Which one of the thermal approaches (heating DNA or cells) enhances the gene expression in mammalian cells?

Alireza Milani · Azam Bolhassani · Fatemeh Rouhollah · Maryam Naseroleslami

Abstract

Objectives Heat treatment as a physical method could increase the cellular uptake of nucleic acids. In this study, the effects of heat shock were evaluated to enhance the transfection efficiency of three plasmid DNAs into HeLa and TC-1 cancerous, and HEK-293 T and Vero non-cancerous cell lines using lipofectamine 2000 reagent.

Methods Two methods of cell- and DNA-based heat treatment were used. Heating DNA solution was performed at 94 °C for 5, 10 and 15 min, and also 72 °C for 30, 60 and 120 min, individually. Moreover, heating the cells was done by incubation at 42 °C for 2 h in different times such as before, during and after DNA transfection.

Results Our data showed that the conformation of plasmid DNAs was changed at different temperatures with increasing time. The heat-treated plasmid DNAs (94 °C for 10 min or 72 °C for 30 min) indicated higher transfection efficiency than untreated plasmid DNAs ($p < 0.05$). Furthermore, heat treatment of cells before and during the transfection was higher than untreated cells ($p < 0.01$). Our results demonstrated that DNA transfection efficiency in cancerous cells was less than non-cancerous cells ($p < 0.01$).

Conclusion Generally, these findings showed that transfection mediated by thermal stimulation could enhance gene transfection in mammalian cell lines.

Keywords Transfection · Gene delivery · Heat treatment · HIV-1 · Nef · Heat shock protein

A. Milani · F. Rouhollah (✉) · M. Naseroleslami
Department of Cellular and Molecular Biology, Faculty of Advanced Sciences and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran
e-mail: panirohollah@yahoo.com

A. Bolhassani (✉)
Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran
e-mail: azam.bolhassani@yahoo.com; A_bolhasani@pasteur.ac.ir

Introduction

Transfection is a form of gene delivery into mammalian cells (Sheikh et al. 2017) for evaluating the function of different DNA sequences, gene therapy, and also DNA vaccine (Liu et al. 2004). As known, the cellular uptake of naked DNA is very low in vitro/in vivo (Dupuis et al. 2000); thus, many techniques were used to solve this problem including chemical, physical, and biological methods (Seow and Wood 2009). Biological methods (*e.g.* virus-mediated method) have high efficiency, but they may not be safe. Chemical methods (*e.g.* calcium phosphate, and cationic polymers/ lipids/ amino acids) are less cytotoxic than biological methods (dependent on dose), but they may not induce specific immunity

(Du et al. 2018). Physical methods (*e.g.* electroporation, microinjection and gene gun) are used due to their minimal side effects, but they are limited in clinical applications (Shinde 2020). Recently, the so-called 'physical–chemical' manipulations could improve the efficiency of gene delivery into the cell and also the nucleus (Mehier-Humbert and Guy 2005). For example, heat treatment as a physical approach increased the total uptake of exogenous nucleic acids (DNA or RNA) into the cell. This approach was mainly focused on direct induction of heat in plasmid DNA or cell. DNA heating could change the DNA structure by separation of the double strands, and then breaking the covalent bonds of DNA single strand. These DNA modifications may influence the transfection efficiency (Hou et al. 2008). Previous in vitro experiments showed that heat treatment of the cells increased lipid-mediated DNA transfection efficiency (Pipes et al. 2005; Takizaki et al. 2017) through caveolar endocytosis, and subsequently escaping from lysosomal digestion (Takizaki et al. 2017).

The most important limitation of human immunodeficiency virus (HIV-1) DNA vaccines is their low permeability and poor immunogenicity. Among different HIV-1 proteins, Nef is an early-expressed protein which plays an important role in the down-regulation of CD4 and MHC class I as a virulence factor for acquired immunodeficiency syndrome (AIDS) pathogenesis (Kaw 2020). Thus, Nef antigen can be considered as a possible attractive target in HIV-1 therapeutic vaccine development. Moreover, co-delivery of antigen with molecular adjuvants such as heat shock proteins (HSPs) is an important approach to improve the immunogenicity of DNA-based vaccines. Our previous study showed that heat shock protein 27 (Hsp27) as a molecular adjuvant could enhance Nef antigen-specific immunity in HIV-1 vaccine development (Milani et al. 2017a).

In this study, the effects of heat shock were evaluated to enhance the transfection efficiency of three plasmid DNAs (pEGFP-N1, pEGFP-Nef and pEGFP-Hsp27-Nef) into HeLa and TC-1 cancerous, and HEK-293 T and Vero non-cancerous cell lines. These data can help us for increasing gene delivery in HIV-1 DNA-based or cell-based vaccine development.

Materials and methods

Preparation of the recombinant plasmids

The pEGFP-N1 vector encoding the enhanced green fluorescent protein (EGFP) was used to monitor the transfection efficiency. The pEGFP-N1 vectors harboring the full length HIV-1 *nef* gene (from HIV-1 vector pNL4-3, Accession No: AF324493.2), and heat shock protein 27 (Accession No: NM_013560) (*hsp27*)-*nef* fusion gene were prepared in our previous study (Milani et al. 2017b). Briefly, HIV-1 *nef* gene was subcloned from pUC19-Nef into the pEGFP-N1 in *Nhe*I/*Pst*I restriction sites. To make pEGFP-Hsp27-Nef, the *hsp27-nef* fusion gene was subcloned from pQE30-Hsp27-Nef into the pEGFP-N1 in *Nhe*I/*Pst*I restriction sites. Herein, the recombinant plasmids (*i.e.* pEGFP-Nef and pEGFP-Hsp27-Nef) were purified by EndoFree plasmid Mega kit (Qiagen) according to manufacturer's instructions. The purity and concentration of the recombinant plasmids were determined by NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific).

Cell culture

HEK-293 T (Human embryonic kidney epithelial cells), Vero (African green monkey kidney epithelial cells), HeLa (Human cervical epithelial tumor cells), and TC-1 (Mouse lung epithelial tumor cells) cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), supplemented with 10% fetal bovine serum (FBS, Gibco), and pen/strep (100U/ml penicillin and 0.1 mg Streptomycin, Gibco). The cells (5×10^4 cells/well) were seeded into 24-well plates 24 h prior to transfection, and incubated under standard cell culture conditions (37 °C, 5% CO_2, humidified air) to achieve approximately 80% confluence.

Transfection

At first, two methods of heat-treated cells and heat-treated DNA were applied under different time and temperature conditions for enhancement of transfection efficiency in HEK-293 T cells. Then, the optimum time and temperature were selected to transfect the Vero, HeLa and TC-1 cell lines.

Transfection using the heat-treated plasmid DNA

Heat-treated plasmid DNAs such as pEGFP-N1, pEGFP-Nef and pEGFP-Hsp27-Nef were prepared by heating DNA solution at 94 °C for 5, 10 and 15 min, and 72 °C for 30, 60 and 120 min, respectively. The heat-treated plasmids were analyzed using electrophoresis on 1% agarose gel, and visualized on a UV *transilluminator*. Untreated DNA was considered as a negative control. For transfection, heat-treated plasmid DNA was immediately quenched on ice. Lipofectamine^{TM} 2000 (Invitrogen, USA) was used for transfection of cell lines by DNA vectors based on the manufacturer's protocols. For production of the lipofectamine-plasmid DNA complex, 50 μl of serum-free medium was mixed with 2 μl of lipofectamine and incubated for 5 min in room temperature. Then, 50 μl incomplete DMEM was mixed with 1 μg of heat-treated plasmid DNAs, added to lipofectamine solution, and incubated for 30 min in room temperature to form the DNA-lipofectamine complexes. After that, the complexes were added to each well, and the medium was replaced after 4 h of incubation at 37 °C with pre-warmed DMEM, 10% FBS and 1/100 penicillin/ streptomycin. Finally, the transfected cells were incubated for 48 h at 37 °C, and transient transfection efficiency of DNA was assessed by fluorescent microscopy and flow cytometry.

Transfection using heat-treated cells

For this purpose, cell plate was covered with parafilm and fully incubated in a water bath at 42 °C for 2 h before, during and after transfection. The cellular transfection was performed for 4 h by adding 1 μg plasmid DNA complexed with lipofectamine according to transfection protocol (as mentioned in above). After that, the transfection medium was replaced with the pre-warmed DMEM *supplemented* with 10% FBS. The controls were handled similarly, but without any heat treatment. Finally, the transfected cells were incubated for 48 h at 37 °C, and DNA transfection was evaluated by fluorescent microscopy and flow cytometry. The schematic model of transfection using heat-treated cells was represented in Fig. 1.

Cell viability

The cytotoxic effects of heat treatment on the cells at 42 °C for 2 h were investigated using MTT assay (MTT assay protocol, Sigma-Aldrich).

Transfection assay

Fluorescent microscopy and flow cytometry techniques were used to detect the uptake of plasmid DNA into different cells. The expression levels of Nef-GFP, Hsp27-Nef-GFP, and GFP proteins were detected after 48 h transfection using fluorescent microscopy (Envert Fluorescent Ceti, Korea), and quantified by flow cytometry (Partec, Germany). For flow cytometry analysis, the cells were harvested by trypsin, and the cell pellets were resuspended in 1 ml PBS (pH = 7.4). The expression of fluorescent genes was measured in the FL1 channel using an excitation filter (485 nm) and emission filter (535 nm). The untransfected and transfected cells with pEGFP-N1 were used as the negative and positive controls, respectively. 10,000 cells were counted in each analysis.

Statistical Analysis

Prism 8.3 software (GraphPad, San Diego, California, USA) was used for statistical analysis. The differences between control and test groups were assessed using Student's *t*-test (GraphPad Prism, GraphPad Software), where $p < 0.05$ was considered statistically significant. Each test was performed in duplicate. The results were determined from two-independent experiments.

Results

Generation of the recombinant plasmids

The DNA constructs encoding *nef* and *hsp27-nef* genes were prepared by EndoFree plasmid kit with high purity. The presence of *nef* and *hsp27-nef* genes in pEGFP-N1 vector was confirmed by restriction enzyme digestion as clear bands of ∼ 648 bp and ∼ 1368 bp on agarose gel, respectively (Fig. 2). The $OD_{260/280}$ ratios of the recombinant plasmids were between 1.8 and 1.9 indicating their purity. The concentration of pEGFP-N1, pEGFP-Nef, and
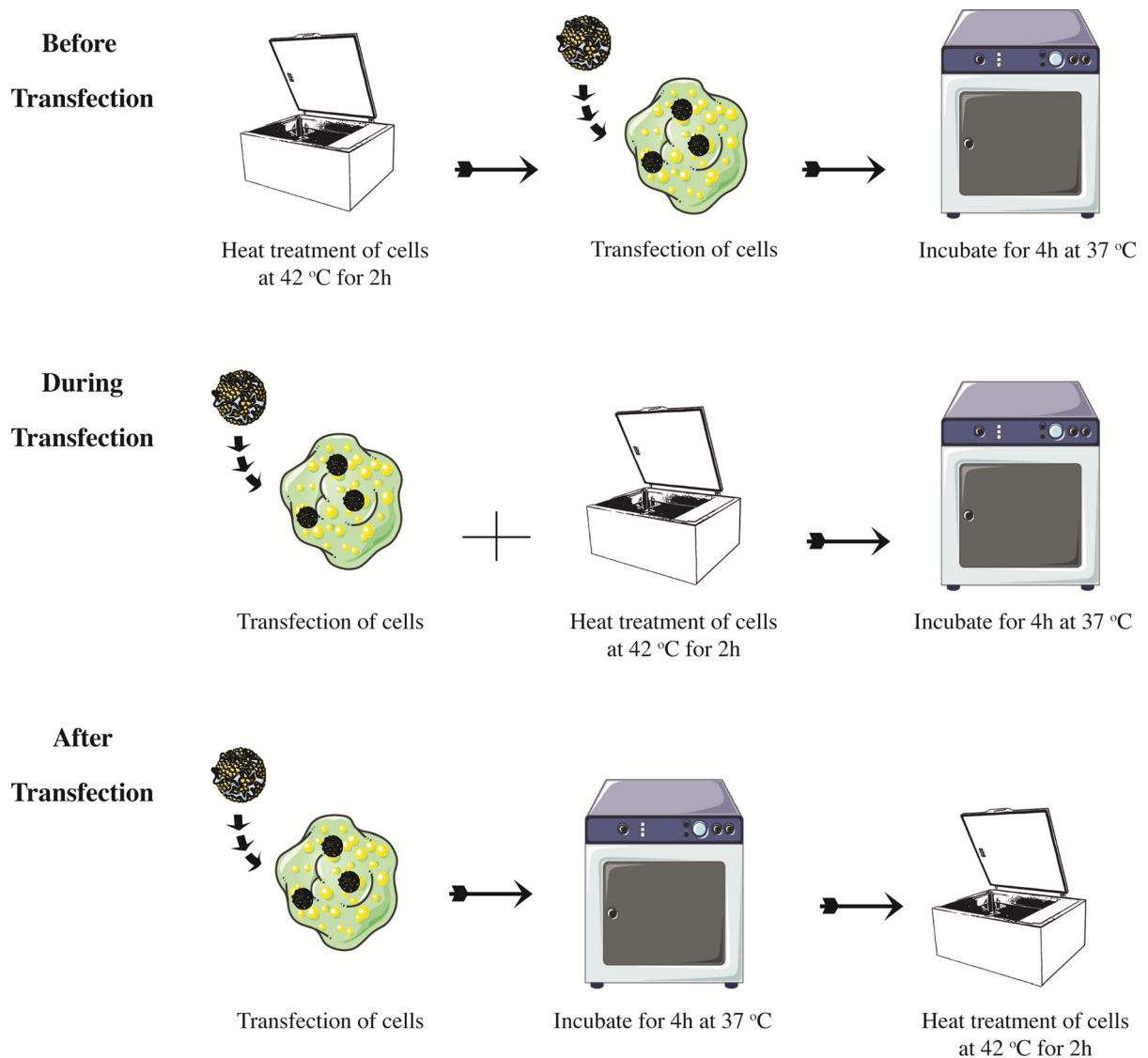
Fig. 1 Schematic representation of transfection using heat-treated cells: The cells were incubated at 42 °C for 2 h before, during and after transfection. The transfection process was done for 4 h by adding 1 µg of plasmid DNA complexed with Lipofectamine 2000 into the cells. After the transfection, the medium was replaced and the cells were incubated for 48 h at 37 °C

pEGFP-Hsp27-Nef constructs were 1420.2, 1380.4, and 1310.1 ng/ µl, respectively.

Monitoring the heat-denatured plasmid DNA

The plasmid DNA extracted from bacterial host consists of open circular DNA, linear DNA, and supercoiled DNA as detected in agarose gel electrophoresis due to their different mobilities (Travers et al., 2005). Herein, after denaturation of DNA at different temperatures and times, an extra band of DNA appeared. This band had higher mobility than supercoiled and open circular. This band was defined as the heat-denatured DNA component. Heat-denatured component appeared at 94 °C after 5 min and at 72 °C after 30 min, and this form gradually continued with increasing time (Fig. 3). It is notable that long-time heating process could break all the covalent bonds between double DNA strands. Thus, heat treatment of DNA at 94 °C for 5 & 10 min, and
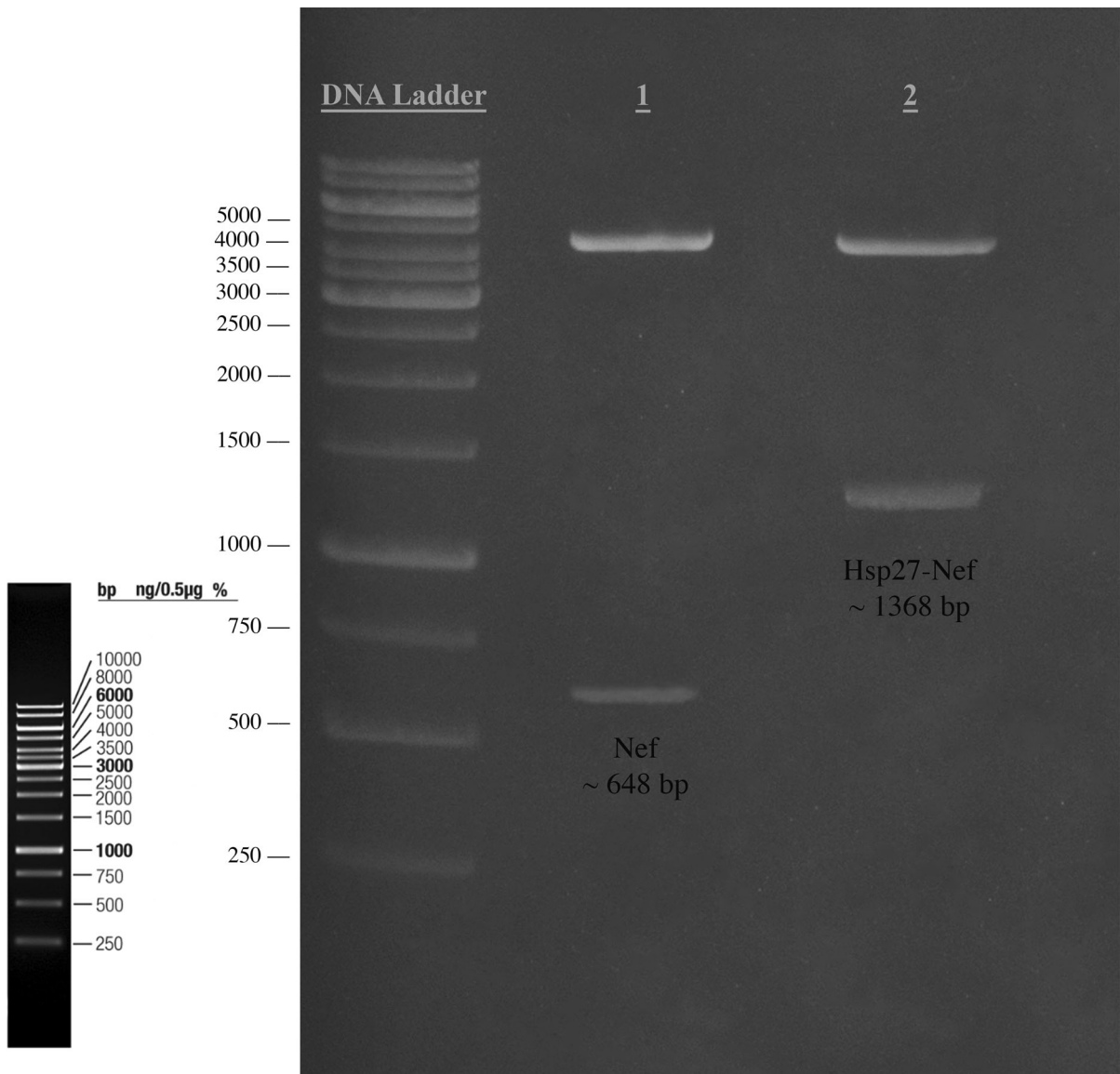
Fig. 2 Confirmation of the recombinant plasmids by double-digestion in gel electrophoresis: The Nef (Lane 1) and Hsp27-Nef (Lane 2) genes were confirmed by restriction enzyme digestion as clear bands of ~ 648 bp and ~ 1368 bp on agarose gel, respectively

72 °C for 30 & 60 min was used for transfection of cells.

Gene transfection experiments

Transfection efficiency of the heat-treated DNA in HEK-293 T

After heat treatment of the plasmid DNA at 94 °C for 5 & 10 min, and 72 °C for 30 & 60 min respectively, the

plasmid DNA was transfected using Lipofectamine^{TM} 2000. The DNA transfection efficiency was determined by the percentage of protein expression using GFP reporter. The transfection efficiency rates of pEGFP-Nef, pEGFP-Hsp27-Nef and pEGFP-N1 were 53.00 ± 0.40, 57.30 ± 0.21 and 69.01 ± 0.30 for treatment at 94 °C for 5 min, and 57.40 ± 0.51, 61.30 ± 0.70 and 74.71 ± 1.02 for treatment at 94 °C for 10 min, respectively. Moreover, the transfection efficiency rates of pEGFP-Nef, pEGFP-
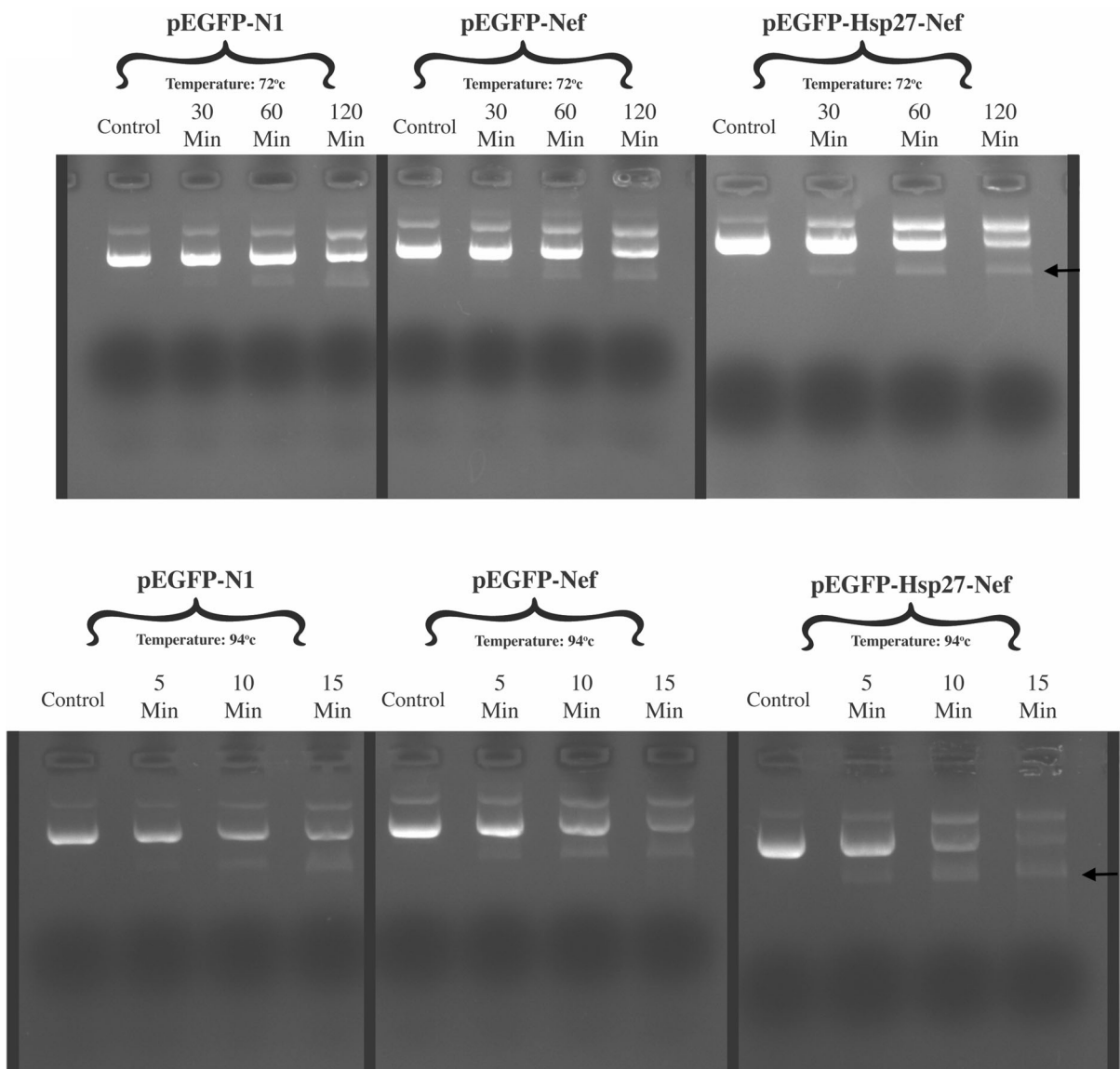
Fig. 3 Monitoring heat-denatured plasmid DNA on agarose gel: After denaturation of DNA at different temperatures and times, an extra band of DNA appeared (shown as arrow). Long-time heating process could break covalent bonds between double DNA strands

Hsp27-Nef and pEGFP-N1 were 60.00 ± 0.53, 67.08 ± 0.70 and 75.10 ± 0.22 for treatment at 72 °C for 30 min, and 58.21 ± 0.60, 65.03 ± 1.01 and 74.10 ± 0.33 for treatment at 72 °C for 60 min, respectively. Based on these results, the heat-treated plasmid DNA at 94 °C for 10 min showed higher transfection efficiency than the heat-treated plasmid DNA at 94 °C for 5 min ($p < 0.05$), and untreated control ($p < 0.01$). No significant difference was detected in the transfection efficiency of heat-treated DNA at 72 °C for 30 and 60 min ($p > 0.05$). Moreover, heat treatment of DNA at 94 °C for 10 min did not show any significant difference with heat treatment of DNA at 72 °C for 30 min ($p > 0.05$). Thus, heat treatment of DNA at 94 °C for 10 min and 72 °C for 30 min was selected to deliver DNA in other cell lines (Fig. 4A).
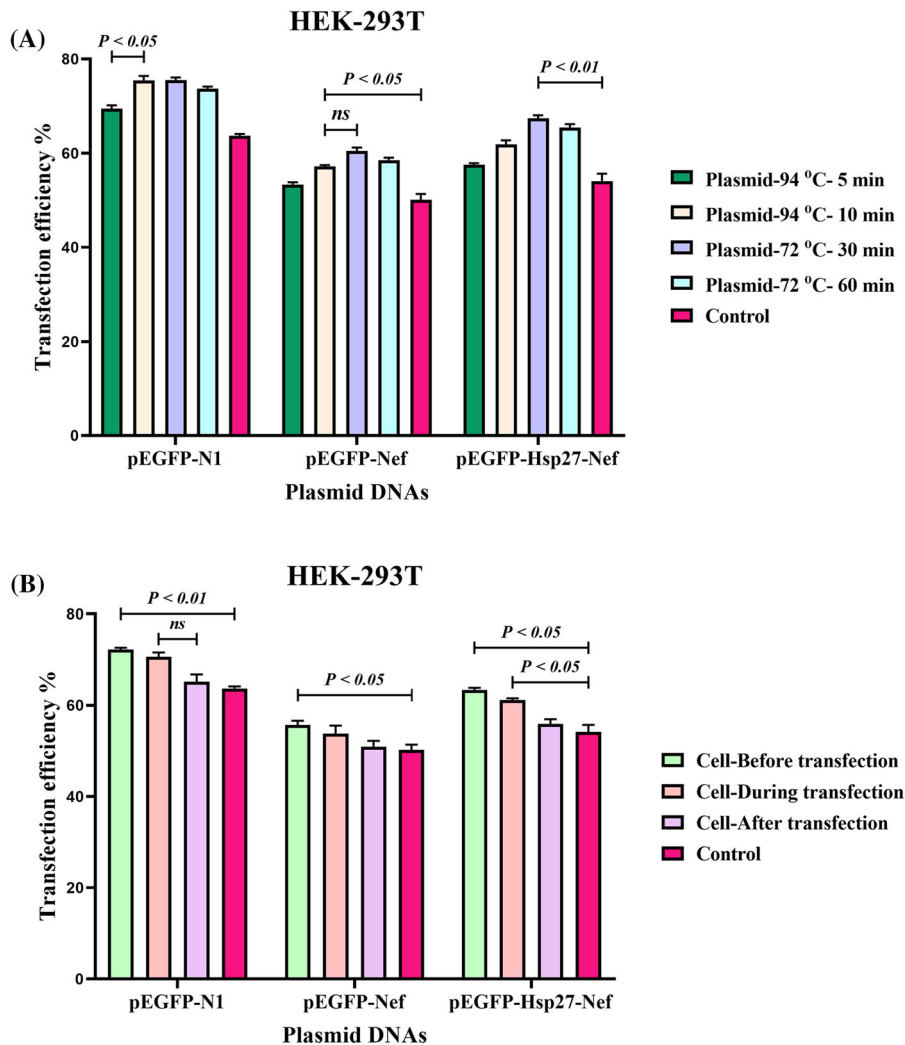
Fig. 4 Comparison of DNA transfection efficiency using thermal methods in HEK-293 T cell lines: **A** Heat-treated plasmid DNAs (94 °C for 10 min or 72 °C for 30 min) indicated higher transfection efficiency than untreated plasmid DNAs; **B** Heat treatment of the cells before transfection showed higher efficiency than untreated cells. The p values less than 0.05 were considered statistically significant; *ns* non-significant ($p > 0.05$)

Transfection efficiency of the heat-treated DNA in Vero, HeLa and TC-1 cell lines

Heat treatment of the plasmid DNA at 94 °C for 10 min and 72 °C for 30 min was selected for transfection of Vero, HeLa and TC-1 cell lines. The flow cytometry analysis of Vero cells showed that the transfection efficiency of pEGFP-Nef, pEGFP-Hsp27-Nef and pEGFP-N1 treated by heat at 94 °C for 10 min was 23.01 ± 0.33, 28.00 ± 0.81 and 40.40 ± 0.52, and at 72 °C for 30 min was 25.21 ± 0.50, 30.82 ± 1.03 and 44.04 ± 0.72,

respectively. These results indicated that heat treatment at 94 °C for 10 min showed no significant difference with heat treatment at 72 °C for 30 min, but it was higher than untreated control ($p < 0.01$). The transfection efficiency of pEGFP-Nef and pEGFP-Hsp27-Nef and pEGFP-N1 treated by heat at 94 °C for 10 min in HeLa cells was 10.52 ± 0.60, 12.71 ± 0.83 and 14.08 ± 0.21, and at 72 °C for 30 min was 23.50 ± 0.33, 24.81 ± 1.10 and 28.03 ± 1.03, respectively. Also, the transfection efficiency of pEGFP-Nef, pEGFP-Hsp27-Nef and pEGFP-N1 treated by heat at 94 °C for 10 min in TC-1 cells was

16.33 ± 0.50, 24.51 ± 0.90 and 24.82 ± 0.72, and at 72 °C for 30 min was 25.82 ± 0.60, 30.22 ± 0.31 and 33.70 ± 0.30, respectively. In comparison with non-cancerous cell lines (HEK-293 T and Vero cells), the transfection efficiency of heat-treated DNA at 72 °C for 30 min was higher than heat-treated DNA at 94 °C for 10 min in HeLa ($p < 0.01$) and TC-1 ($p < 0.05$) cancerous cell lines (Fig. 5).

Transfection efficiency of the heat-treated HEK-293 T cell

At first, the optimal heat shock time was determined to enhance the transfection efficiency of HEK-293 T cells. The cells were incubated at 42 °C for 2 h before, during and after transfection. The percentages of Nef-GFP, Hsp27-Nef-GFP and GFP expression in the heat-treated cells before transfection were 56.31 ± 0.90, 63.70 ± 0.92 and 72.51 ± 0.23, during transfection were 52.51 ± 0.72, 61.40 ± 0.51 and 70.02 ± 0.63, and after transfection were 50.00 ± 0.81, 56.60 ± 0.83 and 66.31 ± 0.62, respectively. Heat treatment of cells before and during transfection had

the same efficiency, and was slightly higher than heat treatment of cells after transfection. However, the transfection efficiency of the heat-treated cells in different conditions was significantly higher than untreated cells ($p < 0.05$). Thus, heat treatment of cells at 42 °C for 2 h before transfection was considered to deliver DNA in other cell lines (Fig. 4B).

Transfection efficiency of the heat-treated Vero, HeLa and TC-1 cell lines

Heat treatment of cells at 42 °C for 2 h before transfection was considered for DNA delivery in Vero, HeLa and TC-1 cell lines. Transfection of DNA constructs encoding Nef-GFP, Hsp27-Nef-GFP and GFP into the heat-treated Vero cells before transfection showed the expression rates of 24.01 ± 1.02, 28.12 ± 0.21 and 42.70 ± 0.52 in comparison with untreated cells. The percentages of Nef-GFP, Hsp27-Nef-GFP and GFP expression were 8.70 ± 0.31, 10.21 ± 0.82 and 11.00 ± 0.62 in HeLa cells, and 15.80 ± 1.10, 16.33 ± 0.42 and 20.50 ± 0.61 in TC-1 cells. These results showed that the transfection
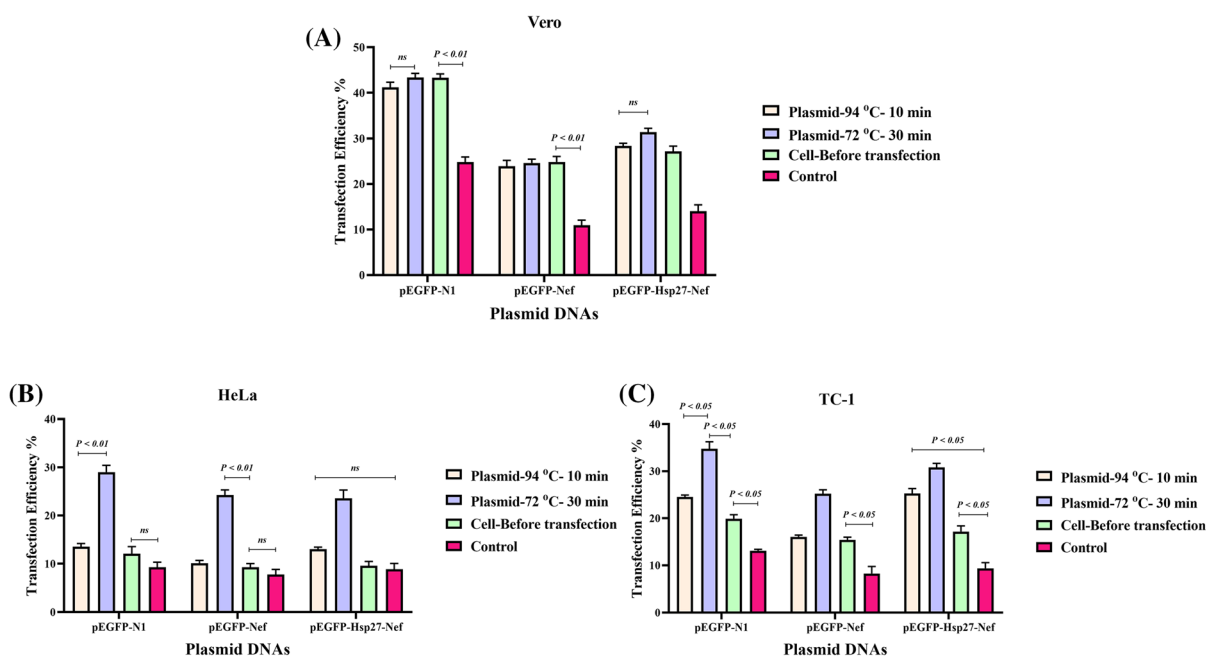

Fig. 5 Comparison of DNA transfection efficiency using thermal methods in Vero (**A**), HeLa (**B**) and TC-1 (**C**) cell lines: Heat-treated plasmid DNAs (94 °C for 10 min or 72 °C for 30 min) indicated higher transfection efficiency than untreated plasmid DNAs. Moreover, heat treatment of the cells

before transfection showed higher efficiency than untreated cells in Vero and TC-1 cell lines. However, DNA transfection efficiency in cancerous cells was less than non-cancerous cells. The p values less than 0.05 were considered statistically significant; *ns* non-significant ($p > 0.05$)

efficiency in cancerous cells was less than non-cancerous cells ($p < 0.05$). In comparison with Vero ($p < 0.01$) and TC-1 cells ($p < 0.05$), heat did not affect the transfection efficiency of HeLa cells. Generally, both methods (heat-treated DNA and heat-treated cells) used to enhance the transfection efficiency of non-cancerous cells showed no significant difference in various conditions ($p > 0.05$). In contrast, the transfection efficiency of heat-treated DNA at 72 °C for 30 min was higher than heat-treated cells for HeLa ($p < 0.01$) and TC-1 ($p < 0.05$) cancerous cells (Fig. 5).

Cell viability

MTT assay was performed to investigate the viability of heat-treated cells versus untreated cells (control). In all experiments, the cell viability rate was between 89 and 93% as compared to control (95–98%). Thus, there was no significant difference between the treated and untreated cells.

Discussion

The major goal of transfection is delivery of genetic material with negative charge into cells through chemical, physical or biological methods (Uddin 2007). There is an urgent *need* to find simple, cost-effective and potent delivery systems that would improve the transfection efficiency using much lower dose of plasmid DNA (Zhao et al. 2011). A number of new techniques have been recently developed to introduce foreign DNA into cells. One approach is non-viral delivery system (Ho et al. 2021). In this study, we focused on a commercial lipid reagent (lipofectamine) along with a physical method (heat shock under various conditions) for gene transfer in four mammalian cell lines with the same tissue origin (epithelial tissue). Several studies indicated the importance of heat shock proteins such as Hsp90, Hsp70 and Gp96 as an adjuvant for enhancement of humoral and cellular immune responses against infections (Corigliano et al. 2021; Dalimi and Nasiri 2020; Gupta et al. 2020). Our previous study showed that Hsp27 could be used as a vaccine adjuvant to enhance HIV-1 Nef antigen-specific immunity in DNA-based immunization (Milani et al. 2017a). Moreover, Hsp27 fused to HIV-1 Nef could increase the Nef expression in vitro (Milani et al. 2017b). Therefore, the transfection efficiency of HIV-1 *nef* and *hsp27-nef* gene constructs linked to *gfp* gene as well as *gfp* gene was studied under different thermal conditions in four mammalian cell lines. These findings can help us to improve therapeutic vaccines against HIV-1 infections.

Different parameters could influence transfection efficiency such as cell type and density, culture conditions, quality of nucleic acid, carrier type, and the ratio of nucleic acid/carrier (Sheikh et al. 2017). For example, HEK-293 T and HeLa cell lines transfected with an EGFP expression vector (pEGFP) using Lipofectamine transfection reagent, showed that the EGFP expression was $\sim$ 86.4% and $\sim$ 52%, respectively (Basirnejad et al. 2018; Reed et al. 2006). In another study, the transfection rates of HeLa cells with pCMV β-Gal using three cationic lipid reagents including FuGENE HD, Lipofectamine and X-treme-GENE were estimated approximately 43%, 31% and 4%, respectively (Asgharian et al. 2014).

Kong et al. reported that the uptake of DNA in cancer cells might be different from normal/non-tumor cells due to gene mutations and the ability of endocytosis in vivo (Kong et al. 2017). Moreover, transfection efficiency of human papillomavirus (HPV) E7 DNA construct using Lipofectamine and TurboFect in non-cancerous HEK-293 T cells (74.43% for Lipofectamine and 80.63% for TurboFect) was significantly higher than cancerous A549 (16.36% for Lipofectamine and 5.95% for TurboFect) and TC-1 cells (8.11% for Lipofectamine and 4.47% for TurboFect) in vitro (Shahbazi et al. 2018). Therefore, transfection efficiency of the DNA/reagent complexes is dependent on the cell type. Chitosan, a cationic polymer, has high potential for DNA delivery into mammalian cells. A previous study indicated that the transfection efficiency of chitosan/DNA microparticles was normally lower than that of lipid/DNA complexes. Moreover, the uptake of chitosan-pSVβ-Gal microparticles in HEK-293 cells was more effective than in HeLa and mouse fibroblastic 3T3 cell lines indicating that transfection efficiency of the DNA/reagent complexes is dependent on the cell type (Dastan and Turan 2004). Our study also indicated that the transfection efficiency of plasmid DNA using Lipofectamine 2000 into the non-cancerous cell lines (HEK-293 T and Vero) was effectively higher than the cancerous cell lines (HeLa and TC-1; $p < 0.01$). The

reports showed that different cell lines may internalize a polyplex through various endocytic pathways leading to a variety of transfection rates (Rejman et al. 2005; Rejman et al. 2005; Von et al. 2006).

A variety of different cell lines were used for transient gene expression. The HEK-293 established by Graham in 1977 (Graham 1987) is a common cell line for evaluation of transfection efficiency. This cell line is usually preferred due to high transfection efficiency and protein expression rate compared to several cell lines such as chinese hamster ovary (*CHO*) cell line. The rates of transient pMAX vector encoding *gfp* gene in adherent cell lines such as Vero and HEK-293 cells were 8.3% and 45%, respectively (Fliedl and Kaisermayer 2011). Our study showed that the transfection rate of pEGFP-N1 in HEK-293 T cells ($\sim$ 64%) was significantly higher than that in Vero cells ($\sim$ 24%).

Heat treatment could alter the structure of the cell membrane in various cell types. For example, amino-acid uptake was impaired in human T-cell line (Kwock et al. 1978), and in rat thymocytes (Lin et al. 1978). Moreover, heat treatment suppressed the facilitated diffusion of thymidine (Slusser et al. 1982) and enhanced the permeability of oligoamines (Gerner et al. 1980) in CHO cells. Heat-induced changes in the membrane potential were determined in normal and transformed hamster lymphocytes. Incubation for 1–2 h at temperatures between 38 °C and 42 °C resulted in a depolarization of normal cells and a hyperpolarization of SV40-transformed cells (Mik-kelsen and Koch 1982). Giovanella et al. showed that malignant cells (*e.g.* transformed 3T6 cells) were more thermosensitive than normal cells (*e.g.* untransformed 3T3 cells) leading to cell membrane permeabilization (likely due to an increased intracellular Ca^{+2} concentration), and finally cell death (Giovanella et al. 1979; Hayat and Friedberg 1986). These differences between the transformed and non-transformed cells might be due to differences in the cell membrane composition and topology (Hayat et al. 1986). In 2017, Tkizaki et al. reported that gene transfection could be enhanced by heat treatment. Moreover, heat treatment before transfection showed a higher increase in gene delivery than during or after transfection. Indeed, heat treatment increased the total uptake of plasmid DNA due to enhancing caveolar endocytosis and subsequently escaping from lysosomal digestion (Takizaki et al. 2017). In another study, human lung carcinoma

cell line (A549), human colon carcinoma cell line (SW480), human breast carcinoma cell line (MCF-7), murine melanoma cell line (B-16) and murine mammary tumor cell line (4T1) were treated for 10 min at 42 °C immediately following lipid DMRIE-C transfection. Transient transfection efficiency was increased by heat treatment in A549, SW480 and MCF-7 cells. Heat shock likely influences the cells through an increase in the number of cells that uptake the plasmid (*i.e.* change in fluidity of the cell membrane), and/ or an increased stable integration rate (*i.e.* change in fluidity of the nuclear membrane or in chromatin structure) (Pipes et al. 2005). In our study, heat treatment of the cells at 42 °C for 2 h before transfection could increase transfection efficiency and gene expression in both non-cancerous ($p < 0.01$) and cancerous ($p < 0.05$) cell lines. It may be likely related to the induction of heat shock protein 56 (Hsp56), cholesterol uptake and finally an enhanced caveolar endocytosis (Takizaki et al. 2017). Also, the effects of heat treatment on DNA structures and its bioactivities were studied by Hou et al. The heat-treated pEGFP-C2 vector at 94 °C for 3, 9, 15, 21 and 27 min showed that the covalent bonds of DNA strands were broken by long-time heating process. The time of 4 min was selected to balance between the denatured DNA and the destroyed DNA for evaluation of the transfection efficiency of heat-treated DNA. It was observed that the transfection efficiency of DNA in HEK-293 T was improved about 10% after heat treatment (Hou et al. 2008). Our data also showed that heat-denatured plasmid DNA appeared at 94 °C after 5 min and at 72 °C after 30 min, and this rate gradually continued with increasing time. Our results showed that the transfection rate of heat-treated DNA at 72 °C for 30 min was significantly higher than that at 94 °C for 10 min in cancerous HeLa ($p < 0.01$) and TC-1 ($p < 0.05$) cell lines. Heat treatment of plasmid DNA at 72 °C for 30 min, and heat treatment of cells before transfection could enhance the transfection efficiency in comparison with untreated control in HEK-293 T, Vero and TC-1 cell lines.

There is a correlation between the cell type and transcription machinery that influences transfection efficiency. Mammalian expression vectors containing the simian virus 40 (SV40) enhancer regions promote the constitutive DNA replication in most mammalian cells leading to higher levels of expression. However,

the ability of cell lines is different for promotion of SV40 T antigen transactivation. The efficiency of transactivation is strongly related to the level of replicated DNA in the cells. In this regard, the potency of the SV40 enhancer region in HEK-293 and HeLa cell lines showed that the ability of HEK-293 cells to support SV40 T antigen was approximately three- to four-fold higher than HeLa cells. Moreover, DNA replication in HEK-293 cells was more effective than that in HeLa cells (Kelly et al. 1989). The delivery of plasmids harboring genes under the control of CMV promoter (*e.g.* pEGFP, pcDNA) could effectively force the translation of gene products in HEK cells and other transformed cell lines (*e.g.* COS & CHO). Thus, the difference between cells is due to the cellular environment (Thomas and Smart 2005). Herein, we used pEGFP vector as an eukaryotic expression vector and HEK-293 T cell as a cellular vehicle. Our data confirmed the best gene expression in HEK-293 T cell as compared to Vero, HeLa and TC-1 cells. Lipoplexes (Cationic lipid-DNA complexes) are normally formed by direct mixing between cationic liposomes and plasmid DNA (Khalil et al. 2006; Felgner et al. 1995; Rejman et al. 2005). Herein, we used lipofectamine 2000 reagent for lipoplex formation, as well. However, different uptake approaches (direct penetration or clathrin- and caveolae-mediated endocytosis) influence transfection efficiency and gene expression in various cell lines (El-Sayed et al. 2013; Khalil et al. 2006; Billiet et al. 2012; El-Sayed and Harashima 2013).

The enhancement of DNA transfection in the cells using different approaches can be used in cell-based vaccines such as dendritic cell-based or tumor cell-based vaccines (Calmeiro et al. 2020; Copier et al. 2006). The cells expressing the gene of interest in vitro can be injected to increase immune responses in preclinical and clinical trials. In addition, heat-treated plasmids can be applied as DNA-based vaccine in vivo.

In summary, effective non-viral transfection in cell-based biotechnology techniques such as drug screening, cell therapy and/ or vaccine development will likely require reagents and culture conditions. An increased gene transfection into mammalian cell lines was detected by heat-treated plasmids or cells. Heat-treated plasmids at 94 °C for 10 min and 72 °C for 30 min showed higher transfection efficiency than untreated control. Also, heat-treated cells at 42 °C for

2 h before transfection could increase transfection rate and gene expression in both non-cancerous and cancerous cell lines. In addition, the insert gene could affect the transfection efficiency. Some mechanisms were involved for thermal effects by different researches. Heat treatment/shock may change the fluidity of the cell membrane, intracellular processes (*e.g.* endosomal escape and delivery to the nucleus), and transcription and translation processes. However, further studies will be required to reveal the actual mechanism of heat shock in future.

Declarations

References

Asgharian A, Banan M, Najmabadi H (2014) Optimizing a lipocomplex-based gene transfer method into hela cell line. Cell J (yakhteh) 15(4):372

Basirnejad M, Bolhassani A, Sadat SM (2018) The distinct role of small heat shock protein 20 on HCV NS3 expression in HEK-293T cell line. Avicenna J Med Biotechnol 10(3):152

Billiet L et al (2012) Gene transfer by chemical vectors, and endocytosis routes of polyplexes, lipoplexes and lipopolyplexes in a myoblast cell line. Biomaterials 33(10):2980–2990

Calmeiro J et al (2020) Dendritic cell vaccines for cancer immunotherapy: the role of human conventional type 1 dendritic cells. Pharmaceutics 12:158

Copier J et al (2006) Overview of tumor cell-based vaccines. Int Rev Immunol 25(5–6):297–319

Corigliano MG et al (2021) Heat shock proteins 90 kDa: Immunomodulators and adjuvants in vaccine design against infectious diseases. Frontiers in Bioengineering and Biotechnology 8:1532

Dalimi A, Nasiri V (2020) Design, construction and immunogenicity assessment of pEGFP-N1-KMP11-GP96 (Fusion) as a DNA vaccine candidate against leishmania major infection in BALB/c mice. Iran J Parasitol 15(1):11

Dastan T, Turan K (2004) *In vitro* characterization and delivery of chitosan-DNA microparticles into mammalian cells. J Pharm Pharm Sci 7(2):205–214

Du X et al (2018) Advanced physical techniques for gene delivery based on membrane perforation. Drug Delivery 25(1):1516–1525

Dupuis M et al (2000) Distribution of DNA vaccines determines their immunogenicity after intramuscular injection in mice. J Immunol 165(5):2850–2858

El-Sayed A, Harashima H (2013) Endocytosis of gene delivery vectors: from clathrin-dependent to lipid raft-mediated endocytosis. Mol Ther 21(6):1118–1130

Felgner PL et al (1995) Improved cationic lipid formulations for *in vivo* gene therapy. Ann N Y Acad Sci 772(1):126–139

Fliedl L, Kaisermayer C (2011) Transient gene expression in HEK293 and vero cells immobilised on microcarriers. J Biotechnol 153(1–2):15–21

Gerner EW, Holmes DK, Stickney DG, Noterman JA, Fuller DJ (1980) Enhancement of hyperthermia-induced cytotoxicity by polyamines. Can Res 40(2):432–438

Giovanella BC, Stehlin JS, Shepard RC, Williams LJ (1979) Hyperthermic treatment of human tumors heterotransplanted in nude mice. Can Res 39(6):2236–2241

Graham F (1987) Growth of 293 cells in suspension culture. J Gen Virol 68(3):937–940

Gupta A, Narayan B, Kumar S, Verma SK (2020) Vaccine potential of a recombinant bivalent fusion protein LcrV-HSP70 against plague and yersiniosis. Front Immunol 11:988

Hayat H, Friedberg I (1986) Heat-induced alterations in cell membrane permeability and cell inactivation of transformed mouse fibroblasts. Int J Hyperth 2(4):369–378

Ho W et al (2021) Next-generation vaccines: Nanoparticle-mediated DNA and mRNA Delivery. Adv Healthcare Mater 10(8):2001812

Hou S, Yang K, Liu Z, Feng XZ (2008) A method to increase the bioactivity of plasmid DNA by heat treatment. J Biochem Biophys Methods 70(6):1066–1072

Kaw S et al (2020) HIV-1 infection of CD4 T cells impairs antigen-specific B cell function. EMBO J 39(24):e105594

Kelly JJ, Munholland JM, Wildeman AG (1989) Comeasurement of simian virus 40 early and late promoter activity in HeLa and 293 cells in the presence of T antigen. J Virol 63(1):383–391

Khalil IA, Kogure K, Akita H, Harashima H (2006) Uptake pathways and subsequent intracellular trafficking in non-viral gene delivery. Pharmacol Rev 58(1):32–45

Kong Y, Zhang X, Zhao Y, Xue Y, Zhang Y (2017) Uptake of DNA by cancer cells without a transfection reagent. Biological Research 50

Kwock L, Lin PS, Hefter K, Wallach DF (1978) Impairment of Na+-dependent amino acid transport in a cultured human T-cell line by hyperthermia and irradiation. Can Res 38(1):83–87

Lin PS, Kwock L, Hefter K, Wallach D (1978) Modification of rat thymocyte membrane properties by hyperthermia and ionizing radiation. Int J Radiat Biol Relat Stud Phys Chem Med 33(4):371–382

Liu D, Chia EF, Tian H (2004) Chemical methods for DNA delivery. Gene Delivery to Mammalian Cells 3–23

Mehier-Humbert S, Guy RH (2005) Physical methods for gene transfer: improving the kinetics of gene delivery into cells. Adv Drug Deliv Rev 57(5):733–753

Mikkelsen RB, Koch B (1982) Membrane potential thermosensitivity of normal and simian virus 40-transformed lymphocytes. In Third International Symposium, Cancer Therapy by Hyperthermia, Drugs, and Radiation: A Symposium Held at Colorado State University, Fort Collins,

Colorado; Sponsored by the National Cancer Institute 61 (82): 89–91

Milani A et al (2017a) Small heat shock protein 27: An effective adjuvant for enhancement of HIV-1 Nef antigen-specific immunity. Immunol Lett 191:16–22

Milani A, Bolhassani A, Heshmati M (2017b) Delivery of HIV-1 Nef linked to heat shock protein 27 using a cationic polymer is more effective than cationic lipid in mammalian cells. Bratisl Lek Listy 118(6):334–338

Pipes BL et al (2005) Brief heat shock increases stable integration of lipid-mediated DNA transfections. Biotechniques 38(1):48–52

Reed SE, Staley EM, Mayginnes JP, Pintel DJ, Tullis GE (2006) Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. J Virol Methods 138(1–2):85–98

Rejman J, Bragonzi A, Conese M (2005) Role of clathrin-and caveolae-mediated endocytosis in gene transfer mediated by lipo-and polyplexes. Mol Ther 12(3):468–474

Seow Y, Wood MJ (2009) Biological gene delivery vehicles: beyond viral vectors. Mol Ther 17(5):767–777

Shahbazi S, Haghighipour N, Soleymani S, Nadji SA, Bolhassani A (2018) Delivery of molecular cargoes in normal and cancer cell lines using non-viral delivery systems. Biotech Lett 40(6):923–931

Sheikh S, Coutts AS, La Thangue NB (2017) Transfection. Basic science methods for clinical researchers (pp. 191-209): Elsevier

Shinde P et al. (2020) Physical approaches for drug delivery: an overview. Delivery of Drugs 161–190

Slusser H, Hopwood LE, Kapiszewska M (1982) Inhibition of Membrane Transport by Hyperthermia 1, 2. In Third International Symposium, Cancer Therapy by Hyperthermia, Drugs, and Radiation: A Symposium Held at Colorado State University; Sponsored by the National Cancer Institute 1(82):85

Takizaki M et al (2017) Enhancing mechanism of gene transfection by heat shock. Chem Lett 46(8):1158–1160

Thomas P, Smart TG (2005) HEK293 cell line: a vehicle for the expression of recombinant proteins. J Pharmacol Toxicol Method 51(3):187–200

Travers A, Muskhelishvili G (2005) DNA supercoiling-a global transcriptional regulator for enterobacterial growth? Nat Rev Microbiol 3(2):157–169

Uddin SN (2007) Cationic lipids used in non-viral gene delivery systems. Biotechnol Mol Biol Rev 2(3):058–067

Von Gersdorff K et al (2006) The internalization route resulting in successful gene expression depends on both cell line and polyethylenimine polyplex type. Mol Ther 14(5):745–753

Zhao K et al (2011) Preparation and immunological effectiveness of a swine influenza DNA vaccine encapsulated in chitosan nanoparticles. Vaccine 29(47):8549–8556