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Mini Review

Gene transfer to skeletal muscle by site-specific delivery of electroporation and ultrasound

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ABSTRACT

Transfecting foreign genes into target cells require certain vectors and specific techniques. With more new genes and gene targets for gene therapy are continually being discovered, which provide useful clues for the study of gene function and gene therapy for human disease. However, there still remain a number of important unresolved problems associated with the use of viral and non viral vectors or techniques, such as secondary toxicity, immune response, or low gene transfer efficiency. Therefore, efficient and safe approaches of gene delivery in vivo still need to be found for medical applications. Electroporation or ultrasound (US), which involving electrical pulses or a US field to increase cell membrane permeability, has shown to be an efficient, safe and simple non viral physical method of DNA delivery in vivo and alternative technique in the field of gene therapy. However, the high field strength or energy often required for electroporation or US can result in tissue damage, thus limit their widely clinical applications. In recent years, site-specific gene delivery by electroporation or US has aroused much attention, because of optimized protocols and novel devices using a lower field strength than conventional methods, which has shown high transfection efficiency with minimal tissue damage. In this paper, we reviewed the advancement in the field of electroporation and US for gene delivery, particularly by site-specific delivery into skeletal muscle for gene therapy and their applications in Alzheimer's disease (AD)'s treatment.

1. Gene therapy and delivery system in vivo

Gene therapy, the use of DNA as a pharmaceutical agent to treat disease, is a promising treatment option for a number of diseases, including inherited disorders [1], some types of cancer [2], neurodegenerative disease [3], and certain viral infections [4]. DNA that encodes a therapeutic protein is packaged within a "vector", which is used to transport the DNA inside cells within the body. Once inside, the DNA is expressed by the cell machinery, resulting in the production of the therapeutic protein, which in turn treats the patient's disease. Gene therapy was first conceptualized in 1972, the authors urging caution before commencing gene therapy studies in humans [5]. Since that time, clinical trials have been widely researched, and over 1,700 clinical trials have now been conducted using a number of techniques for gene therapy [6]. Although early clinical failures led many to dismiss gene therapy, clinical successes in 2009–2012 have bolstered new optimism. These include successful treatments of patients with retinal disease [7], Leber's Congenital Amaurosis [8], X-linked SCID [9], ADA-SCID [10], adrenoleukodystrophy [11], heart failure [12], AD [13] and PD [14]. These recent clinical successes have aroused a renewed interest in gene therapy, with several articles in scientific and popular publications calling for continued investment in the field [15,16]. However, successful gene therapy largely depends on delivery systems which are safe, easy to apply and provide efficient transgenic expression in vivo.

In vivo gene delivery systems are classified as viral vectors and non viral vectors. Viral vectors typically offer higher transduction efficiency and long-term gene expression, but may be associated with toxicity, immunogenicity, restricted target cell specificity and high cost, thus limit their widespread clinical applications [17]. The death of a patient in an adenoviral-mediated gene therapy trial in 1999 was associated with a massive stimulation of the patient's innate immune system, causing disseminated

Abbreviations: US, ultrasound; hNEP, human(h) neprilysin; CNS, central nervous system; AD, Alzheimer's disease; PD, Parkinson's diseasex; EBD, Evans Blue dye.

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intravascular coagulation and multiple organ failure [18]. Although viral vectors can be genetically modified to remove viral components that trigger an immune response, these modified viruses are often difficult to produce in high titer.

In contrast, non viral vectors, which offer improved safety profiles, capacity to transfer large genes, site-specificity and their non-inflammatory, non-toxic and non-infectious properties compared to viruses, represent a promising alternative approach for gene therapy applications. However, the clinical usefulness of non-viral vectors is limited by their low transfection efficiency and relatively poor transgene expression [17]. Injection of naked DNA has been characterized as the simplest and safest method for non viral gene delivery. Mice injected with naked DNA can produce long-term therapeutic protein expression [19,20]. However, this method has many limitations, including gene expression that is too low for the treatment of various human disease states, resulting in its limited use in genetic immunization studies. In addition, interindividual variability in gene expression and unknown reasons for lack of reproducibility may present a predicament in clinical settings [21]. However, research efforts focusing on improving the efficiency of naked DNA uptake have yielded several effective methods, such as electroporation and sonoporation [22,23].

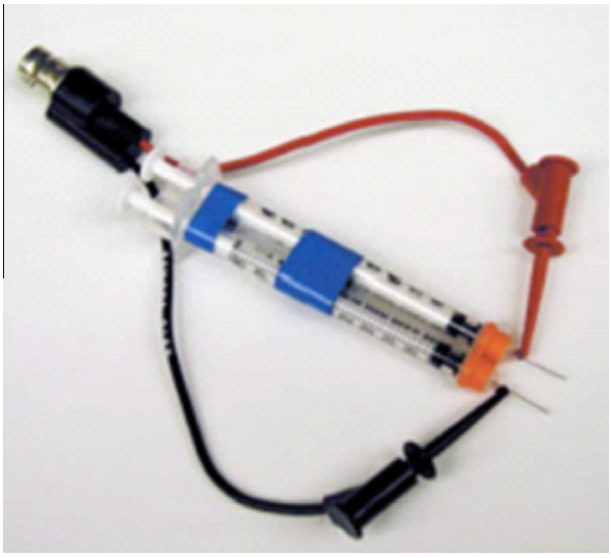

Fig. 1. The syringe electrode. Electric pulses are delivered through two needle electrodes. The gap distance between the two needles is 0.6 cm. Plasmid DNA is injected by one of the needles [32].

2. Site-specific gene delivery in vivo by electroporation

Electroporation, the process of using electric pulses to enhance cellular uptake of molecules, has recently been investigated as an effective physical method for gene delivery in vivo [24]. This electrical stimulus destabilizes the cellular membrane and leads to the subsequent formation of nanometer-sized pores allowing the entry of macromolecules including DNA, enzymes, and antibodies [25]. Electrotransfer can achieve long-lasting expression and may be used in different tissue types in various species, particularly in skeletal muscle [26]. Skeletal muscle has the largest potential for electroporation as genes are not rapidly lost through significant cell replacement [27], transgene expression may persist for long periods [28], and the muscle has an abundant vascular supply, allowing for the efficient secretion of therapeutic proteins into the circulation [29].

The transfection efficiency of electroporation is much greater than naked DNA injection and has reduced interindividual variability. However, the efficiency of electrotransfer depends on the strength of the electric field. If the field strength is too low, the cellular plasma membrane is not altered enough to allow the passage of DNA. Irreversible tissue damage has been observed at field strengths of greater than 100 V/cm, although maximum levels of transient gene expression have been historically obtained with an electric field ranging from 250 to 750 V/cm. The potential of irreparable damage via heat shock or burn injury induced by electroporation is a major barrier for its clinical application in gene therapy [30,31].

2.1. Site-specific delivery to skeletal muscle using a much lower electric field strength

The present method of site-specific electroporation has evolved to decrease the electric field strength to reduce cellular injury. A syringe electrode has been developed to site-specific electrotransfer application with a design allowing for the highest electric field intensity at the genes injected site (Fig. 1). Additionally, this electric field rapidly decreases with increasing distance from the electrodes, confining the injected DNA to the high-intensity region of the field and minimizing damage. Thus, the syringe electrode can use a much lower field strength than conventional electrodes as low as 50 V/cm, resulting in minimal tissue damage while maintaining equivalent transfection efficiency [32].

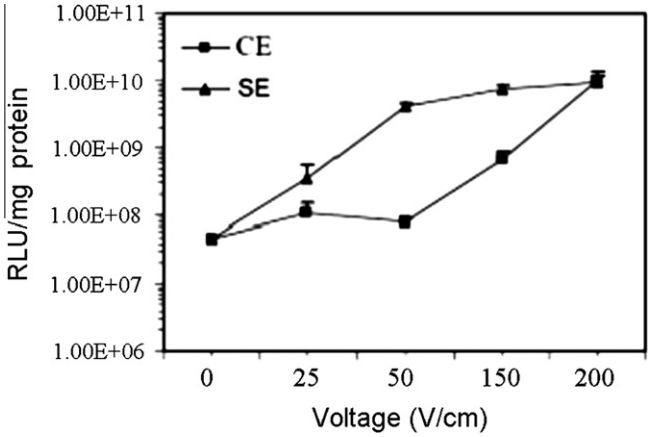

Fig. 2. Transfection efficiency of electrical pulses at various field strengths. Electric pulses were delivered through either the syringe electrode (SE) or caliper electrode (CE) after DNA injection [32].

Reporter and therapeutic genes have been successfully delivered in animal models using the syringe electrode (Fig. 2). At a field strength of 50 V/cm, luciferase activity was 50 times greater than with the caliper electrode, while similar luciferase expression was achieved with both electrodes at a field strength of 200 V/cm. Field strengths greater than 250 V/cm resulted in decreased gene expression with both electrodes, presumably due to tissue damage. Currently, standard electrotransfer conditions are defined as 6 pulses, 20 ms per pulse, and 50 V/cm for the syringe electrode or 200 V/cm for the caliper electrode [32]. An accumulation of EBD following electrotransfer by the caliper electrode under standard conditions was observed via myocyte staining and in quadriceps and gastrocnemius muscles photographed with a fluorescence microscope. In contrast, no accumulation of EBD was noted in muscles following electrotransfer with the syringe electrode. Therefore, gene transfer may occur at a lower field strength and with lower potential for muscle damage [32].

2.2. Site-specific delivery to skeletal muscle using sine-wave current pulses

Previously, all commercial electropulsators were designed to convey direct current square-wave pulses [33]. Direct current square-wave pulses require relatively high field strengths and therefore can result in irreversible tissue damage [34]. Human volunteer studies of direct current square-wave electroporation in skin [35] and muscle [36] also have reported increased pain sensation. Efficient gene transfer may be safely achieved with pulses of alternating current sine-waves with a frequency of 60 Hz. Compared to conventional direct current square-wave pulses, the field strength can be decreased to as low as 20 V/cm and electrogene transfer increased greater than 10-fold with less toxicity (Fig. 3). Initially, the effect of field strength was examined with alternating current sine-wave and direct current square-wave pulses. When field strength varied from 10 to 30 V/cm with a fixed pulse strength of 600 ms, all groups tested with alternating current sine-wave pulses had a significant improvement in gene expression ($p < 0.01$) resulting in a 10- to 20-fold increase in luciferase expression. The current through needle electrodes with alternating current sine-wave pulses was 11 to 13 mA as measured with an Extech digital thermometer.

Alternating current sine-wave gene transfer is further characterized by DNA dose, gene expression kinetics, and transfer to various tissues. Using this approach, significant levels of luciferase expression in muscle can be detected by as little as 1 μg DNA and reach a plateau when injected with 5 μg DNA. Gene expression can also be detected as early as 6 h after injection of luciferase DNA to at least 80 days with peak levels of approximately 900 ng luciferase at day 7. Peak levels can be regained in the muscle by a second electrogene transfer in the same muscle at day 80 [37]. Alternating current sine-wave pulses can also be used to efficiently transfer naked DNA into additional tissues besides muscle. Gene expression was detected 6 h after alternating current sine-wave or direct current pulse electrogene transfer in the liver and skin or after 20 h in a HPV16E7 + tumo. An 8-fold increase in gene expression in the liver and 10-fold increase in skin and tumor occurred in tissues treated with alternating current sine-wave pulses when compared to direct current square-wave pulses. Alternating current sine-waves were also used to transfer plasmid DNA to the skin of neonatal mice as gene therapy protocols for the induction of immune tolerance in the neonate population [37].

3. Site-specific gene delivery to skeletal muscle by US

In recent years, application of US in the enhancement of drug delivery, controlled drug release, and site-specific delivery has been a topic of extensive research [38]. US-enhanced drug and gene delivery is shown to be another promising non-viral physical method for gene therapy and treatment of various human diseases [39,40]. This enhancement is believed to be due mostly to cavitation activity and streaming [41]. US, especially combination with microbubble, can largely enhance transfection and more safe [42], which involves using a US field to permeabilize cell membranes and facilitate exogenous polynucleotide transit across the cytoplasmic membrane. The technique is painless, mechanical, and non-invasive, thus has shown a great promise for clinical gene therapy [43]. In addition, US can facilitate a number of different therapeutic strategies with low toxicity and low immunogenicity in different target tissues [44]. US-mediated gene transfer has recently shown potential for treating a wide range of therapeutic applications, especially neuronal diseases [45].

However, exist US technologies for gene transfer to skeletal muscle generally have several technical limitations, including higher ultrasound energies lead to microbubble destruction [44] and tissue damage. Microbubble stability is a major determinant of the efficiency of ultrasound and microbubble mediated in vivo gene transfer [46]. Thus, low-frequency ultrasound is a promising approach for gene delivery in vivo [47]. In previous studies, much attention has been focused on US protocols [48] and few on improve the US device for increase DNA uptake. As skeletal muscle owns a rich blood supply and long life span, thus a particularly excellent host tissue for delivery to the circulatory system. A novel syringe-focused US device for site-specific DNA delivery has been developed for gene transfer to skeletal muscle, which combines DNA injection simultaneously with US irradiation (Fig. 4) [49]. This technique can achieve high DNA uptake while lowering the incidence of tissue injury. The syringe-focused US device can target and streamline US energy precisely on the site of DNA injection and uptake. When plasmid DNA injection and US stimulation take place concurrently, the uptake of plasmid into cells is much higher than separated conventional approaches. High US energy is mostly focused on the tip of the syringe, thus reducing the needed US energy and dose of drugs in addition to allowing for easier manipulation and avoiding high-strength US field. This targeting also creates more experimental efficiency, as it is more convenient to control drug release in a specific site with easily manipulable tool. Uptake of EBD to evaluate tissue damage was very little and showed only at the site where the US needle tip focused emitted energy [49].

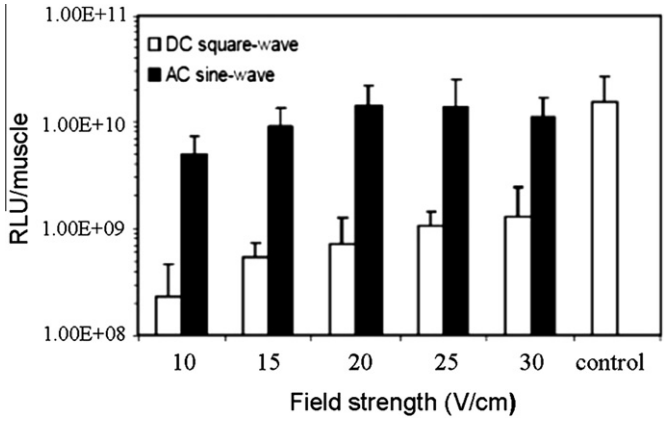

Fig. 3. Electrogene transfer into quadriceps of mice with alternating current (AC) sine-wave and direct current (DC) square-wave pulses. The muscle in the control was transferred with a caliper electrode with direct current square-waves (6 pulses, 200 V/cm, 20 ms pulse duration) [37].

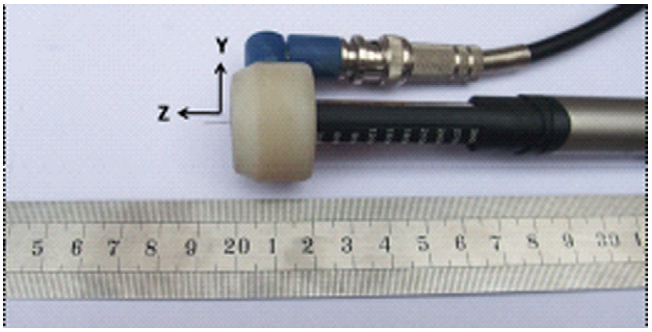

Fig. 4. The Syringe Focused US Device: It combines a Novo insulin syringe (the length of the exposed needle is $Z = 0.9$ cm and the radius of US probe is $Y = 1$ cm) [49].

4. Site-specific delivery of naked plasmid of hNEP into skeletal Muscle by electroporation and US: implications in gene therapy for AD

CNS-targeted gene therapy can be accomplished by introducing genes encoding neurotrophic growth factors or corrective enzymes to injured or diseased neurons [50]. In several animal models of neurodegenerative disease, such as AD or PD, gene therapy has achieved dramatic pathologic and functional improvements [51]. As an alternative to direct CNS injection, delivery vectors could be administered peripherally and routed to the CNS post-injection [52].

Much attention has focused on the possibility of increasing neprilysin (NEP) levels as a therapeutic approach to AD [53]. Direct delivery into the brain using lentivirus vectors expressing hNEP has been attempted [54]. Intravenous transplantation of genetically modified cells gave results similar to those obtained with direct gene delivery [55]. More recently, a novel method of lentivirus-mediated mouse NEP gene delivery into skeletal muscle was proposed, and there was no evidence of adverse effects via interference with the processing of other physiological peptide targets for NEP [56]. However, viral vectors have drawbacks include anti-vector immunity and secondary toxicity. The non viral approaches have been demonstrated to be a safe, efficient, simple and inexpensive method of gene transfer in vivo [57]. Considerable attention has been focused on the possibility of using physical methods, such as electric or US to deliver therapeutic molecules directly to tissues and organs.

By site-Specific gene delivery, the syringe-focused US device has been used to specifically introduce hNEP to mouse hindlimb muscle with US protocol (1.7 MHz, 1 W/cm^2, 1 min exposure time). hNEP expression has been successfully observed in skeletal muscle, blood serum, and brain tissue with minor tissue injury [49]. The syringe-focused US device enabled simultaneous combination of DNA transfer and US stimulation, giving rise to two months of gene expression [49]. While using the syringe electrode to transfect the naked plasmid hNEP to the Hind limb of mice with the protocol (50 V/cm, 6 pulses, 20 ms per pulse), a large amount of hNEP protein was detected in the local muscle, serum and brain of treated mice at 7, 14 and 30 days post-transfer with minor tissue damage. These results demonstrate that site-specific DNA delivery to muscle can give rise to therapeutic protein for CNS therapy, such as AD [58]. This technique provides safe and efficient non-viral methods for in vivo gene delivery with potential applications in both basic research and in gene therapy of neuronal disease.

5. Concluding remarks

Considering the numerous barriers encountered in the application of viral vectors or conventional physical methods of gene delivery to skeletal muscles, the design of effective non viral vehicles are required. Site-specific DNA delivery using electroporation or US techniques allows gene transfer at much lower threshold values. This design is based on the principles of field strength distribution, reducing the field strength to decrease toxicity while maintaining efficiency. Site-specific DNA delivery using the syringe electrode and syringe-focused US device has therefore been shown to be simple, safe, and effective for gene therapy application. At present, a US company has made a very professional syringe electrode for site-specific DNA vaccine delivery to muscle (http://www.inovio.com/technology/intramusculardelivery.htm). Hopefully, new generations of devices or optimal protocols will continue to be developed, so that more researchers can use this technology to study gene function, gene transfer and gene therapy for human diseases.

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