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Optimization of Gene Delivery Mediated by Lipoplexes and Electroporation into Mouse Mesenchymal Stem Cells

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Recently, mesenchymal stem cells (MSCs) began to be utilized as a vehicle for *ex vivo* gene therapy based on their plasticity. Effective and safe transfection of therapeutic genes is a critical step for genetic modification of MSCs. Therefore, optimization of *in vitro* gene delivery into MSCs is essential to provide genetically modified stem cells. In this study, various cationic liposomes, O,O'-dimyristyl-N-lysyl aspartate (DMKD), DMKD/cholesterol, O,O'-dimyristyl-N-lysyl glutamate (DMKE), DMKE/cholesterol, and N-[1-(2,3-dioleoyloxy)]-N,N,N-trimethylammonium propane methyl sulfate (DOTAP)/cholesterol, were mixed with plasmid DNA encoding luciferase (pAAV-CMV-Luc) at varied ratios, and then used for transfection to MSCs under varied conditions. The MSCs were also transfected by electroporation under varied conditions, such as voltage, pulse length, and pulse interval. According to the experimental results, electroporation-mediated transfection was more efficient than cationic liposome-mediated transfection. The best MSC transfection was induced by electroporation 3 times pulses for 2 ms at 200 V with 10 seconds of a pulse interval.

Key Words: Mouse mesenchymal stem cells, Cationic liposome, Electroporation, Gene delivery

INTRODUCTION

Recently, the stem cells rise as vehicles for an *ex vivo* gene therapy based on their plasticity. Especially, mesenchymal stem cells (MSCs) are able to differentiate into adipocytes, osteoblasts, chondrocytes, neuronal cells, cardiomyocytes, and hepatocyte-like cells (Kassem, 2004; Dezawa et al., 2004; Miyahara et al., 2006; Lee et al., 2004). Moreover, they are relatively easier to be isolated and cultured than other stem cells. Over the past few years, there have been many studies about the mesenchymal stem cell-based gene therapy for neurological disorders, blood disorders, vascular diseases, musculoskeletal diseases, and cancers (Reiser et al., 2005).

There are two different types of gene delivery strategies for genetic modification of stem cells, gene transfection mediated by viral and non-viral vector systems (Conrad et al., 2007). The viral vectors such as lentivirus, adenovirus, and adeno-associated virus have a strong advantage of high transfection efficiency while they have disadvantages in host immune stimulation, infection, insertional mutagenesis (Tian and Andreadis, 2009; Secchiero et al., 2008; Ren et al., 2008). On the other hand, non-viral gene transfection mediated by cationic liposomes, cationic polymers, electroporation or ultrasound has been considered to be convenient and safe, but relatively inefficient in transgene expression, compared with the viral vectors (Yang et al., 2007; Corsi et al., 2003; Peister et al., 2004; Ebisawa et al., 2004). Selection of an appropriate gene-transferring system should be dependant on target diseases, and functional characteristics and therapeutic mechanisms of transgenes.

Until now non-viral gene delivery systems have not been as widely applied as viral gene delivery systems for genetic modification of stem cells. Previously, we reported that two different types of cationic liposomes consisting of O,O'-dimyristyl-N-lysyl aspartate (DMKD) or O,O'-dimyristyl-

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N-lysyl glutamate (DMKE) were efficient gene-transferring vehicles for *in vitro* and *in vivo* transgene expression (Kim et al., 2006). In this study, we have tried to provide optimal conditions of transfection mediated by cationic liposomes or electroporation. Various formulations of cationic lipoplexes consisting of DMKD, DMKE, and N-[1-(2,3-dioleoyloxy)]-N,N,N-trimethylammonium propane methyl sulfate (DOTAP) were prepared and utilized for *in vitro* transfection to MSCs. At the same time, MSCs were transfected by electroporation under varied conditions of pulse magnitude, duration, and repetition. Their gene-transferring efficiencies and cytotoxicities were compared to each other. Based on those results, we suggested optimal conditions for effective and safe gene transfection to MSCs.

MATERIALS AND METHODS

Isolation and culture of mouse mesenchymal stem cells

Mesenchymal stem cells (MSCs) were isolated from the bone marrow of limb bones C57BL/6 mice (Orient Bio., Korea) with syringes and purified on the basis of their ability to adhere to the plastic tissue culture dish. The cells were cultured in a complete culture medium, Dulbecco's modified Eagle medium (Gibco, USA) containing D-glucose (1 g/L), L-glutamine (584 mg/L), sodium pyruvate (110 mg/L), 10% inactivated fetal bovine serum, and 1% penicillin (10,000 units)/streptomycin (10 mg) at 37℃ in a humidified atmosphere containing 5% carbon dioxide. When the cells reached about 90% confluency, they were harvested by trypsinization with the trypsin-EDTA solution and then subcultured for a further experiment.

Identification of mouse mesenchymal stem cells

Adipocyte differentiation from mesenchymal stem cells was induced by dexamethasone (1 μM, Sigma, USA), 3-isobutyl-1-methylxanthine (0.5 mM, Sigma, USA), insulin (10 μg/ml, Sigma, USA) and indomethacin (100 μM, Sigma, USA) (Dennis et al., 1999). Adipocytes differentiated from mesenchymal stem cells were shown by Oil red O staining (Dennis and Caplan, 1996) on the 19th day post treatment.

Osteoblast differentiation from mesenchymal stem cells was induced in a collagen-coated plate by dexamethasone (0.1 μM), ascorbic acid 2-phosphate (0.2 mM, Sigma, USA) and glycerol 2-phosphate (10 mM, Sigma, USA) (Jaiswal et al., 1997). Osteoblasts differentiated from mesenchymal stem cells were proved by detection of calcium deposits. The calcium deposits were shown by Alizarin Red S staining on the 24th day (Claudhary et al., 2004).

MSC transfection mediated by cationic liposomes

The cationic lipids, N-[1-(2,3-dioleoyloxy)]-N,N,N-trimethylammonium propane methyl sulfate (DOTAP, Avanti, USA), O,O'-dimyristyl-N-lysyl aspartate (DMKD), or O,O'-dimyristyl-N-lysyl glutamate (DMKE), were mixed with cholesterol (Chol) at 1:1 molar ratio in the chloroform-methanol solution (2:1, volume ratio). The organic solvent was vaporized by N_2 gas in a rolling tube. The dried lipid films were desiccated under vacuum to remove residual organic solvent. The lipid films were hydrated in deionized water by vigorous mixing for 5 minutes.

The prepared plasmid DNA encoding luciferase, pAAV-CMV-Luc (Kim et al., 2006) and cationic liposomes were mixed together in the culture medium at varied pDNA : lipid weight radios. The resulting mixtures were incubated for 1 hour at room temperature to stabilize the pDNA-liposome complexes (lipoplex). The stabilized lipoplex solutions were added to the primary cultured MSCs in 24-well plates (1 μg DNA each well). The cells were then incubated at 37℃ in a humidified atmosphere containing 5% CO_2 for 4 hours. After incubation, the lipoplex media were replaced by the culture media containing 10% FBS and the transfected cells were further incubated for 24 hours.

MSC transfection mediated by electroporation

The primary cultured MSCs were resuspended in the culture medium at the concentration of 1×10^6 cells/ml. The cell suspension (200 μl) was mixed with 10 μg of pDNA in a sterile electroporation cuvette of 4-mm gap width. The electroporation cuvettes with the MSCs were incubated on ice for 10 minutes before electroporation. To find an optimal electroporation condition, the voltage was varied from 100 to 1,200 V and the length of pulse was varied from 10 to 3,000 μs. The pulse was repeated 1~10

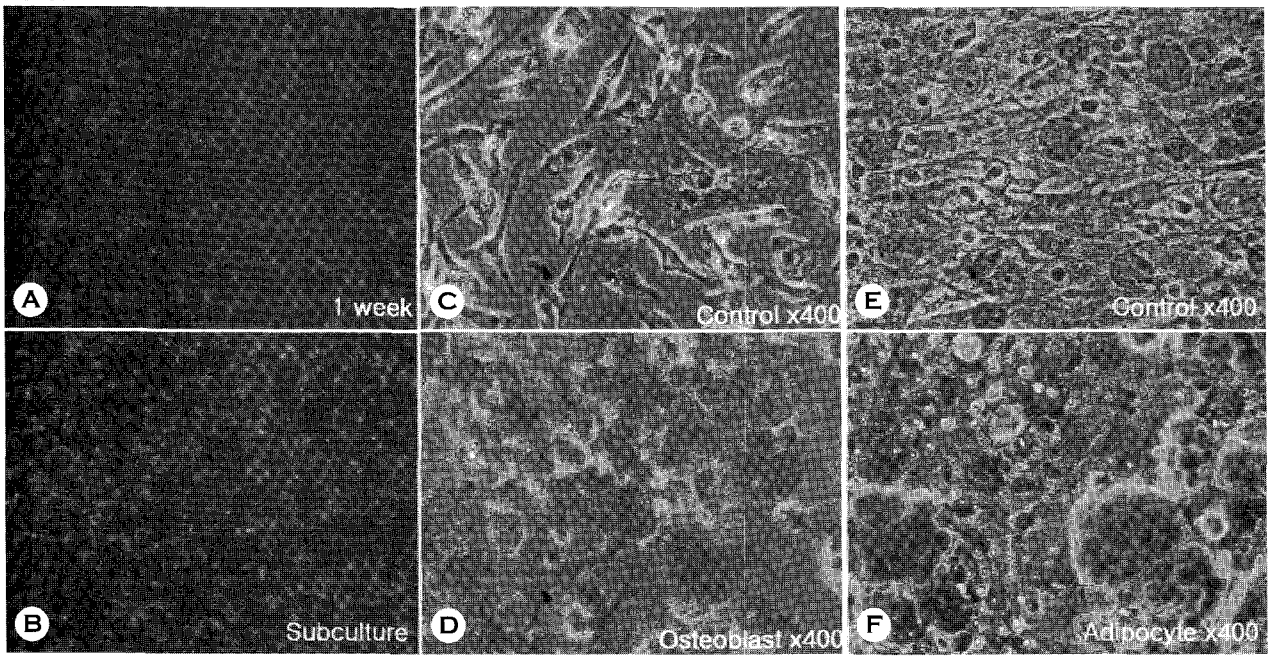


Fig. 1. Isolation and identification of mouse mesenchymal stem cells. The mesenchymal stem cells isolated from the C57BL/6 cells **(A)** were subcultured **(B)** in a complete medium. The subcultured cells were stimulated with dexamethasone, 3-isobutyl-1-methylxanthine, insulin, and indomethacin. The treated **(D)** and untreated control cells **(C)** were observed after Oil red O staining on the day 19 post stimulation with a light microscope. At the same time, the cells were treated with dexamethasone, ascorbic acid 2-phosphate, and glycerol 2-phosphate. On the day 24 post stimulation, the treated **(F)** and untreated control cells **(E)** were observed after staining with the Alizarin Red S solution.

times with 10 second intervals. After electroporation, the transfected MSCs were incubated for 10 minutes at room temperature and then transferred to the complete culture medium and further incubated for 48 hours.

Assays of transfection and cell viability

The transfection efficiency was measured by the luciferase assay described earlier (Kim et al., 2004). The transfected MSCs with pAAV-CMV-Luc under various conditions were lysed in a lysis buffer (0.1 M Tris-HCl, 2 mM EDTA, and 0.1% Triton X-100, pH 7.8). Luciferase activities of the samples were measured by a luminometer (MiniLumat LB9506, EG&G Berthold, Germany) with a luciferase assay kit (Promega, USA). To calculate the relative light unit (RLU), the protein concentration in the sample was also measured by the DC protein assay kit (BioRad, USA).

Viability of the transfected MSCs was measured by the MTT assay (Camichael et al., 1987). The transfected cells were incubated in the MTT solution (Sigma, USA) for 4 hours. Then, the MTT solution was replaced by DMSO to dissolve MTT formazan crystals. Absorbance of the resulting solution was measured with a spectrophotometer (λ=595 nm) (TECAN GENios, MTX Lab System, USA). The assays of transfection and viability were independently repeated twice with triplicate or quintuplet samples. The statistical analysis was done by Student's *t* test.

RESULTS AND DISCUSSION

Preparation and identification of mouse mesenchymal stem cells

Mouse mesenchymal stem cells were isolated from the bone marrows of mouse limb bones. A number of cells suspending in the medium began to adhere and show short spindle shape or asteroid shape one week after cell planting (Fig. 1A). Another one week later, the adherent cells populated to 90% confluence, which were hard to be distinguished in a single cell from each other. Appearance of the subcultured cells was the same with their parent cells (Fig. 1B).

Differentiation of the cultured cells to adipocytes and

osteoblasts confirmed that the MSCs were successfully isolated (Fig. 1C-F). On day 10 post treatment with dexamethasone, 3-isobutyl-1-methylxanthine, insulin, and indomethacin, the cells began to differentiate to adipocytes with the round vacuoles. On the 19th day, these cells were stained by the Oil red O staining reagent (Dennis and Caplan, 1996). The vacuoles in the cells were stained with red color resulting from Oil red O staining of lipidic substances in their inside (Fig. 1D). This result implied that the isolated cells were the MSCs in the beginning and then successfully differentiated to adipocytes.

The same cells were also successfully differentiated to another cell type, osteoblasts by treatment with a different set of chemical inducers consisting of dexamethasone, ascorbic acid 2-phosphate, and glycerol 2-phosphate. On the 24th day post treatment with chemical inducer treatment, the cell differentiation was confirmed by staining with the Alizarin Red S solution (Fig. 1F). This result also implied that the isolated cells were the MSCs, capable of differentiating into osteoblasts.

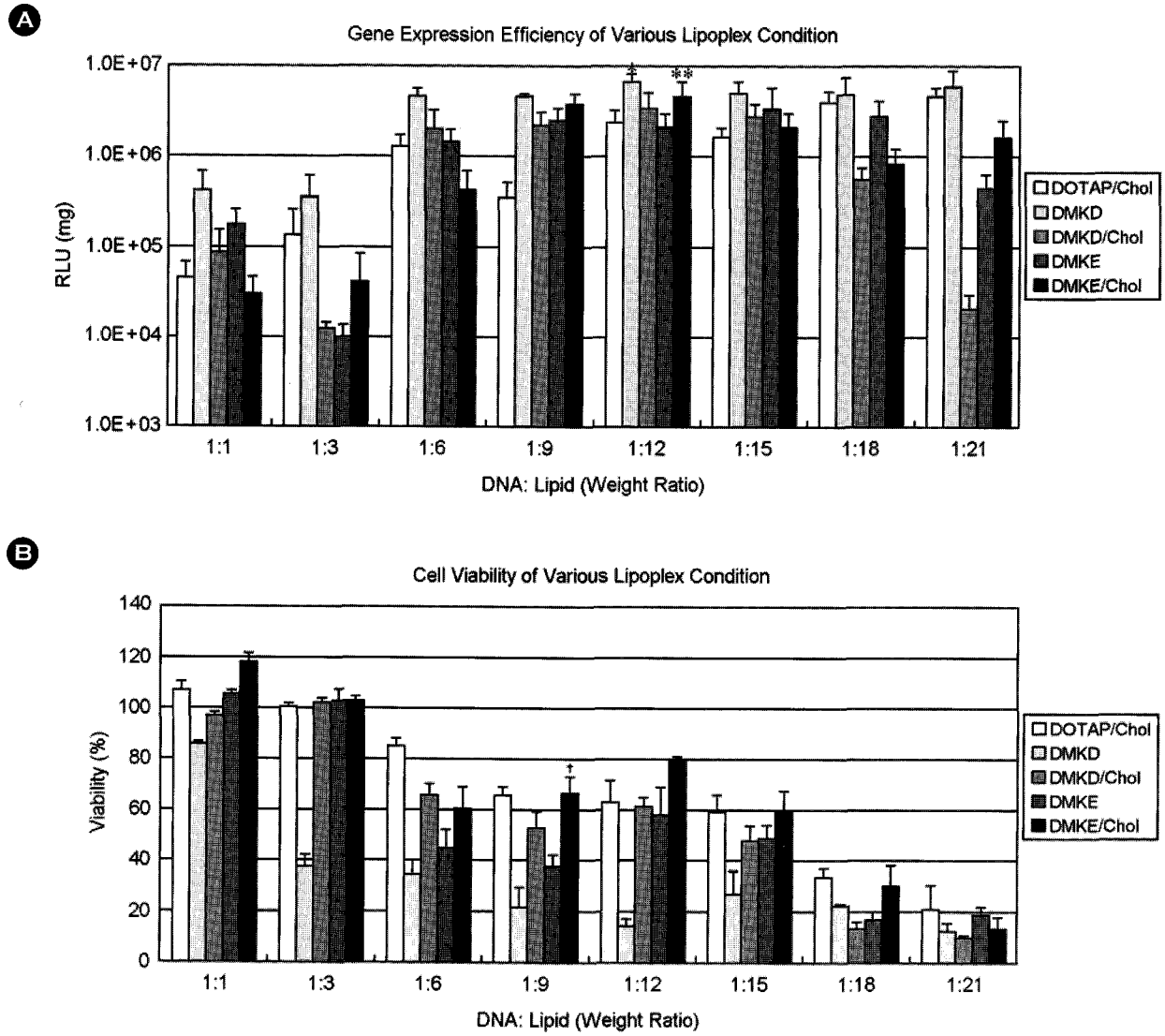
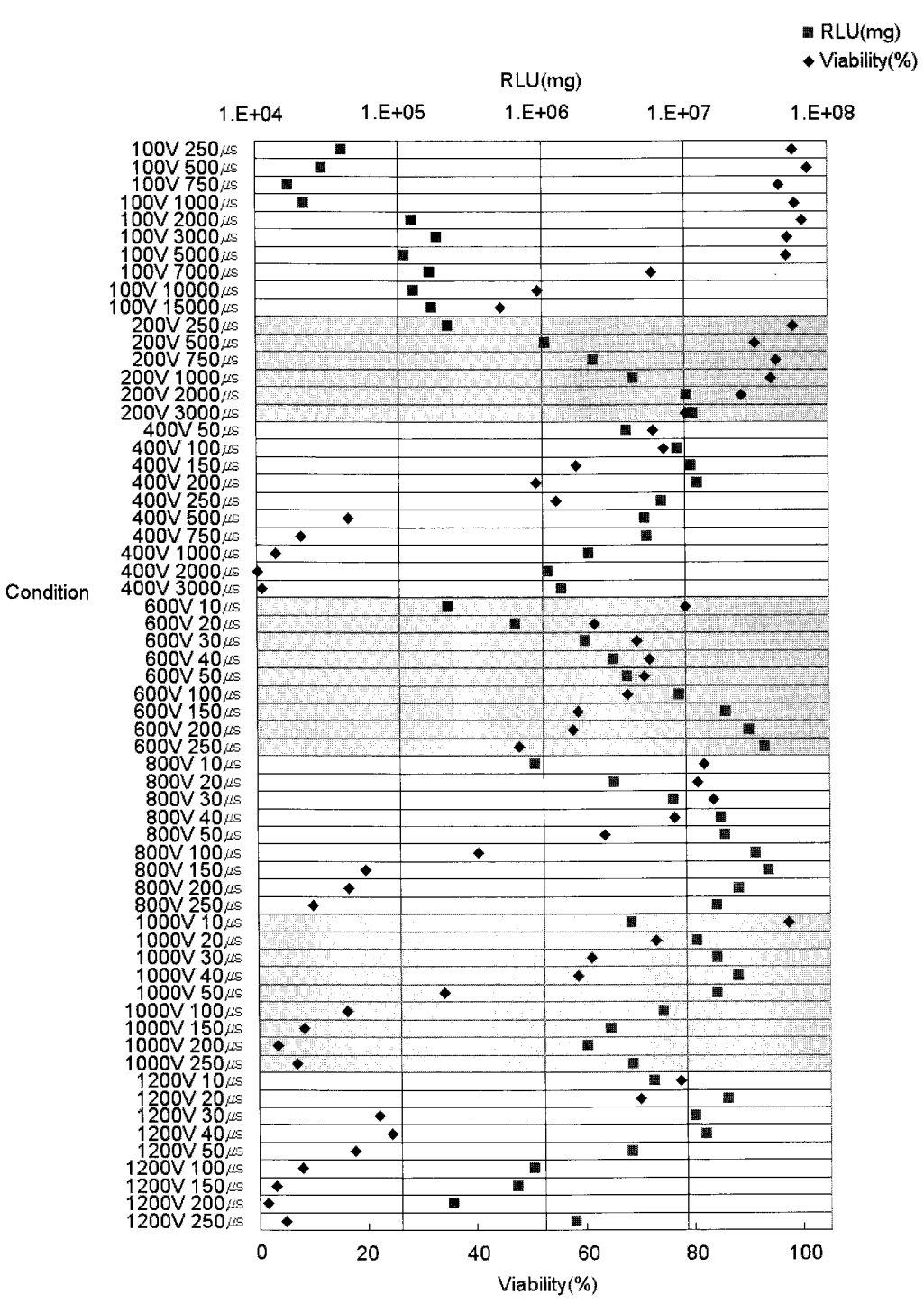


Fig. 2. Gene expression efficiency and cell viability after liposome-mediated transfection. The prepared MSCs were transfected with lipoplexes consisting of varied weight ratios of pAAV-CMV-Luc and cationic liposomes, DOTAP/Chol, DMKD, DMKD/Chol, DMKE, and DMKE/Chol. Luciferase activities of the cell lysates were measured by a luminometer using a luciferase assay kit **(A)**. At the same time, viability of the transfected MSCs was measured by the MTT assay **(B)**. The X-axis represents the weight ratios of pDNA:lipid. The Y-axis represents RLU per mg of total proteins (n=5) and cell viability % (n=3). $^{*}P<0.05$ versus DMKE/Chol, $^{**}P<0.05$ versus DOTAP/Chol at 1:12 ratio. $^{\dagger}P<0.05$ versus DMKD at 1:9 ratio.

***In vitro* gene transfection mediated by cationic liposomes or electroporation to mouse mesenchymal stem cells**

Prior to selection of the gene-transferring vectors for the purpose of gene therapy, there are a number of considerations such as a type and state of disease, therapeutic mechanisms and characteristics of expressed transgenes, and so on. Even when we develop a gene-transferring procedure using non-viral systems for transient expression of transgenes, we have to consider at least two different parameters, transfection efficiency and cell toxicity. These



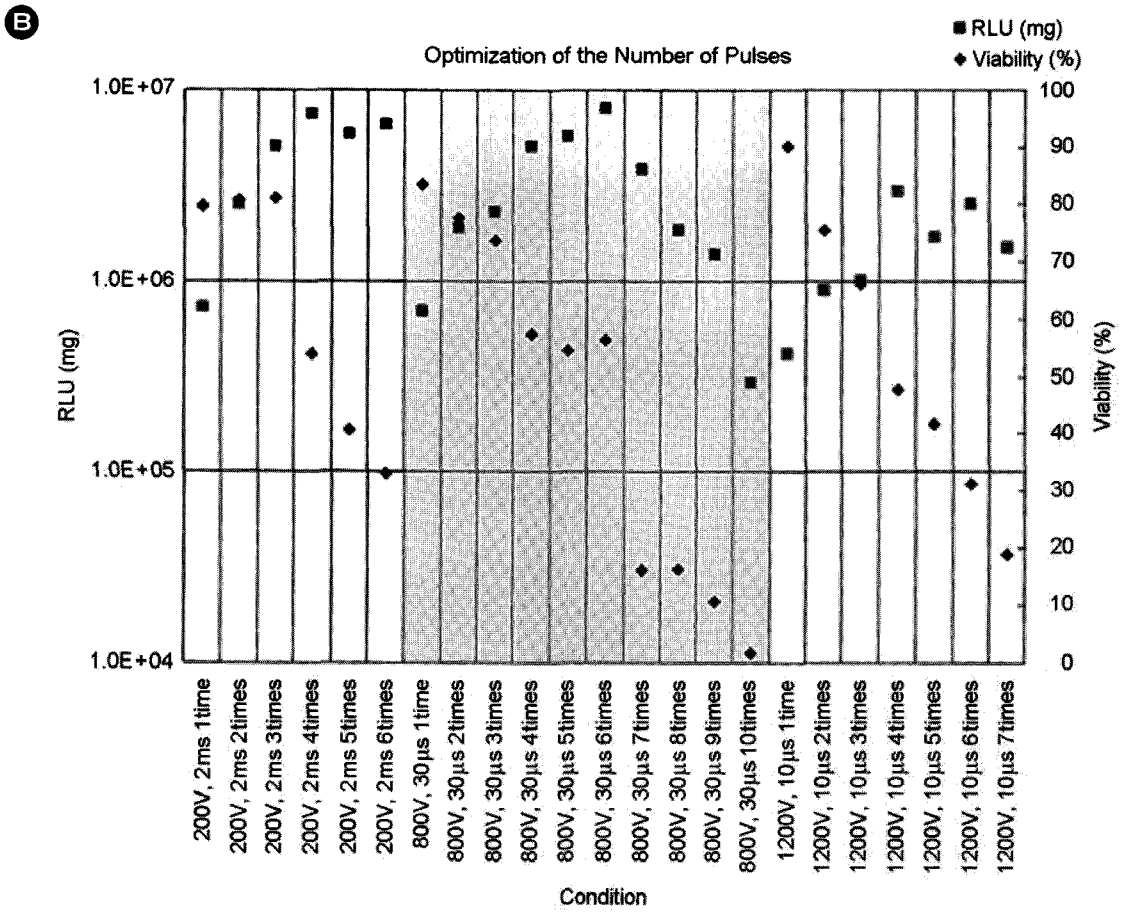


Fig. 3. Gene expression efficiency and cell viability after electroporation-mediated transfection. The prepared MSCs were transfected by electroporation under varied conditions of voltage and pulse duration with 10 sec of pulse interval **(A)** After selection of optimal voltage and pulse duration, the MSCs were electroporated with varied repetition of pulse under the optimal conditions **(B)**. The Y-axis represents RLU per mg of total proteins (n=5) and cell viability % (n=3).

two parameters have to be compromised to induce the most effective expression of therapeutic transgene. In this study, transgene expressions mediated by lipofection (liposome-mediated gene transfection) and electroporation were compared to each other. At the same time, viabilities of the cells transfected by the two different methods under varied conditions.

First of all, the prepared MSCs were transfected with various types of lipoplexes containing luciferase genes. Generally the luciferase expression mediated by lipofection was increased according to the increase of added cationic liposomes regardless types of liposomes (Fig. 2A). At higher than 1:12 weight ratio of pDNA and lipid, the gene expression was saturated in all types of lipoplexes. Among the lipoplexes, the DMKD lipoplex exhibited the highest gene expression at the all ratios of pDNA and lipid. The DMKE/Chol lipoplex showed a better gene expression than the DOTAP/Chol lipoplexes up to 1:12.

Meanwhile, the viability of transfected cells was rapidly diminished at ratios higher than 1:6 (Fig. 2B). Among the lipoplexes, the DMKD lipoplex was the most cytotoxic formulation while it was the most effective in transgene expression (Fig. 2A). The DOTAP/Chol and DMKE/Chol lipoplexes were relatively less toxic. Up to 1:16 ratios of pDNA and lipid, the cells transfected with the either lipoplex were survived over 60%. In general, the DMKE/Chol lipoplex appeared to be less toxic than the DOTAP/Chol lipoplex. In order to find the most effective lipofection condition, the cytotoxicities and transgene expression efficiencies of all the lipoplexes are simultaneously considered. Based on these results, the best formulation of lipoplexes for transfection to the MSCs was the DMKE/

Chol lipoplexes at 1:12 weight ratio of pDNA and lipid.

At the same time, the MSCs were transfected by electroporation under varied conditions. The electroporation voltage, duration time, number of pulse, and pulse interval time were varied to find an optimal condition for efficient and safe gene transfection. Generally the transgene expression increased according to electronic voltage, but concomitantly cell damage increased as well (Fig. 3A). At low voltage (up to 200 V), the longer electroporation induced the higher transgene expression. However, at voltages higher than 200 V, longer electroporation not always exhibited better transfection. This data suggested that there are optimal ranges of electrical power combining voltage and pulse duration. The highest and safe gene expression was obtained under the conditions of 200 V for 2 ms, 800 V for 30 μs, and 1,200 V for 10 μs. For this experiment, the number and interval of pulse were kept at 3 and 10 seconds, respectively.

To find an optimal pulse number, the same experiment was repeated under the conditions of varied pulse number with the voltage and pulse duration optimized above. Under the same experimental condition, increase of pulse number enhanced transgene expression, but concomitantly damaged the transfected MSCs (Fig. 3B). Especially, pulse repetition at high voltage (800 V and 1,200 V) seriously diminished the cell viability. The highest transgene expression and cell viability were obtained under the electroporation condition of 3 repeated pulses at 200 V for 2 ms with 10 seconds of pulse interval. Pulse intervals from 2 to 60 sec little affected transgene expression and cell viability under the optimized condition (data not shown).

According to the lipofection experiment, the mouse MSCs in 24 well plates were the most efficiently and safely transfected with the lipoplexes consisting of 1 μg pDNA and 12 μg the DMKE/Chol liposomes each well. The most efficient and safe transfection to the same MSCs in a 200 μl cuvette of 4-mm gap was obtained by 3 repeated electroporation pulses at 200 V for 2 ms with 10 seconds of pulse interval. Under both optimized transfection conditions, transgene expression by electroporation was relatively higher than that by lipofection.

Efficient and safe gene delivery is a critical step for genetic modification of a variety of cells including MSCs. This study clearly shows that efficiency transgene expression and viability of transfected cells can be significantly changed depending upon transfection conditions. An optimized condition of lipofection or electroporation would provide the best outcomes from the genetically modified MSCs.

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