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OPTIMIZATION OF TRANSFECTION METHODS FOR Huh-7 AND VERO CELLS: A COMPARATIVE STUDY

Availability of an efficient transfection protocol is the first determinant in success of gene transferring studies in mammalian cells which is accomplished experimentally for every single cell type. Herein, we provide data of a comparative study on optimization of transfection condition by electroporation and chemical methods for Huh-7 and Vero cells. Different cell confluencies, DNA/reagent ratios and total transfection volumes were optimized for two chemical reagents including jetPEI™ and Lipofectamine™ 2000. Besides, the effects of electric field strength and pulse length were investigated to improve electroporation efficiency. Transfection of cells by pEGFP-N1 vector and tracking the expression of GFP by FACS and Fluorescence Microscopy analysis were the employed methods to evaluate transfection efficiencies. Optimized electroporation protocols yielded 63.73 ± ± 2.36 and 73.9 ± 1.6 % of transfection in Huh-7 and Vero cells respectively, while maximum achieved level of transfection by jetPEI™ was respectively 14.2 ± 0.69 and 28 ± 1.11 % for the same cells. Post transfectional chilling of the cells did not improve electrotransfection efficiency of Huh-7 cells. Compared to chemical based reagents, electroporation showed the superior levels of transfection in both cell lines. The presented protocols should satisfy most of the experimental applications requiring high transfection efficiencies of these two cell lines.

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Introduction. The primary stage of every successful gene expression study for investigations such as functional genomics, gene therapy and gene regulation or pathway analysis requires an efficient and optimized method of transfection of nucleic acids (usually in the form of plasmid DNA) into specified mammalian cells [1, 2]. Although currently several biological (virus mediated) and non-biological (including chemical and physical) transfection methods are available but for every individual cell type the supreme method which should be efficient and reproducible with minimum cell toxicity, needs to be optimized and selected experimentally through comparative studies [2]. Chemical transfection protocols are the most widely used methods in research which employ cationic polymers, calcium phosphate and cationic lipids with almost similar functional mechanisms [3]. Briefly, positively charged chemicals make complex with negatively charged nucleic acids and the complex subsequently is fused to the negatively charged cell membrane followed by passing through it via endocytosis or phagocytosis [1, 2]. Cell confluency and reagent/DNA ratio are two main parameters that should be optimized for the cell types under study when chemical transfection methods are being employed [4]. Besides, the total volume of the transfection medium is suggested to have some minor effects on the efficiency of transfection when chemical methods are being used [4]. The physical transfection methods such as gene gun or electroporation (which is the most widely used technique) make use of physical tools to deliver nucleic acids into cells [1]. Although the exact mechanism of electroporation is unknown but it is supposed that short electrical pulse makes some holes in the cell membrane through which nucleic acids can go across [5]. Since the electrical pulse induces a high degree of cell death, having minimal amount of cell death as well as highest gene transfer efficiency are provided by optimization of the electric field strength and pulse length for each cell type [6]. In addition, some prior studies indicated that post-transfectional chilling of the cells to 0 °C [6] or utilization of electroporation buffers containing dimethyl sulphoxide DMSO (1.25 %) during and for 24 h after the pulse [7] may improve transfection efficiencies.

Huh-7 is an immortal and well differentiated hepatocyte-derived cellular carcinoma cell line that was originally taken from a liver tumor [8].

These cells have application in various studies, especially understanding of hepatic processes at the cellular and molecular levels [9, 10]. The Vero cell line was initiated from the kidney of a normal adult African green monkey. These cells are being used in different studies such as; screening for the toxin of *Escherichia coli* [11], as host cells for a wide range of viruses including polio, arboviruses and retroviruses as well as host cells for eukaryotic parasites [12, 13].

Although, a few comparative studies have already addressed the transfection efficiency of several chemical reagents to the electroporation using numerous cell types such as primary and transformed airway epithelial cells, primary fibroblasts, lymphoblasts, HEK 293, primary hematopoietic stem cells [4], embryonic stem cells [14–15] and primary human corneal endothelial cells [16] but there is no report (to our knowledge) on optimization and comparing efficiency of electroporation to chemical transfection systems for Huh-7 and Vero cell lines.

In the present study, we provide data of a detailed study on optimization of transfection condition (based on transfection of a Green Fluorescent Protein (GFP) harboring plasmid DNA; pGFPN1) for two chemical transfection reagents including one cationic polymer based reagent (jetPEI™) and one cationic lipid based reagent (Lipofectamine™ 2000) compared to electroporation in Huh-7 and Vero cell lines and present an efficient transfection protocol for these two cell types.

Material and method. *Cell culturing.* The human hepatoma (Huh-7) (Invitrogen, USA) and African green monkey kidney (Vero) cell lines (National Cell Bank of Iran, Tehran) were grown in Dulbecco's Modified Eagle Medium (DMEM) (Biosera) supplemented with 10 % heat-inactivated fetal bovine serum (Gibco, Invitrogen, USA) and antibiotics (0.1 mg/mL streptomycin and 100 U/mL penicillin) (Biosera). Cells were cultured at 37 °C in a humidified incubator supplied with 5 % CO_2.

Plasmid preparation. To monitor the efficiency of transfection, pEGFP-N1 plasmid DNA (NRGB, Pasteur Institute of Iran, Tehran) encoding Enhanced Green Fluorescent Protein was employed through all experiments of this study. The vector was purified from cultured transformed bacteria using plasmid purification Maxi kit (QIAGEN, USA) according to the manufacturer's protocols. The mean A260/280 ratio of the DNA samples isolated from both cell lines was 1.8 ± ± 0.045 (n = 3).

Transfection methods. Two commercially available transfection reagents including jetPEI™ (Polyplus, France) and Lipofectamine™ 2000 (Invitrogen, USA) were used for transfection of cell lines by pEGFP-N1 vector based on the manufacturer's protocols. Each experiment was performed three times.

After washing two times in ice-cold phosphate-buffered saline (PBS), cells were resuspended at $1.1 \cdot 10^8$/ml in RPMI 1640 + 10 mM HEPES. 5 μg of DNA was mixed with 0.09 ml of Huh-7 or Vero cell suspensions, transferred to a 4-mm gapped cuvette and immediately pulsed using a Gene Pulser Xcell (BioRad, Germany). The cells were subsequently diluted into 10 ml prewarmed complete growth media (DMEM-15% FBS) and plated in 75 cm^2 flask and incubated at 37 °C, 5 % CO_2 for 48 hours [4]. For all studies, untransfected cells (mock) were included as negative (reference) control. Each experiment was performed three times.

Evaluation of transfection efficiency. To determine the proportion of fluorescent cells (i.e.: transfected cells), the cells were sorted by a Partec PAS instrument (Germany) and samples were evaluated by FlowMax software (Tree Star Inc., Ashland, OR). Briefly, twenty-four hours after transfection via chemical based reagents or 48 h after transfection by electroporation, the cells were harvested by trypsinization. After centrifugation, the cells were resuspended at $1 \cdot 10^6$ cells/ml in DMEM medium. To identify the GFP positive cells, untransfected cells (mock) were used as the GFP negative control to set the gates. Subsequently the gated region was analyzed for green fluorescence (488–508 nm) on a Partec PAS instrument supplied with a 6 W argon laser tuned to 488 nm at 100 mW output power. The percentage of the total cells displaying GFP emission in the gated region was expressed as transfection efficiency.

GFP fluorescent images of transfected cells were acquired using an inverted fluorescence microscope (Olympus BX51, London, UK) with a 10× lens and digital images were collected using a digital camera.

Results. *Optimization of transfection efficiency via chemical based reagents.* To obtain the optimized cell confluency for the highest transfection efficiency 40, 50, 60, 70 and 80 · 10^3 Huh-7 cells were seeded per well (in 24-well plates) twenty four hours before transfection. Different reagent/DNA ratios (based on the manufacturers' recommendation) were also considered to screen the highest efficiency. As shown in Fig. 1, *a*, the highest transfection efficiency using Lipofectamine™ 2000 was obtained when 60 · 10^3 cells were seeded and the employed transfection reagent/DNA ratio was 4 µl/0.8 µg (results for other cell confluencies are not shown). In this optimized condition, the highest transfection efficiency was 12.2 % for Huh-7 cells (Fig. 1, *b*).

Different cell confluencies for Huh-7 cells (40, 50, 60, 70 and 80 · 10^3) and Vero cells (10, 20, 30, 40, 50, 60 and 80 · 10^3 cells) were seeded per well (in 24-well plates) twenty four hours before transfection. Different reagent/DNA ratios (based on the manufacturers' recommendation) were also considered to screen the highest efficiency. As shown in Fig. 1, *c*, *e* the highest transfection efficiency using jetPEI™ was obtained when 60·10^3 and 20·10^3cells were seeded in case of Huh-7 and Vero cells respectively while the employed transfection reagent/DNA ratio was 4 µl/2 µg for both cell lines (results for other cell confluencies are not shown). In this optimized condition, the highest transfection efficiencies were 14.2 and 28 % for Huh-7 and Vero cells, respectively (Fig. 1, *d*, *f*).

We also investigated if the total volume of medium per well during chemical transfection would affect the efficiencies for Huh-7 and Vero cells as suggested by manufacturers (www.polyplus-transfection.com). To this end the transfection efficiencies among wells containing 500, 250, 150 µl media during transfection were compared. Interestingly when results showed no significant differences in case of Lipofectamine™ 2000, in case of JetPEI™ the highest transfection efficiencies for Huh-7 and Vero cells were obtained in wells containing 500 and 150 µl of total medium respectively with almost 10 % variation of efficiencies within the range of the measured volumes (500, 250, 150 µl; data not shown). These results showed that the effect of total volume on transfection efficiency may depend on both the type of reagent and the cell line under study.

Optimization of transfection efficiency via Electroporation. According to prior studies, application of the square wave and low field strengths (500–2000 V/cm) together with long time constants (10–30 ms) provided the highest transfection efficiencies in case of mammalian cell types [6]. Besides, low-resistance buffers like PBS or culture medium (itself) are shown to be the optimal media for efficient electrotransfection of these cell types [6]. Accordingly, to obtain the condition in which cell death and electroporation efficiency are approximately equilibrated, we employed square wave form and evaluated different low field strengths (400, 425, 450, 625, 750 V/cm corresponding to: 160, 170, 180, 250, 300 volts (for a 4-mm gapped cuvette), respectively. as well as two different long pulse lengths (20 and 40 ms) for both cell lines (Fig. 2, *a*). Results indicated that the optimal electroporation condition for Huh-7 cells (electric field strength and pulse length of 425 V/cm and 40 ms respectively) led to 63.73 ± 2.36 % transfection efficiency while the optimal condition for Vero cells (625 V/cm and 20 ms) resulted in 73.9 ± 1.6 % transfection efficiency (Fig. 2, *b*, *c*). Furthermore the cell viability 24 h after plasmid delivery was similar and more than 50 % for both cell lines (data not shown).

To investigate the temperature effect on the electroporation efficiency of Huh-7 cells, the cuvette containing the electroporated cells was incubated on ice for 5 min before transferring to the growth medium. After 48 h, green fluorescence was detected through flow cytometry and compared with those cells transferred to the medium immediately after pulsing. Interestingly, our results demonstrated that chilling the Huh-7 cells to 0 °C post-transfectionally led to a significant decrease in cell viability by 40 % (data not shown). Moreover, to evaluate the effect of DMSO on electroporation efficiency, a final concentration of 1.25 % was included into electroporation buffer during and for 24 h after the application of the pulse. Results showed a decrease in electroporation efficiency and cell survival of Huh-7 cells by 60 % and 50 % respectively (data not shown).

Discussion. Huh-7 and Vero cells are two immortalized cell systems in two different mammalian species (human and monkey respectively)

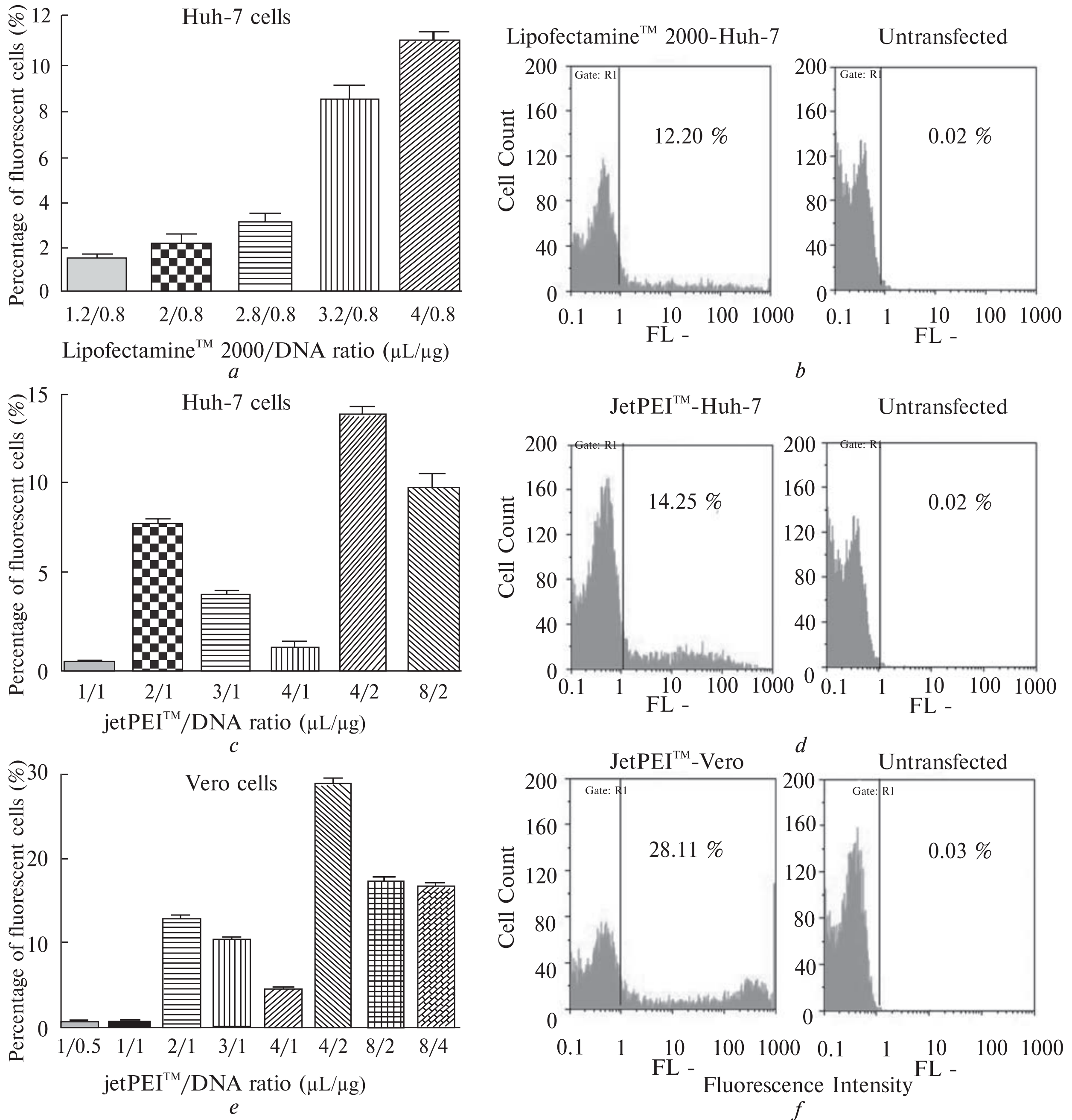

Fig. 1. Optimization of chemically based transfection conditions in Huh-7 and Vero cells and Flow cytometry plots of the transfected cells: *a* – different transfection reagent/DNA ratios using pEGFP plasmid shown for Lipofectamine™ 2000 in Huh-7 cells also jetPEI™ in Huh-7 and Vero cells. Data represent the mean ± S.D. ($n = 3$); *b* – the highest efficiency obtained when $60 \cdot 10^3$ cells were seeded per well and ratio was 4 μl (Lipofectamine™ 2000)/0.8 μg (DNA) and 4 μl (jetPEI™)/2 μg (DNA) in Huh-7 cells. Also the highest efficiency obtained when $20 \cdot 10^3$ cells were seeded per well and ratio was 4 μl (jetPEI™)/2 μg (DNA) in Vero cells. Transfection efficiencies were expressed as the ratio of the GFP-positive cells to total cells in the gated region

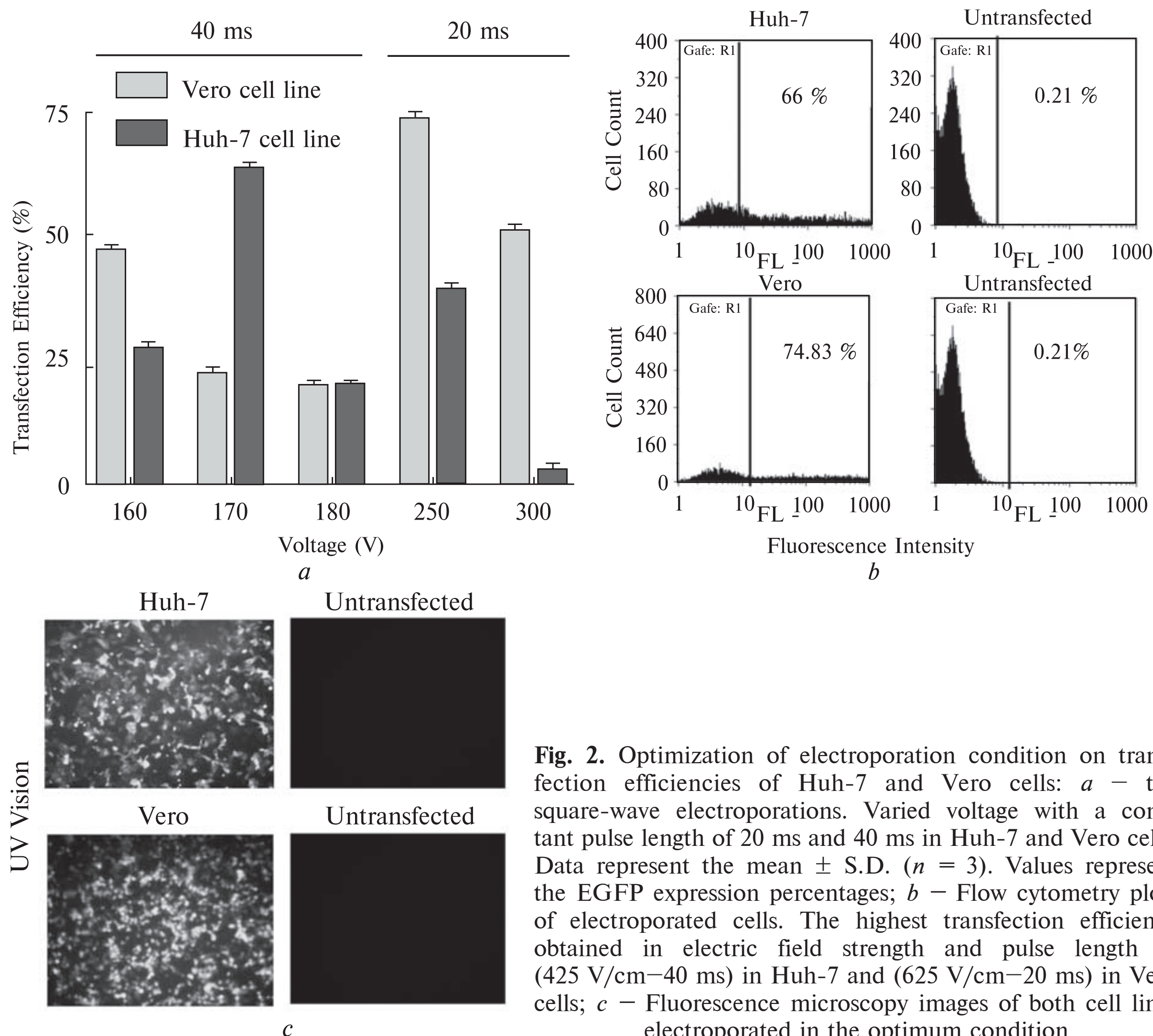

Fig. 2. Optimization of electroporation condition on transfection efficiencies of Huh-7 and Vero cells: *a* — the square-wave electroporations. Varied voltage with a constant pulse length of 20 ms and 40 ms in Huh-7 and Vero cells. Data represent the mean ± S.D. (n = 3). Values represent the EGFP expression percentages; *b* — Flow cytometry plots of electroporated cells. The highest transfection efficiency obtained in electric field strength and pulse length of (425 V/cm—40 ms) in Huh-7 and (625 V/cm—20 ms) in Vero cells; *c* — Fluorescence microscopy images of both cell lines electroporated in the optimum condition

that are particularly important for characterization of hepatitis viruses molecular mechanisms (Huh-7) [8, 10, 17] or as host cells for a wide range of viruses and eukaryotic parasites (Vero cells) [11—13]. Although Huh-7 and Vero cell lines have been employed in number of prior studies and were transfected via different transfection reagents or electroporation in separate set of experiments but, none of the previous reports provided an optimized transfection protocol for these important cell types based on a comparative study [18—22]. This is specially important for Huh-7 which is generally known as a difficult to transfect cell line [23] and when high rates of transfection efficiencies are determinant for the aim of the study. Previously the elegant study of Uchida and co-workers [24] based on a comparative analysis for the transfection efficiency and cytotoxicity of six non-viral chemical reagents (Lipofectin, Lipofectamine plus, SuperFect, Effectene, DMRIE-C and DOTAP) intended for a wide range of human cells including Huh-7 was reported. Results of this prior study indicated efficient transfection of Huh-7 cells by «Lipofectamine plus» and «Effectene» compared to other reagents (with some strong cytotoxicity for «Effectene»). However there is no report to compare the efficiency of transfection between chemical methods and electroporation in Huh-7 and Vero cells and to provide an optimized and

practical protocol of transfection for these cell types.

In the present study we compared two commercially available transfection reagents including «Lipofectamine™ 2000» (as a cationic liposome) and «jetPEI™» (as a cationic polymer). Optimization of the parameters in both cell lines resulted in the transfection efficiencies of 12.2 ± ± 0.6 % in case of «Lipofectamine™ 2000» for Huh-7 cells and 14.2 ± 0.69 and 28 ± 1.11 % in case of «jetPEI™» for Huh-7 and Vero cells, respectively. Although these transfection efficiencies are comparable with the results reported by Uchida et al. [24] but the slightly lower transfection efficiency of Huh-7 cells using «Lipofectamine™ 2000» (12.2 ± 0.6 %) in our study compared to «Lipofectamine plus» used by Uchida et al. might be relevant to the addition of the DNA-compacting agent to cationic lipids in «Lipofectamine plus» composition [24]. In agreement to this suggestion, addition of DNA compacting peptides derived from human sequence to cationic lipid agents were previously shown to enhance the gene transfer efficiencies *in vitro* and *in vivo* [25].

Endocytosis is considered as the preferred route of cell entry of nonviral complexes and cargos via chemical transfection methods [26]. The efficiency of cargo transfer by endocytic pathway itself (and therefore the transfection efficiency of chemical methods) is highly dependent on the cell line type and specific characteristics of the chemical reagent (polyplex or lipoplex) used as carrier [27, 28]. Different transfection efficiencies obtained in our study for Huh-7 and Vero cells (14.2 ± 0.69 % versus 28 ± 1.11 % respectively) in spite of employing the same reagent (jetPEI™ as a polyplex reagent) clearly indicated the effect of the cell type in utilizing different endocytic pathways. Indeed, previous findings suggested that the same chemical reagent (polyplex) may be internalized by different cell lines through various endocytic pathways leading to different transfection efficiencies. Of note, it is suggested that in Hela and 293 cells the caveolae-dependent endocytic pathway leads to effective transfection by polyplex reagent [29] while clathrin-mediated endocytosis is the main endocytic pathway in Huh-7 and COS-7 cells when the same chemical reagent (polyplex) is employed for transfection [30]. Therefore, our results for the observed differences in the transfection efficiencies between Vero and Huh-7 cells in case of polyplex reagent may be related to the different fates of this reagent through endocytic pathways in these two cell lines.

We also improved electroporation protocols for gene delivery into these two cell lines by optimizing the electric field strength and pulse length. In accordance with previous reports the square wave form provides higher mammalian transfection efficiencies and is widely used for *in vitro* and *in vivo* applications [31]. Therefore, square wave pulsing was employed in our study. In addition Reports using electroporation to transform mammalian cells either employed high field strengths and short pulse length or using lower field strengths and longer pulse length [31]. However, later studies showed that higher levels of expression could be obtained by the latter [6]. Our results showed that high efficiencies as well low cell death were obtained with low field strengths and long time constants condition in both cell lines.

Effect of cold treatment on electrotransfection efficiency of Huh-7 cell was also addressed in our study. Few prior reports have indicated that post-transfectional chilling of the cells to 0 °C increase the transfection efficiency of some cell types (apparently due to keeping the membrane pores open for longer time which subsequently provides more time for DNA to enter the cell) [6] when on the contrary immediate transferring of electroporated cells to pre-warmed medium have been suggested for other cell types to increase cell survival (probably via helping the pores created to be resealed) [6]. Our results in accordance with the later report demonstrated that post-transfectionally chilling of the Huh-7 cells increased cell death by 40 %. We also assessed the potential role of dimethyl sulphoxide (DMSO) on electrotransfection efficiency of Huh-7 cell in our study. DMSO has been reported to enhance the transfection efficiencies of DNA introduced by polycations including polybren and DEAE [32, 33] as well as calcium phosphate method [34]. It is suggested that DMSO enhances the uptake of DNA by augmenting cell membrane permeabilization and the osmotic shock [34]. However, the concentration of DMSO for efficient transfection

is critical and should be determined experimentally for each cell line. In fact optimal concentration of DMSO for one cell line may be toxic for another one. This fact was previously reported for chemical transfection of human epidermal keratinocytes and muine NIH-3T3 cells by polybrene reagent in which the optimal concentration of DMSO for highest transfection efficiencies of the cells were 27 and 15 % respectively [35]. According to a prior study, improvement in electrotransfection efficiency and cell viability in four different cell lines (HL60, TR146, COS-7 and L132) using electroporation buffer containing DMSO (1.25 %) during and for 24 h after the pulse was reported [7]. Accordingly in our study Huh-7 cells were electroporated in buffer (media) containing 1.25 % DMSO when other parameters were kept in optimized condition. However this modification decreased the electroporation efficiency and cell survival of Huh-7 cells by 60 and 50 % respectively (data not shown). This result was not surprising and may indicate the toxic effect of 1.25 % DMSO on Huh-7 cells. Further experiments are required to access the specific concentration of DMSO for Huh-7 cells in which cell permeability increase is balanced with cell viability decrease. In addition, concentration of plasmid DNA might be an important parameter for electrotransfection efficiencies that should be determined experimentally for each cell type. Application of 1–10 μg plasmid was suggested in different electrotransfection studies [6, 36]. In our study 5 μg of plasmid DNA (as a mean concentration and starting point) was used throughout all experiments. Although, it may be possible to further increase the transfection efficiencies obtained in this study by optimizing DMSO and plasmid DNA concentrations, but the achieved efficiencies (63.73 ± 2.36 and 73.9 ± 1.6 % for Huh-7 and Vero cells respectively) are good enough to satisfy most of the experimental applications requiring high transfection efficiencies of these two cell types.

Finally, in complete agreement with a recent report by Maurisse et al. [4] which indicated the superiority of electroporation/nucleofection to chemical methods for DNA delivery into primary and transformed mammalian cells from different lineage, our results also showed that electrotransfection was the suprime method for Huh-7 and Vero cells. The mechanism for this superiority of electroporation compared to chemical methods should lie on direct delivery of the plasmid DNA into the cytosol and passing though the endocytic pathways as previously suggested [37].

In summary, to our best of knowledge, we provided data of the first comparative study on optimization of transfection condition by electroporation and chemical methods for Huh-7 and Vero cells. These data indicated the superiority of electroporation to chemical methods for DNA delivery into Huh-7 and Vero cells. According to our results, application of $60 \cdot 10^3$ Huh-7 cells (confluency) and transfection reagent/DNA ratios of 4 μl/0.8 μg and 4 μl/2 μg in a total volume of 500 μl provided transfection efficiencies of 12.2 % ± 0.6 and 14.2 % ± 0.69 in case of «Lipofectamine™ 2000» and «jetPEI™» reagents respectively, while application of the later reagent provided 28 % efficiency using $20 \cdot 10^3$ Vero cells and transfection reagent/DNA ratio of 4 μl/2 μg in a total volume of 150 μl. Alternatively, electroporation of both cells at a density of $1.1 \cdot 10^8$/ml in RPMI+10 mM HEPES buffer in the presence of 5 μg of plasmid DNA and application of electric field strength and pulse length of «425 V/cm and 40 ms for Huh-7» cells and «625 V/cm–20 ms for Vero cells» resulted to transfection efficiencies of 63.73 ± 2.36 and 73.9 ± 1.6 % for these two cell lines respectively. The presented protocols should satisfy most of the experimental applications requiring high transfection efficiencies of these two cell types.

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ОПТИМИЗАЦИЯ МЕТОДОВ ТРАНСФЕКЦИИ КЛЕТОК Huh-7 И VERO: СРАВНИТЕЛЬНЫЙ АНАЛИЗ

Наличие эффективного протокола трансфекции является первым условием успешных исследований по переносу генов в клетки млекопитающих, что достигается экспериментально для каждого конкретного типа клеток. Здесь мы приводим

данные сравнительного исследования по оптимизации условий трансфекции клеток Huh-7 и Vero с помощью электропорации и химическими методами. Для двух химических соединений, jetPEI™ и Lipofectamine™ 2000, были оптимизированы сочетания различных клеток, соотношения ДНК/реагент и общие объемы трансфекции. Кроме того, для улучшения эффективности электропорации было изучено влияние силы электрического поля и длины импульса. Трансфекция клеток с помощью вектора pEGFP-N1, определение экспрессии GFP с помощью FACS и флюоресцентная микроскопия были использованы для оценки эффективности трансфекции. В оптимизированных протоколах достигалась трансфекция на уровне 63.73 ± 2.36 и 73.9 ± 1.6 % в клетках Huh-7 и Vero соответственно, в то время как максимальный уровень трансфекции с помощью jetPEI™ составлял 14.2 ± 0.69 и 28 ± 1.11 % для тех же клеток. Охлаждение клеток после трансфекции не улучшало эффективность электротрансфекции клеток Huh-7. В обеих клеточных линиях электропорация позволила достичь более высокого уровня трансфекции по сравнению с использованием химических реагентов. Представленный протокол может быть пригодным для большинства экспериментальных манипуляций, которые требуют высокого уровня трансфекции исследуемых клеточных линий.

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