Optimization of an electroporation protocol using the K562 cell line as a model: role of cell cycle phase and cytoplasmic DNAses

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Abstract The improvement of gene therapy protocols is intimately related to the establishment of efficient gene transfer methods. Electroporation has been increasingly employed in *in vitro* and *in vivo* protocols, and much attention has been given to increasing its transfection potential. The method is based on the application of an electric field of short duration and high voltage to the cells, forming reversible pores through which molecules can enter the cell. In this work, we describe the optimization of a protocol for the electroporation of K562 cells involving the combination of electric field, resistance and capacitance values. Using RPMI 1640 as pulsing buffer and 30 μg of pEGFP-N1 plasmid, 875 V cm^{-1} , 500 μF and infinite resistance, we achieved transfection rates of $82.41 \pm 3.03\%$, with $62.89 \pm 2.93\%$ cell viability, values higher than those reported in the literature. Analyzing cell cycle after electroporation, with three different electric field conditions, we observed that in a

heterogeneous population of cells, viability of G_1 cells is less affected by electroporation than that of cells in late S and G_2/M phases. We also observed that efficiency of electroporation can be improved using the DNase inhibitor Zn, immediately after the pulse. These results can represent a significant improvement of current methods of electroporation of animal and plant cells.

Keywords Cell cycle · DNase inhibitors · Electroporation · Gene transfer · K562 cells

Introduction

The improvement of gene therapy protocols is intimately related to the establishment of efficient gene transfer methods. Viral vectors have been shown to present great efficiency. Non-viral vectors, however, are safer so that great effort is dedicated to the development of methods to improve their efficiency.

Electroporation, or electropermeabilization, is one of the techniques employed for the transfer of non-viral vectors, as well as for the introduction of other molecules (drugs, RNA or protein) into the target cells. The method is based on the application of an electric field of short duration and high voltage to the cells. When the electric pulse surpasses the membrane capacitance, its stability is affected and transient, reversible pores

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are formed through which molecules can enter the cell (reviewed in Gehl 2003).

Although the method is being largely employed *in vitro* and *in vivo*, much attention has been given to increasing its transfection potential. Different parameters are analyzed, such as the conductivity and osmolarity of the electroporation buffer (Pucihar et al. 2001), the cell cycle stage at which target cells are during electroporation (Takahashi et al. 1991; Golzio et al. 2002), or the effects of centrifugation immediately after the electric pulse is applied (Li et al. 1999). Particular care must be taken with the viability of the transfected cells, since parameters, which increase transfection efficiency generally result in higher cell death rates.

The K562 cell line was established in 1975, from a patient with chronic myeloid leukemia (CML) in acute phase (Lozzio and Lozzio 1975). This cell line is very useful for the study of the pathways involved in CML development as well as of erythroid differentiation and the patterns of globin gene expression, due to its capacity to produce embryo and fetal hemoglobin. It has been largely employed in the investigation of alternative treatments for thalassemias and sickle cell disease, as well as the pathways involved in the development of CML (Park et al. 2001; Rodrigue et al. 2001; Haynes et al. 2004).

Most of the studies in which K562 cells are electroporated do not aim at the improvement of the method itself, so that the protocols employed present great variation. The highest transfection efficiency described in the literature, with conventional electroporation, is 40% with 49% cell viability (Van Tendeloo et al. 2001). A new methodology, called nucleofection, which results in high levels of transfection and low cell death indices ($74.7 \pm 8.0\%$ and $5.4 \pm 0.2\%$, respectively), was recently described (Schakowski et al. 2004). These results, however, are not reproducible using conventional approaches, since electric pulse conditions are not informed by the company which markets the technology.

In this work, we describe the optimization of a protocol for the electroporation of K562 cells with better results than those previously described. The results are even superior to those obtained with nucleofection, although with slightly lower cell viability. Our results also

show that the cells most affected by electroporation are those in advanced cell cycle phase, and that Zn^{2+} can be used as an inhibitor of intracellular nucleases, increasing the efficiency of electroporation.

Materials and methods

Plasmid

The plasmid pEGFP-N1 (Clontech Lab, Palo Alto, CA, USA) contains the sequence coding for the EGFP protein under the control of the human cytomegalovirus (CMV) promoter. It also presents a gene for kanamycin/neomycin resistance ($\text{kan}^R/\text{neo}^R$), regulated by the early promoter and enhancer from SV40 virus. The plasmid was extracted by alkaline lysis from competent *Escherichia coli*, DH5- α strain, selected with kanamycin, and purified with the Plasmid Maxi-Prep Kit (Qiagen, Valencia, CA, USA). Plasmid preparations were quantified by spectrophotometry.

Cells

The K562 cell line (ATTC CCL 243) was maintained in logarithmic growth in RPMI 1640 medium (Cultilab, Campinas, Brazil) complemented with 10% fetal calf serum (FCS) (Cultilab) and 60 $\mu\text{g}/\text{ml}$ gentamycin (Schering-Plough S/A, Rio de Janeiro, Brazil). The cells were cultured at 37°C in a humidified chamber with 5% CO_2 in air, and passaged 1:10 twice a week. For some of the experiments, medium was complemented with 80 μM ZnSO_4 (Reagen, Rio de Janeiro, Brazil), and 1 mM EDTA or EGTA (Sigma, St. Louis, MO, USA).

Electroporation

The cells were suspended in RPMI 1640 without FCS or antibiotics, at a concentration of 10^7 cells ml^{-1} . A volume of 0.3 ml was transferred to a sterile electroporation cuvette (Bio-Rad Gene Pulser cuvette, 0.4 cm), and kept at room temperature for 15 min in presence of different concentrations of pEGFP-N1 to analyze the

efficiency of transfection, or $15 \mu\text{g ml}^{-1}$ propidium iodide (PI) (Calbiochem, La Jolla, CA, USA) to analyze the efficiency of electroporation. Electroporation was performed with a Bio-Rad Gene Pulser® Transfection Apparatus, in different conditions. Other types of pulsing buffer were also analyzed, with different osmolarity (O1, O2 and O3, Golzio et al. 1998) and conductivity (M1, M2 and M3, Pucihar et al. 2001), as well as combinations of phosphate buffered saline (PBS) with or without HEPES (Sigma) and/or sucrose (Reagen).

After receiving the electric pulse, the cells were treated according to the protocol suggested by Li et al. (1999). Briefly, cells were centrifuged at $13,000g$ for 30 s and maintained pelleted for 20 min. The electroporation medium was then removed and the pellet was suspended in 3 ml of RPMI 1640 with 10% FCS and gentamycin, transferred to 12-well culture plates (Corning Star, Acton, MA, USA) and incubated for 1 h at 37°C in a humidified atmosphere of 5% CO_2 . Viability was determined by Trypan blue exclusion and viable cells were isolated by centrifugation through Ficol-Paque™ PLUS (Amersham Pharmacia Biotech AB, Uppsala, Switzerland). Live cells were suspended in RPMI 1640 with 10% FCS at $5\text{--}7 \times 10^5 \text{ cells ml}^{-1}$, and maintained at 37°C in a humidified atmosphere of 5% CO_2 until analysis.

Efficiency of electroporation and transfection

Fluorescence of the cells was analyzed within the viable cell fraction, 24 and 48 h after electroporation, by examination in an inverted microscope (Axiovert 25 CFL, Carl Zeiss, Germany), with filter set 9 (excitation: BP 450–490, beamsplitter: Ft 510, emission: LP515) and by flow cytometry, using a FACScalibur (Becton Dickinson, San Jose, CA, USA) and the CellQuest software (Becton Dickinson). For analysis of transfection efficiency (expression of the EGFP gene), data were collected with the fluorescence peak of 20,000 pulses in FL1 (FL1H), and logarithmically amplified, within a gate established in a FSC $\times$ SSC plot. Electroporation efficiency (presence of membrane pores, allowing entry of PI) was analyzed by similar procedures but using the

red-orange (FL2H) fluorescence detector. Negative control was represented by non-electroporated K562 cells.

Cell cycle analysis

The method described by Overton and McCoy (1994) for analysis of cell cycle was used, with slight modification. One hour after electroporation, around 5×10^5 live cells were pelleted and suspended in a solution containing 50 μg PI, 10 mM Trizma base (Sigma), 10 mM NaCl, 0.7 U RNase (Invitrogen Carlsbad, CA, USA), and 0.01% NP-40 (USB, Switzerland). After 15–30 min, the cells were analyzed by flow cytometry with collection of data representing width (FL2W) and area (FL2A) of 20,000 FL2 (red-orange) pulses, that were analyzed with the ModFit software (Verity Software House, Topsham, ME, USA). A sample of cells submitted to all steps except electroporation was used as negative control.

Statistics

All experiments were repeated at least three times in different days, and results are expressed as mean $\pm$ standard deviation of the results obtained in independent experiments. Statistical analysis was done with the Student's *t*-test, and results were considered significantly different when $p < 0.01$.

Results

Optimization of electroporation conditions

Different electroporation conditions were tested, including variations in electric field (500, 625, 750, 875, 1000 and 1125 V cm^{-1}), resistance (100, 200, 400 ohms and infinite) and capacitance (250, 500 and $960 \mu\text{F}$). In each reaction, $37.5 \mu\text{g ml}^{-1}$ of the pEGFP-N1 plasmid was used. The best results, represented by $71.39 \pm 4.61\%$ EGFP-positive cells, were obtained in the combination 875 V cm^{-1} , $500 \mu\text{F}$ and infinite resistance. Cell viability

under these conditions was $65.22 \pm 3.89\%$, and the time constant was between 21.0 and 22.5 ms.

Having defined these conditions, we further tested different amounts of the plasmid. Electroporation was thus performed with 875 V cm^{-1} , $500 \mu\text{F}$, ∞ ohms, and with 25, 50 or $75 \mu\text{g ml}^{-1}$ of the pEGFP-N1 plasmid. As shown in Table 1, transfection efficiency was directly proportional to plasmid concentration. Viability was not significantly different, but a tendency for a decrease in cell viability with higher plasmid concentrations was observed. This analysis showed also two positive cell subpopulations, according to the fluorescence level, more easily seen when treatment was done with $75 \mu\text{g ml}^{-1}$ plasmid (Fig. 1).

The use of other types of pulsing buffer did not result in higher transfection efficiency. Cell viability was greatly reduced in media O1, O2, O3, M1, M2 or M3 ($<20\%$), hampering the recovery of viable cells through Ficoll-Paque centrifugation and posterior analysis by flow cytometry. The use of PBS resulted in high cell viability (85.67%) but low transfection efficiency (15.78%), a result not significantly affected by the addition of HEPES and/or sucrose (data not shown).

Analysis of cell cycle after electroporation

To evaluate the effect of electroporation on cells in different phases of the cell cycle, K562 cells were electroporated in RPMI 1640 medium without FCS or antibiotics (10^7 cells/ml), with $15 \mu\text{g PI/ml}$, using three different electric field conditions (875 , 1000 or 1125 V cm^{-1}). Resistance was set on infinite and capacitance on $500 \mu\text{F}$.

Table 1 Effect of different plasmid concentrations on electroporation efficiency

Plasmid concentration ($\mu\text{g/ml}$)	Cell viability (%)	Electroporation efficiency (%)
Control	>99	–
25	66.23 ± 2.51	45.25 ± 3.81
50	64.06 ± 3.82	71.08 ± 2.16
75	62.89 ± 2.93	82.41 ± 3.03

Cell viability (% viable cells) was assessed by Trypan blue exclusion and transfection efficiency (% EGFP-positive cells) by flow cytometry

One hour later, cell viability was assessed by Trypan blue exclusion, viable cells were isolated as previously described and analyzed for PI staining and cell cycle. As shown in Table 2, whereas the electroporation efficiency was directly proportional to the electric field, cell viability was inversely proportional. Compared to the control samples, electroporated K562 cells presented higher frequency of cells in the G_1 phase, and lower frequency of S/ G_2/M cells.

Effect of DNase inhibitors in the electroporation process

To test the effect of nuclease inhibitors on the electroporation process, K562 cells (10^7 cells ml^{-1}) were electroporated with $37.5 \mu\text{g ml}^{-1}$ plasmid at 875 V cm^{-1} , $500 \mu\text{F}$ and infinite resistance, in the presence of $80 \mu\text{M ZnSO}_4$ and 1 mM EDTA or EGTA . The treatment was done in four different conditions: 15 min before electroporation and until analysis of cell viability (pre), 15 min before electroporation and continued until analysis of transfection efficiency 48 h later (pre/cont), immediately after electroporation and until analysis of cell viability (post) and immediately after electroporation with maintenance of the reagents until analysis of transfection efficiency 48 h later (post/cont). Transfection efficiency was evaluated 48 h after electroporation, by analysis of the expression of EGFP by fluorescence microscopy and flow cytometry.

The results (Table 3) showed that transfection was more efficient when ZnSO_4 was added after electroporation, independent of its maintenance until the moment of analysis, but not when ZnSO_4 was added at the moment of electroporation. The Ca^{2+} chelator EGTA, on the other hand, did not affect the electroporation process, but cell viability decreased with the treatment. Treatment with EDTA, a Ca^{2+} and Mg^{2+} chelator, resulted in lower transfection efficiency and cell viability.

Discussion

This work describes the optimization of an electroporation protocol for K562 cells, involving

Fig. 1 Electroporation of K562 cells with increasing plasmid concentration results in an increase in the frequency of EGFP-positive cells. **(a)** negative control—electroporation without plasmid; **(b)** $25 \mu\text{g ml}^{-1}$; **(c)** $50 \mu\text{g ml}^{-1}$; **(d)** $75 \mu\text{g ml}^{-1}$ of plasmid pEGFP-N1

the combination of electric field, resistance and capacitance values, combining standard electroporation processes with the post-pulse manipulation described by Li et al. (1999). The *egfp* reporter gene has been widely used to analyze the efficiency of gene transfer protocols, and the pEGFP-N1 plasmid was previously shown to transfect K562 cells with use of the polycationic compound SuperFect (Qiagen) (Teixeira et al. 2001). We obtained transfection rates of

$82.41 \pm 3.03\%$, with $62.89 \pm 2.93\%$ cell viability. These values are higher than those reported with standard electroporation protocols, of which the most efficient was described by Van Tendeloo et al. (2001) and resulted in 40% efficiency with 49% cell viability. The main differences presented by our protocol are the culture medium employed (RPMI 1640 versus X-vivo 15 medium) and the electroporation conditions (which in that study were electric field of 625 V cm^{-1}

Table 2 Effect of electroporation on cells in different phases of the cell cycle

Electric field	Cell viability (%)	Cell cycle phase (%)			Electroporation efficiency (%)
		G ₁	S	G ₂ /M	
Control	>99	36.4	55.5	8.0	—
875 V	65.22 ± 1.89	$42.9 \pm 0.62\%$	53.0 ± 1.04	4.1 ± 0.88	71.39 ± 4.61
1000 V	40.51 ± 0.83	$45.0 \pm 0.89\%$	50.1 ± 0.46	4.9 ± 1.21	78.09 ± 3.77
1125 V	27.08 ± 1.46	$48.4 \pm 1.21\%$	46.0 ± 0.96	5.6 ± 2.53	89.30 ± 2.56

Cell viability (% viable cells) was assessed by Trypan blue exclusion; cell cycle phase (% cells in each stage) and transfection efficiency (% EGFP-positive cells) were assessed by flow cytometry

Table 3 Cell viability (% viable cells) assessed by Trypan blue exclusion 1 h after medium change, and transfection efficiency (% EGFP-positive cells) analyzed by flow cytometry after electroporation done in presence of ZnSO₄, EGTA or EDTA

Treatment	Cell viability (%)	Transfection efficiency (%)
None	69.62 ± 1.89	63.36 ± 2.19
ZnSO ₄ pre	46.29 ± 3.89	66.30 ± 1.25
ZnSO ₄ pre/cont		66.42 ± 1.89
ZnSO ₄ post	62.79 ± 2.03	75.97 ± 2.36
ZnSO ₄ post/cont		74.70 ± 1.90
EGTA pre	41.70 ± 2.28	60.21 ± 1.71
EGTA pre/cont		62.45 ± 0.86
EGTA post	59.39 ± 1.25	62.90 ± 3.22
EGTA post/cont		61.27 ± 0.73
EDTA pre	21.65 ± 3.02	16.75 ± 3.08
EDTA pre/cont		17.29 ± 2.44
EDTA post	27.97 ± 2.92	38.21 ± 2.18
EDTA post/cont		36.89 ± 1.50

and capacitance of 1500 μ F). The transfection efficiency obtained in the present study is even higher than that reported with the new electro-transfer technology known as nucleoporation (Schakowski et al. 2004), which resulted in 74.7 ± 8% positive cells. Cell viability in the present study is also higher than that in the report by Van Tendeloo et al. (2001), but lower than the viability reported by Schakowski et al. (2004), which resulted in 5.4 ± 0.2% dead cells. A comparison of our protocol with the nucleoporation method is, however, difficult because the conditions of electric field, resistance and capacitance, and the pulsing buffer composition are not described in that report.

The increase in transfection efficiency with lower cell viability, observed in higher electric field condition during the optimization experiments, has also been observed in other studies. In addition, our results show that among viable cells, isolated one hour after electroporation, viability of G₁ cells is less affected by electroporation than that of cells in late S and G₂/M phases. Similar results were reported by Golzio et al. (2002), but in that study purified cell populations were analyzed in G₁, S or G₂/M. This correlation between increased mortality among cells in the late phases of the cycle can be explained by the same formula, which explains the correlation between increase in

the electric field intensity and electroporation efficiency. According to this formula, $\Delta V_m = fE_{\text{ext}}r \cos\Phi$ electroporation begins when ΔV_m exceeds the resting membrane potential (V_m), which has similar magnitude (around 1 V) in several cell types. In the formula, f is a factor which most authors describe as 1.5, E_{ext} is the electric field used during electroporation, r represents the cell radius and Φ is the polar angle with respect to the external electric field; for spherical cells this value is zero (Pucihar et al. 2001; Gehl 2003). The voltage which will exceed the resting membrane potential of a spherical cell will thus be: $E_{\text{ext}} > 1 \text{ V} (1.5r \cos 0)^{-1} = 1 \text{ V} \times 1.5r^{-1}$.

For K562 cells, which have a 20 μ m radius (Koeffler and Golde 1980), E_{ext} is thus 1 V $(1.5 \times 20 \mu\text{m})^{-1}$ or 330 V cm⁻¹. As the cell doubles its volume at late S/G₂ phases, its radius becomes 26% larger (according to the trigonometrical properties of spheres), so that for efficient electroporation the electric field must be higher than 1 V $(1.5 \times 25.2 \mu\text{m})^{-1} = 260 \text{ V cm}^{-1}$. This represents a 70 V cm⁻¹ difference in the electric field necessary to exceed the membrane resting potential in G₁ or G₂ cells. In other words, submitted to the electric field necessary for generating a change in the potential of a G₁ cell, G₂ cells would have a $\Delta V_m = 330 \text{ V cm}^{-1} \times 1.5 \times 0.00252 \text{ cm} = 1.247 \text{ V}$, which is almost 25% higher. Treatment of G₂ cells with the electroporation conditions which represent the optimum for G₁ cells would thus produce irreversible membrane lesions, and as a consequence the number of live G₁ cells after electroporation would be proportionally higher. This situation emphasizes the importance of using homogeneous populations for transfection, and suggests that the increased efficiency observed when cells are electroporated in the S (Takahashi et al. 1991) or G₂/M (Goldstein et al. 1989; Golzio et al. 2002) phases is due to the adjustment of the electric field intensity which is applied, rather than to an intrinsic advantage of the phase in which the cells are.

Although G₁ cells seem to have been less affected, the results suggest that they are efficiently transfected, since only 3–5 days after electroporation normal cell proliferation was observed (unpublished results). Although the study by Golzio et al. (2002) focused on the

increased efficiency of transfection of G₂/M cells, transfection of G₁ cells was also observed. Dean (1997) showed that G₁ cells can be transfected, as long as the plasmid presents a nuclear localization sequence (NLS). Plasmids containing the region of SV40 virus genome which includes the replication origin, early-late promoter and enhancer, were shown to be able to enter the nucleus of G₁ cells; the most potent NLS in the sequence is the 72 bp repetition which forms the enhancer (Dean et al. 1999). This particular sequence is present in the plasmid used in the present work, as well as in the plasmid pEGFP-C1 used by Golzio et al. (2002). These observations suggest that the presence of this sequence is important in any vector used for gene transfer, particularly when the target cells of the transfection process are in G_{0/1} as happens in *in vivo* electroporation processes, as proposed by Blomberg et al. (2002).

The low proliferation rates observed after electroporation could be due to degradation of the plasmids by cytoplasmic nucleases. In this case, nucleotides derived from plasmid degradation would be incorporated in the cell dNTP pool (Lechardeur et al. 1999; Bureau et al. 2004). Depending on the plasmid sequence, this incorporation can cause an imbalance in the dNTP pool, which results in blockage of the cell cycle and/or cell death due to errors in DNA replication or DNA repair (Kunz and Kohalmi 1991).

Nucleases are responsible for plasmid degradation before their entry into the nucleus (Lechardeur et al. 1999; Bureau et al. 2004). To test the impact of this phenomenon on the results of transfection, we tested the effect of three reagents able to inhibit nucleases. EDTA, a chelator of Ca²⁺ and Mg²⁺ (necessary for the enzymatic activity of DNases) decreased electroporation efficiency and resulted in lower cell viability. Neumann et al. (1996) suggested that Ca²⁺ and Mg²⁺ are important for electroporation due to their interaction with the plasmid DNA, decreasing its negative charge and facilitating its interaction with the cell membrane. The results of post-pulse treatment in the present work, similar to those of pre-pulse treatment, suggest however that in electroporation these ions are more important for intra-cellular processes such as the

transport of the plasmid through the nuclear pores. Our results with EGTA, used in a concentration able to chelate double of the Ca²⁺ present in RPMI 1640 medium, did not affect transfection efficiency. Cell viability was also not affected, showing that Ca²⁺ does not influence cell viability during electroporation as already proposed (Gehl 2003). The use of ZnSO₄, on the other hand, induced significant increase in transfection efficiency, so that 15 µg plasmid in the presence of ZnSO₄ had results similar to those obtained with 30 µg without the compound. These results emphasize the positive role of Zn²⁺, which is known to directly block DNase activity (Mastrangeli et al. 1994). The effect was more visible when ZnSO₄ was used after the electric pulse, suggesting a possible interference of this reagent during the pulse.

It is possible that the use of more potent DNase inhibitors, such as the aurintricarboxylic acid or its combination with Zn²⁺ can further increase the efficiency of transfection through electroporation, similar to the effect described on the transfection of salivary glands when naked DNA was used (Niedzinski et al. 2003). Finally, it is important to stress that in the present study the possibility of gene transfer mediated by Zn²⁺ is excluded by the fact that no significant differences were observed between the post (40 min exposure) and the post/cont (48 h exposure) Zn²⁺ treatments.

This study showed that modifications of the protocol for electroporation of K562 cells result in higher efficiency as compared to conventional methods. The cells most affected by electroporation were those in advanced cell cycle phase, and the use of Zn²⁺, inhibiting intracellular nucleases, increased the efficiency of electroporation. These results can represent a significant improvement of current methods of electroporation of animal cells.

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