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Original Article

Enhancement effect of urea toward electroporation-mediated plasmid transfection efficiency in the HEK-293 cell line

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Abstract

Background and purpose: Intracellular delivery is crucial in biological and medical studies. Although many molecular tools have been created for cell-based gene therapies, it remains challenging to introduce external molecules into cells. As one of the most popular non-viral transfection methods, electroporation induces transient pores in the cell membrane by applying an external electric field. Unsatisfactory transfection efficiency and low cell viability are the major drawbacks of electroporation. To overcome these issues, the current study investigated the effect of urea on electroporation-mediated transfection efficiency.
Experimental approach: Three voltages of electroporation, including 100, 120, and 140 V, and 3 concentrations of urea buffer, including 0.25%, 0.5%, and 1% W/V, were considered as variables in this study. The HEK-293 cell line was used for transfection, and green fluorescent protein (GFP) expression was evaluated using flow cytometry and fluorescence microscopy.
Findings/Results: The results showed that the combination of electroporation and urea increased electroporation efficacy, but the effect depended on voltage and urea concentration. When different concentrations of urea were added to HEK-293 cells at a voltage of 100 V, the number of cells transfected by pEGFP-N1 increased (from 12.3 ± 0.2% in untreated cells to 17.35 ± 0.55%, 23.3 ± 0.3%, and 14 ± 0.1% at urea concentrations of 0.25%, 0.5%, and 1% W/V, respectively). The electroporation buffer containing 0.5% W/V urea showed the highest EGFP expression (23.3 ± 0.3%) and high cell viability (over 90%).
Conclusion and implications: This research offers a new perspective for improving gene transfection efficiency once electroporation is utilized.

Keywords: Chemical enhancers; Electroporation; Gene delivery; HEK-293 cells; Mammalian cells; Urea.

INTRODUCTION

An efficient entry of foreign genes into mammalian cells, generally called transfection, is essential for gene function study and genetic modifications, especially cell-based gene therapy (1). In recent decades, studies on improving cell function by replacing defective genes or inducing alternations in desired gene expression by introducing exogenous genetic material have become increasingly attractive (2). The cell membrane consists of tightly packed lipids in an organized lamellar structure with an outer hydrophilic portion and an inner lipophilic portion with a good diffusional and partitioning barrier, especially against large polar molecules such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (3). For this reason, effective and non-destructive transfection is one of the main challenges in gene therapy and biotechnology. Hence, various delivery systems have been devoted to improving this process.

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Nowadays, gene delivery methods are mainly categorized into viral and non-viral (including chemical and physical) methods (4). There are special advantages and drawbacks to each of these methods. Accordingly, the procedure is selected based on the type of cell line, experiment purpose, and accessibility (1). In the early 1980s, physical methods were introduced as a new method to overcome viral and chemical system weaknesses. Physical methods quickly became a common tool for transfection due to their greater safety, simplification, and inexpensiveness. Another advantage of physical delivery is the ability to control the parameters involved in transfection according to various conditions (5). Electroporation is one of the most widely used physical transfection methods in which applying high-voltage electrical pulses to the cell for a short duration of time diminishes the required energy for the penetration of water molecules and forms hydrophilic pores in the phospholipid bilayer, which can last for a few milliseconds to minutes. These pores facilitate the passage of polar and non-polar molecules into the cytoplasm (5).

Although electroporation is a fast, safe, and reproducible method that can be used in most cell lines and at any stage of the cell cycle, it has some limitations such as the high percentage of cell death and low transfection efficiency, especially in primary cells, indivisible cells, and immune cells. The transfection efficiency of electroporation is adjustable by arranging the effective parameters, such as maximum voltage, pulse duration, and electric current intensity. Optimization aims to achieve the maximum quantity of gene delivery and minimize damage to the cell (6). Applying chemical enhancers is one of the other strategies taken into account to improve the electroporation process, possibly by elevating the fluidity of the cell membrane. Utilizing chemical penetration enhancers as electroporation buffers have been explored to increase the transfection rate while preserving cell survival (7-9). The commercially available buffers used for enhancing electroporation efficiency are expensive. So, urea which is readily available in the lab, easy to apply, and cheaper, is considered for the first time in this study for developing an effective and low-cost electroporation buffer. Until now, few studies have been done on the effects of urea on the cell membrane. Saffari *et al*. showed that urea caused membrane fluidity on the surface of A549 lung carcinoma cells and thus could increase the passage of cationic liposomes through the cell membrane (10). Although the mechanism of action of urea is not exactly clear, it is known to permeate both artificial and natural membranes, causing destabilization through hydrophobic interactions (11-13). Moreover, research using protein-free liposomal systems as models for cell membranes has suggested that urea reduces the organization of fluid-phase microdomains within lipid bilayers (12). Furthermore, the actions of urea in fluidizing membranes may also manifest in biological environments, either by disrupting the hydrophobic core of membrane bilayers or by affecting integral membrane proteins (12,14).

For the first time, in the current study, we investigated the effect of a combination of urea (as a low-cost chemical enhancer) with the electroporation procedure on transferring the pEGFP-N1 reporter plasmid into the HEK-293 cell line.

MATERIALS AND METHODS

Plasmid preparation

The pEGFP-N1 plasmid, which encodes an Enhanced green fluorescent protein (EGFP), was a kind gift from the Department of Molecular Genetics of Tarbiat Modares University. The plasmid was transformed into the competent *Escherichia coli* DH5a strain. Transformed cells were then grown in Luria-Bertani broth comprising 0.5% yeast extract, 1% tryptone, 1% NaCl, and 25 µg/mL kanamycin at 37 °C and 180 rpm for 16 h. The plasmid extraction was performed using a commercial kit (GeneAll Company, South Korea) following the manufacturer’s instructions.

Cell culture

The HEK-293 cell line was obtained as a gift from the cell bank of Tarbiat Modares University, Tehran, Iran. Cells were grown in Dulbecco's modified eagle's medium (DMEM; Inoclon, Iran) with high glucose containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (Biosera, France) and maintained in a 97.5% relative humidified atmosphere at 37 °C with 5% CO_2.

Electroporation buffer preparation

Urea was used here as a permeation enhancer to amplify the effect of electroporation, possibly through membrane fluidization. To prepare the electroporation buffers with final urea concentrations of 0.25%, 0.5%, and 1% W/V, the required amounts of urea powder (Merck, Germany) were dissolved in distilled water and filtered *via* 0.22 µm polyvinylidene fluoride syringe filters. DMEM high glucose medium was added to the urea solutions to obtain the final concentrations during the electroporation process. Undoubtedly, in this study, a concentration of urea should be chosen that is not toxic to the HEK-293 cells. Therefore, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used in this study to investigate urea cytotoxicity. To this end, cytotoxicity was measured after 24 and 48 h of treatment with urea at the concentrations of 0.25, 0.5, 0.75, 1, and 2% W/V, and no toxicity was observed (unpublished results). However, due to the MTT assay results showing that treating cells with urea at concentrations below 2% for 24 or 48 h did not negatively affect cell viability; concentrations below 1% were used for the current investigation. Notably, in the current design, the effect of urea on transfection efficiency was examined when cells were in the vicinity of urea only during the electroporation pulses (urea was only present in the electroporation buffer and was removed immediately after the pulse by changing the culture medium).

Electroporation

Cells with 80% confluency at their second passage number were used for all experiments. Cells (1.5×10^6) were re-suspended in 90 µL electroporation buffer and 10 µL plasmid solutions containing 1 µg plasmid in distilled water. The samples were transferred to 4 mm electroporation cuvettes and incubated for 5 min on ice. Afterward, cuvettes were exposed to a single 25-ms square-wave pulse with an indicated voltage generated by a Gene Pulser Xcell System (Bio-Rad, USA). After the pulse induction, transfected cells were incubated on ice for 10 min and then transferred to the wells of 6-well plates. Initially, in the absence of urea in the electroporation buffer, 3 voltages of 100, 120, and 140 V were investigated to optimize the electroporation efficiency. According to the results, both 100 and 120 V were used for further experiments. In each of these voltages, cell samples were subjected to 5 experimental conditions of electroporation including negative control (without plasmid and urea), untreated (urea-free), and urea in 3 different concentrations (0.25%, 0.5%, and 1% W/V). Each of these experimental conditions was tested in 2 independent experiments.

Cell viability

To evaluate the viability of the cells after the electrical pulse, a 10 µL sample was taken from the wells 2 h after the electroporation process. Two technical replicates were included for each well. Cells were mixed and stained with 10 µL of 0.4% trypan blue dye solution (Sigma, USA). The ratio of the number of survived cells to the total number of cells was then counted under a light-inverted microscope (Ceti, Belgium) using a Neubauer chamber (Marienfeld Superior, Germany).

Fluorescence microscopy and flow cytometry

To evaluate the transfection efficiency, cells were imaged by the inverted fluorescence microscope (IX53, Olympus Co., Japan) 48 h after electroporation. Afterward, the accurate percentage of pEGFP-expressing cells was detected by a fluorescence-activated cell sorting (FACS) flow cytometer (BD Biosciences, San Jose, CA, USA). Following the manufacturer's instructions, cells were harvested and re-suspended in 1000 µL phosphate-buffered saline to be prepared for FACS analysis.

Statistical analysis

Data were analyzed by GraphPad Prism 7 software and presented as the mean ± SD from 2 independent experiments. For evaluating the effect of voltage on cell viability and transfection efficiency, statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Tukey post-test. The effect of urea concentrations on cell viability and the percentage of pEGFP-expressing cells at the voltages of 100 and 120 V was analysed by two-way ANOVA followed by Tukey post-test. Also, for comparing 2 factors including GFP expression and viability between the voltages of 100 and 120 V in the urea concentrations of 0.25%, 0.5%, and 1% W/V, two-way ANOVA followed by Sidak post-test was applied. $P < 0.05$ was considered statistically significant.

RESULTS

Optimizing electroporation voltage

Before the usage of urea in our electroporation buffer and to optimize the electroporation voltage along with high cell survival, the electroporation procedure was performed at 3 different voltages using 1.5×10^6 cells. HEK-293 cells were electroporated with a single square wave pulse of 100, 120, or 140 V with a duration of 25 ms. The cell viability of each condition was determined 2 h after electroporation using the trypan blue staining method. The calculated cell viability for 100, 120, and 140 V electroporation conditions were 94.2 ± 0.2%, 73.25 ± 0.3%, and 53.25 ± 2.75%, respectively (Fig. 1A). The cell viability results revealed a significant decrease with the elevated voltages (Fig. 1A). Due to the low cell viability of the cells transfected with 140 V square wave pulse, this voltage was not used in the later steps of this study.

To show the EGFP expression transfected cells were observed by fluorescent microscopy 48 h post-transfection (Fig. S1). Flow cytometric analysis was used to compare the transfection efficiency (Fig. S2). As shown in Fig. 1B, the results of flow cytometry analysis demonstrated the percentages of transfected cells at the voltages of 100, 120, and 140 V as follows: 12.3 ± 0.2%, 18.75 ± 0.15%, and 12.4 ± 1.8%, respectively. The voltage changes from 100 to 120 V led to an increase in the transfection efficiency; as the voltage increased to 140 V, this efficiency declined (Fig. 1B).

Synergistic effect of urea and electroporation on transfection efficiency and cell viability at voltage 100 V

Cell viability of the electroporated cells (at the voltage of 100 V) either treated with urea at the concentrations of 0.25%, 0.5%, and 1% W/V or untreated was compared with the negative control, which had neither urea nor vector. According to the results, the survival of cells treated with urea at the concentration of 0.25% (93.1 ± 0.1%) did not show a significant loss, however, it decreased significantly at the concentrations of 0.5% (90.9 ± 0.3%) and 1% (74.6 ± 1.1%) (Fig. 2A). The images taken by fluorescence microscopy and the results from flow cytometry analysis were presented in Fig. S3 and Fig. S4, respectively. The pEGFP transfection efficiency was then compared with the untreated sample. According to the flow cytometry results, the addition of urea resulted in a significant increase in transfection efficiency (from 12.3 ± 0.2% in untreated cells to 17.35 ± 0.55%, 23.3 ± 0.3%, and 14 ± 0.1% in the concentrations of 0.25%, 0.5%, and 1% W/V, respectively). The electroporation buffer containing 0.5% W/V urea showed the highest EGFP expression (Fig. 2B).

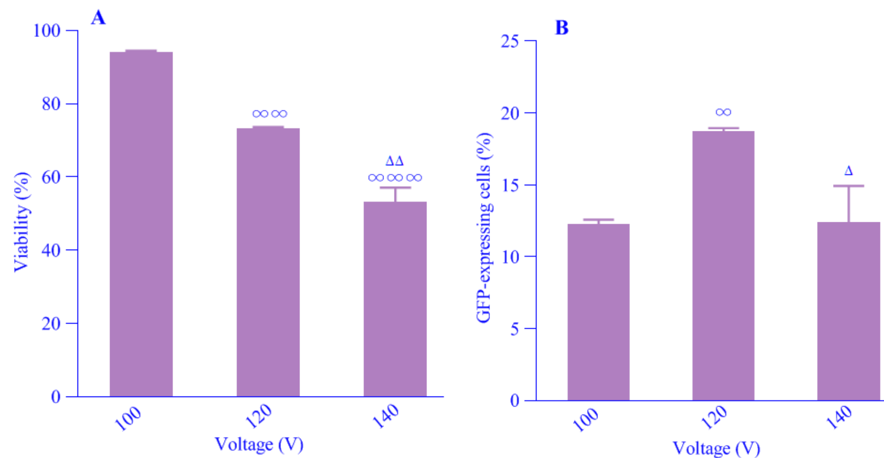

Fig. 1. (A) Cell viability and (B) gene expression in electroplated cells at 100, 120, and 140 V. Cell viability was determined 2 h after electroporation. pEGFP expression was evaluated by flow cytometry after 48 h. Data were presented as mean ± SD, n = 2. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post-test. $^{\infty}P < 0.05$, $^{\infty\infty}P < 0.01$, and $^{\infty\infty\infty}P < 0.001$ demonstrated significant differences compared with voltage 100 V; $^{\Delta}P < 0.05$ and $^{\Delta\Delta}P < 0.01$ versus voltage of 120 V. GFP, Green fluorescent protein; EGFP, enhanced green fluorescent protein.

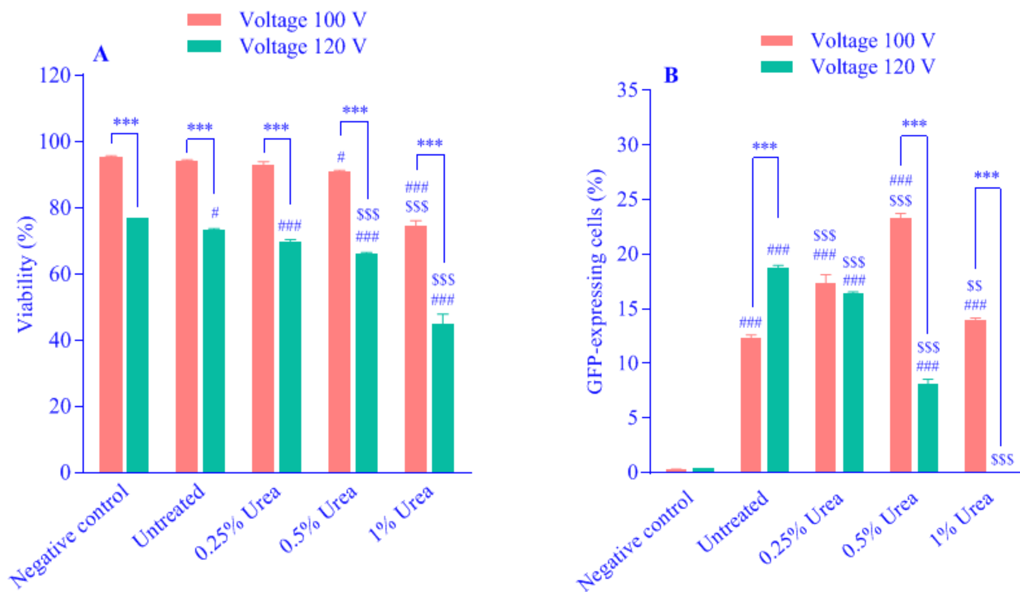

Fig. 2. Effects of urea concentrations (0.25%, 0.5%, and 1% W/V) on (A) cell viability and (B) percentage of pEGFP-expressing cells at the voltages of 100 and 120 V. All cells were electroporated at the voltages of 100 and 120 V. Negative control was subjected to cells without receiving GFP and urea. Untreated cells (urea-free) and cells receiving urea concentrations were exposed to the plasmid. Cell viability was determined 2 h after electroporation. pEGFP expression was evaluated by flow cytometry after 48 h. Data were presented as mean ± SD, n = 2. Data were analyzed using a two-way ANOVA followed by Sidak's test. $^{***}P < 0.001$ demonstrated a significant difference compared with the respective sample. Data were analyzed using two-way ANOVA followed by Tukey's post-test. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ demonstrated significant differences compared with the respective negative control; $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$ versus the respective untreated sample. GFP, Green fluorescent protein; EGFP, enhanced green fluorescent protein.

Synergistic effects of urea and electroporation on transfection efficiency and cell viability at voltage 120 V

The cell viability of test samples including untreated and treated with electroporation urea buffers containing 0.25%, 0.5%, and 1% W/V was compared with the negative control samples. At voltage 120 V, increasing urea concentrations showed a significant loss in cell viability (69.95 ± 0.35%, 66.10 ± 0.3%, and 44.9 ± 2.1% for 0.25%, 0.5%, and 1% W/V of urea, respectively) compared to negative control (77.0%) (Fig. 2A). Also, at the concentration of 1%, the cell viability reached below 50% which could not be detected by flow cytometry, which may be due to the weakness of the cell membrane in this sample. The images taken by fluorescence microscopy and the results from flow cytometry analysis were provided in Fig. S5 and Fig. S6, respectively. Similar to voltage 100 V, pEGFP transfection efficiency in voltage 120 V was compared to the untreated samples. According to the flow cytometry results, the concentration elevation of urea showed an inverse relationship with GFP transfection. The addition of urea resulted in a significant decrease in transfection efficiency (from 18.75 ± 0.15% in untreated cells to 16.45 ± 0.5% and 8.12 ± 0.29% in the concentrations of 0.25% and 0.5%, W/V, respectively) (Fig. 2B). Transfection efficiency and cell viability of the 2 voltages of 100 and 120 V were also compared in Fig. 2, showing a higher survival reduction at the higher voltage. According to our results, the voltage of 120 V and 1% urea concentration induced a more destructive effect on the electroporated cells than the voltage of 100 V and similar urea concentration.

DISCUSSION

Efficient gene delivery into a living cell, such as electroporation, is a key step in gene modification and cell biology studies. In recent decades, physical transfection methods have emerged as an alternative to viral and chemical vectors in several situations to overcome their disadvantages (5). On the other hand,

electroporation is associated with limitations such as high cell mortality and low efficiency. In this state, chemical enhancers play an important role in overcoming weaknesses (15). However, in many cases, electroporation is preferable to other selective carriers due to its rapidity and low toxicity, no restriction on carrying large genes, and wide range of applications in a diversity of cell lines (3). Accordingly, researchers have studied the effects of in-house buffers as a chemical penetration enhancer and electroporation to improve its efficiency (16). Over the years, commercial nucleofection systems have been developed based on a combination of an optimized square-wave electrical pulse and cell-type-specific buffer kits. As Iversen *et al*. have reported using electroporation *via* the nucleofector system for delivering reporter plasmids to human smooth muscle cells and endothelial cells could be 10-20 folds more efficient than a chemical transfection method (17). However, the high cost of commercial buffer kits may restrict extensive and large-scale experiments. In a recent study on T lymphocytes in humans and mice, Chicaybam *et al*. showed that using low-cost in-house buffers with the nucleofector device could also produce genetically modified lymphocytes with high efficiency as well as proper cell survival (18). In another study aimed at increasing the electroporation efficiency, in human chronic leukemia K562 cells, Zu *et al*. used gold nanoparticles in different sizes and concentrations in an electroporation buffer to increase the pEGFP transfection efficiency while preserving cell survival (19). In all of the mentioned studies, based on the type of cell line and the chemical penetration enhancer used, the ultimate goal was to increase gene delivery into the cell while maintaining adequate cell viability. In the same way, in our current study, the principals were evaluated.

Urea is known as a non-expensive and accessible chemical compound. Saffari *et al*. indicated the effectiveness of urea in increasing the fluidity and permeability of cell membranes in human lung adenocarcinoma A549 cells. They showed that urea could enhance the delivery of cationic liposomes containing antisense oligonucleotides (10). The current results were in agreement with Saffari's study. The present findings suggested an increase in the permeability of HEK-293 cells in the presence of urea, which might be due to fluidizing the cell membrane and extending the life of the pores created by electroporation. Over the past 2 decades, some authors have examined and evaluated the synergy between electroporation and pre- or co-treatment with chemical penetration enhancers (8,9). Sen *et al* investigated the combined effect of lipids and electroporation and suggested that, as opposed to liposomes, utilizing lipid dispersions in conjunction with skin electroporation is a more straightforward and economical technique. Particular anionic lipids such as dioleoylphosphatidylglycerol could improve the transport of fluorescent molecules such as protoporphyrin IX through the epidermis. The observed enhanced effectiveness implied that incorporating exogenous lipids into the skin during electroporation could result in more pores, larger pores, or longer-lasting pores (9). Moreover, the combined effects of electroporation and electrolytes on the penetration of calcein through hairless rat skin were studied, and found that adding either $CaCl_2$ or $MgCl_2$ considerably increased the calcein permeation through the skin. There were 83.3- and 54.7-fold increases in penetration as compared to electroporation without these electrolytes. The findings demonstrated that the pores in the skin formed during electroporation in the presence of electrolytes stay open for a long time, maybe several hours (8).

Previous studies have indicated that increasing the electroporation voltage unless it causes further cell destruction, is directly related to DNA delivery into the cell and inversely related to cell survival (6,20). In this regard, our results showed that cell viability at the voltage of 120 V at all concentrations of urea was lower than at the voltage of 100 V. Interestingly, the results demonstrated that treatment of cells with a high concentration of urea (1%) led to a further decrease in cell survival at a voltage of 120 V than at a voltage of 100 V. A comparison of the effects of urea on the pEGFP entry into cells at the voltages

of 100 and 120 V showed that although increasing the voltage without the presence of urea could increase the percentage of gene transfer (from the voltage of 100 V to 120 V with the values of 12.3 ± 0.2% and 18.75 ± 0.15%, respectively), it led to a significant decrease in cell survival (from the voltage of 100 V to 120 V with the values of 94.2 ± 0.2% and 73.25 ± 0.3%, respectively). Therefore, to reach more efficiency in the electroporation process, it was suitable to add urea in a concentration of 0.5% to the cells at a voltage of 100 V (with a transfection amount of 23.3 ± 0.3%) instead of increasing the voltage. In this way, due to the increased pEGFP transfection, higher cell viability was also achieved (over 90%).

Since this study assessed the effect of voltage and urea concentration on transfection efficiency, other electroporation parameters such as pulse type, the number of pulses, and pulse duration could be considered for further analysis. Ultimately, considering the urea concentration and voltage electroporation device settings, urea was suggested as a suitable option for use in the HEK-293 cell line.

CONCLUSION

For the first time, the current study used urea to increase the efficiency of the electroporation process. The findings showed that the addition of different concentrations of urea to HEK-293 cells at the voltage of 100 V could increase the number of cells transfected by pEGFP-N1, and the concentration of 0.5% urea had the highest transfection efficiency (approximately 2-fold increase compared to untreated cells) as well as high cell viability (over 90%). On the other hand, adding 3 concentrations of urea at the voltage of 120 V decreased transfection efficiency. Urea at both studied voltages reduced the cell survival percentage, which was inversely related to the urea concentration. At 1% urea concentration and the voltage of 120 V, the significant reduction in cell viability revealed the destructive effect of synergism between high voltage and a high concentration of urea on the cell membrane. Nevertheless, the results proposed urea as a proper chemical penetration enhancer to increase electroporation efficiency in the HEK-293 cell line.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

M. Mowla wrote the main manuscript text and prepared figures; A. Hashemi and H.R. Moghimi made substantial contributions to conception and design, organized, and supervised the whole project; M. Mowla and G. Gorji-Bahri carried out the experiments and analyzed the data; A. Hashemi, H.R. Moghimi, and G. Gorji-Bahri reviewed and edited the manuscript for spelling, grammar, and intellectual content; A. Hashemi provided the facilities and materials required for the project. All authors read and approved the finalized article.

Supplementary materials

The supplementary materials for this article can be found online at: https://github.com/FatemehEshraghiJazi/SUP.

REFERENCES

1. Kim TK, Eberwine JH. Mammalian cell transfection: the present and the future. Anal Bioanal Chem. 2010;397(8):3173-3178. DOI: 10.1007/s00216-010-3821-6.
2. Rahimmanesh I, Totonchi M, Khanahmad H. The challenging nature of primary T lymphocytes for transfection: effect of protamine sulfate on the transfection efficiency of chemical transfection reagents. Res Pharm Sci. 2020;15(5):437-446. DOI: 10.4103/1735-5362.297846.
3. Lambricht L, Lopes A, Kos S, Sersa G, Préat V, Vandermeulen G. Clinical potential of electroporation for gene therapy and DNA vaccine delivery. Expert Opin Drug Deliv. 2016;13(2): 295-310. DOI: 10.1517/17425247.2016.1121990.
4. Rezaei N, Dormiani K, Ghaedi K, Khazaie Y, Lachinani L, Foruzanfar M, *et al*. Construction and efficiency evaluation of replicating nonviral

minicircle for gene therapy. Res Pharm Sci. 2012;7(5):S425.
5. Alsaggar M, Liu D. Physical methods for gene transfer. Adv Genet. 2015;89:1-24. DOI: 10.1016/bs.adgen.2014.10.001.
6. Zhang Z, Qiu S, Zhang X, Chen W. Optimized DNA electroporation for primary human T cell engineering. BMC Biotechnol. 2018;18(1):1-9. DOI: 10.1186/s12896-018-0419-0.
7. Igawa K, Ohara N, Kawakubo A, Sugimoto K, Yanagiguchi K, Ikeda T, *et al*. D-glucosamine promotes transfection efficiency during electroporation. Biomed Res Int. 2014; 2014:1-4. DOI: 10.1155/2014/485867.
8. Tokudome Y, Sugibayashi K. The synergic effects of various electrolytes and electroporation on the *in vitro* skin permeation of calcein. J Control Release. 2003;92(1-2):93-101. DOI: 10.1016/S0168-3659(03)00307-9.
9. Sen A, Zhao Y, Zhang L, Hui SW. Enhanced transdermal transport by electroporation using anionic lipids. J Control Release. 2002;82(2-3):399-405. DOI: 10.1016/S0168-3659(02)00164-5.
10. Saffari M, Tamaddon AM, Hoseini Shirazi F, Oghabian MA, Moghimi HR. Improving cellular uptake and *in vivo* tumor suppression efficacy of liposomal oligonucleotides by urea as a chemical penetration enhancer. J Gene Med. 2013;15(1):12-19. DOI: 10.1002/jgm.2688.
11. Goodman BE. Transport of small molecules across cell membranes: water channels and urea transporters. Adv Physiol Educ. 2002;26(1-4): 146-157. DOI: 10.1152/advan.00027.2002.
12. Barton KN, Buhr MM, Ballantyne JS. Effects of urea and trimethylamine N-oxide on fluidity of liposomes and membranes of an elasmobranch. Am J Physiol. 1999;276(2):R397-R406. DOI: 10.1152/ajpregu.1999.276.2.R397.
13. Mukerjee P, Ray A. The effect of urea on micelle formation and hydrophobic bonding. J Phys Chem. 1963;67(1):190-192. DOI: 10.1021/j100795a046.
14. Yancey PH, Somero GN. Counteraction of urea destabilization of protein structure by methylamine osmoregulatory compounds of elasmobranch fishes. Biochem J. 1979;183(2):317-323. DOI: 10.1042/bj1830317.
15. Alamuru-Yellapragada NP, Kapadia B, Parsa KVL. In-house made nucleofection buffer for efficient and cost effective transfection of RAW 264.7 macrophages. Biochem Biophys Res Commun. 2017;487(2):247-254. DOI: 10.1016/j.bbrc.2017.04.043.
16. Chicaybam L, Barcelos C, Peixoto B, Carneiro M, Limia CG, Redondo P, *et al*. An efficient electroporation protocol for the genetic modification of mammalian cells. Front Bioeng Biotechnol. 2017;4:99,1-13. DOI: 10.3389/fbioe.2016.00099.
17. Iversen N, Birkenes B, Torsdalen K, Djurovic S. Electroporation by nucleofector is the best nonviral transfection technique in human endothelial and smooth muscle cells. Genet Vaccines Ther. 2005;3(1):2,1-13. DOI: 10.1186/1479-0556-3-2.
18. Chicaybam L, Sodre AL, Curzio BA, Bonamino MH. An efficient low cost method for gene transfer to T lymphocytes. PloS One. 2013;8(3):e60298,1-11. DOI: 10.1371/journal.pone.0060298.
19. Zu Y, Huang S, Liao WC, Lu Y, Wang S. Gold nanoparticles enhanced electroporation for mammalian cell transfection. J Biomed Nanotechnol. 2014;10(6):982-992. DOI: 10.1166/jbn.2014.1797.
20. Hyder I, Eghbalsaied S, Kues WA. Systematic optimization of square-wave electroporation conditions for bovine primary fibroblasts. BMC Mol Cell Biol. 2020;21(1):9,1-8. DOI: 10.1186/s12860-020-00254-5.