case, although the prime plus boost protocol was far more robust and more likely to confer long-lasting protection. These data were important to collect, as vaccine schedule compliance is a concern among both patients and physicians. It was therefore important to determine the total HBsAg antibody production in a prime only vaccination protocol, although we suggest in this case the prime boost vaccination protocol would be more effective.

An effective vaccine capable of producing sustained protective antibody titers could eliminate the need for multiple booster vaccinations, an appealing solution for all parties. The current vaccination schedule for HBV in infants recommends a series of three shots: shortly after birth, aged 1–2 months, and 6–18 months.[2,10,11] Our prime boost vaccination protocol consisted of two doses; with antibody titers in our highest GET expressing condition, 45 V 36 pulses with moderate heating, decreasing after 30 weeks. It is therefore likely that additional booster vaccinations may be necessary to prolong HBV protection.

Moderate heating increased the overall expression levels achieved by intradermal GET applied at ambient temperature. Moreover, using moderate heat allows for greater penetration depth for the applied electric fields, creating a situation where the uptake of plasmid DNA by professional antigen presenting cells such as dendritic cells is more likely.[30,35,36] This is an essential aspect of an effective vaccine, mediated by both the cellular and humoral immune systems and dependent upon the specificity for the plasmid-encoded antigen.[30,35–38] Where we have historically viewed an intradermal delivery platform as confined to the epidermis and dermis, with the addition of moderate heating, it could be possible for aqueous delivery via cutaneous GET to the hypodermis and muscle tissues (Edelblute *et al.*, Moderate heat-assisted GET as a potential delivery approach for protein replacement therapy in the skin, submitted; under review, 2021).

The long-lasting HBsAg antibody production we observed after DNA vaccination in our moderately heated GET condition (45 V 36 p+heat) is evidence that muscle delivery with an intradermal approach is not only possible, but also more than likely occurring. This is favorable, as most vaccines require muscle delivery, although it is unclear if their administration is precise as muscle is an underlying unseen tissue that is not so easily monitored. In addition, intramuscular vaccinations typically administered in the deltoid muscle can cause upper arm injury, bursitis, or other shoulder dysfunctions if not carefully administered.[39–42] In infants, intramuscular vaccinations are given in the vastus lateralis located in anterolateral thigh. This site has been deemed the safest intramuscular route, although the middle of the muscle should be the target, not the upper or lower portion, as its adjacency to the muscle branch of the femoral nerve and the lateral circumflex femoral artery.[40,43,44] Intradermal delivery by contrast is visible and therefore easier to ensure correct administration. With moderate heat-assisted GET, there are the advantages of muscle delivery with an intradermal approach.

We have previously shown the utility the MEA device for DNA vaccination with a plasmid encoding protective antigen against *Bacillus anthracis* in a murine model as well as for delivering the same plasmid administered in this work against HBV.[16,17] Here, we expand upon this original platform and present moderate heat-assisted GET as a novel method with a reconfigured device for intradermal DNA vaccination. Our preliminary results demonstrate that moderate heat-assisted GET yields a significantly higher and longer duration of expression compared to needle-fitted syringe injection alone *in vivo*.

We selected HBV to evaluate our novel delivery platform because it is a well-characterized vaccination model. Previous reports have shown HBsAg geometric mean titers in correlation with HBV protective efficacy in mice, rats, guinea pigs, woodchucks, and sheep.[45–47] This suggests that the high HBsAg antibody titers we were able to generate with moderately heated GET to signify humoral immunity would also be in the protective range, as our numbers are in line with these reports. As a proof of principle, we show enduring antibody production against HBV *in vivo* over a period of 30 weeks following moderate heat-assisted GET.

Compared to our original work, adding moderate heat to GET induced antibody production nearly 8-fold over GET using the MEA at ambient temperature.[17] These results indicate the synergy between moderate heat and GET to enhance gene delivery in the skin. Future work should involve characterizing the cellular immune response induced by this approach and a challenge experiment to confirm its protective effects against HBV. Also, these results warrant the consideration of additional vaccinations utilizing plasmid-encoded antigens delivered via moderate heat-assisted GET.

AUTHORS' CONTRIBUTIONS

R.H. conceptualized the experiments. C.E. and R.H. designed the experiments. C.E. wrote the article. C.E. and

C.M. performed the experiments. R.H. supervised the experiments. C.E. analyzed the data. R.H. performed a critical review of the article.

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AUTHOR DISCLOSURE

With respect to disclosure of conflict of interest, R.H. is an inventor on patents covering the technology that was used in the work reported in this article.

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