The effects of antioxidants on gene electrotransfer in vitro

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Abstract

Gene electrotransfer is one of promising non-viral methods for introducing genes into the cell by using high voltage electric pulses. The method enables efficient gene transfer, however high electric pulses can produce free radicals and reactive oxygen species that affect cell survival. Antioxidants are molecules that inhibit oxidation of other molecules and thus protects them from oxidative stress. The aim of our study was to test the effect of two antioxidants on gene electrotransfer efficiency and cell survival. Glutathione, is an important antioxidant present in cells of many organisms. It prevents damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides. The second antioxidant that we have tested is vitamin E. Vitamin E is lipid soluble vitamin, which lipophilic nature allows integration into the cell membrane, where it protects unsaturated fat and other components of cell membranes, which are susceptible to oxidative damage. We used CHO cells, cultured in vitro and performed experiments on plated cells. We analysed effect of two different antioxidants on cell viability after electroporation. We also used two different pulsing protocols consisiting of longer (ms) and shorter(µs) pulses. Our results indicate that both glutathione and vitamin E didn't have much effect on gene electrotransfer efficiency and cell viability. Further investigations are needed to understand the mechanisms behind our findings.

1 Introduction

Gene electrotrasfer is one of the most promising non-viral method for introducing genes in cells in vivo [1]. It uses electroporation of cell membrane for reversibile increase in membrane permeability, which enables transport of molecules and transfer of plasmid DNA in cell. Gene electrotransfer is very promising method for use in gene therapy and first clicnical tests have been succesfully completed [3].

Clinical use of gene electrotransfer is electrogene therapy (EGT), which allows gene transfer or gene silencing in targeted tissue such as: tumor, muscle, skin or other organs. EGT is non-viral gene therapy and it represents safer method of treatment compared to viral therapies [4]. Another promising area of gene electrotransfer is genetic vaccination. In the future, genetic treatments could become effective methode in treatment of degenerative diseases, cancer, cardiovascular diseases, for which there are no currently availible treatments [1]. Gene electrotransfer is a multistep process. Among the factors affecting the final efficiency of electrotransfection are: electric pulse parameters, composition of electroporation medium, plasmid characteristics, cell type and the stage of the cell cycle of the treated cells [1]. Nevertheless the exact mechanisms governing the process are still under investigation.

It was shown by several papers [5,6,7,8,9] that viability of cells after gene electrotransfer is one of crucial factors for efficient transfection. Reactive oxygen species (ROS), such as free radicals in peroxides are very lethal for cells after electroporation. In normal conditions, cells themselves are capable of defense against damage caused by ROS. But when the concentration of antioxidants drops or the production of ROS rises, the balance collapses which leads to oxidative stress. ROS in oxidative stress are capable of damaging DNA, RNA, proteins and lipids, which consequently leads to cell death, inflammation, aging and occurrence of various diseases. That is why antioxidant defense systems are so important.

Glutathione is one of the most important antioxidants. It exists in both reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to other unstable molecules, such as reactive oxygen species. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide (GSSG).

Vitamin E is the best known biological antioxidant. It is a lipid soluble vitamin, which lipophilic nature allows integration into the cell membrane, where it protects unsaturated fatty acids and other components of cell membranes, which are susceptible to oxidative damage. Vitamin E structure is based on eight different natural components (4 tocopherols and 4 tocotrienols). Its function is very important in the last stage of defense against ROS, where it reacts with free lipid radicals and neutralizes/removes free radicals [2].

The aim of our study was to analyse the effect of two antioxidants - glutathion and vitamin E in vitro on plated CHO cells on gene electrotransfer efficiency and cell viability after electroporation.

2 Materials and methodes

2.1. Cell cultures and electroporation medium

Chinese hamster ovary (CHO) cells were grown in 25 cm^2 flasks as a monolayer culture in HAM's cell culture medium supplemented with glutamin, foetal bovine serum and antibiotics at 37°C and 5% CO_2. [1] We have grown them to 70-80% confluence.

2.2. Plasmid DNA

The plasmid pEGFP-N1 (Clontech Laboratories, Inc., Mountain View, CA, USA), which encodes the green fluorescent protein (GFP), was amplified in a strain K12 of *Escherichia coli* and isolated with HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Germany). The concentration of plasmid DNA was then determined spectrophotometrically at 260 nm.

2.3. Preparation of cell culture

After 3-4 days of cell culture, when cells were in logarithmic growth phase, cells were prepared for electroporation. We plated cells in 24 well plates at concentration 4×10^4 cells/ml. Experiments were performed after 24h.

2.4. Electroporation

On the day of the experiment, we removed the culture medium from the wells and then added 200 µL of electroporation buffer, containing plasmid in concentration of 10 µg/ml in each well and incubated for 3 minutes prior to electroporation. Glutathione was added to the cell culture on the day of the experiment and the concentration of glutathione in wells was 5 mM. For electroporation with vitamin E the experiment was the same, except that the vitamin E was added to the cell culture 24 hours prior to the experiment, and the s concentration was 50 µM.

As an electroporation buffer we used NaPB (Na_2HPO_4/ NaH_2PO_4).Electroporation was performed by using Pt/Ir electrodes with 4 mm spacing between the electrodes, wherein the electrodes were placed on the bottom of the well. For electroporation, we used the following parameter pulses: 4× high-voltage µs pulses with a length of 200µs and voltages: E_1 = 1 kV/cm (U=400V), E_2 = 1.4 kV/cm (U=560V) E_3 = 1.8 kV/cm (U=720V) and frequency of 1Hz. We also used 8× low voltage ms pulses with a length of 5 ms and the following voltages: E_1 = 0.4 kV/cm (U=160V), E_2 = 0.6 kV/cm (U=240V) E_3 = 0.8 kV/cm (U=320V) and frequency of 1Hz. After each well was subjected to electroporation, we added 50 µL of FBS serum (fetal bovine serum). After the completion of the electroporation, the cells were placed in the incubator for 5 min so that the cell membrane could reseal. After 5 minutes we added in each well 1 ml of HAM culture medium and we incubated for 24h at 37 °C.

2.5. Microscopy

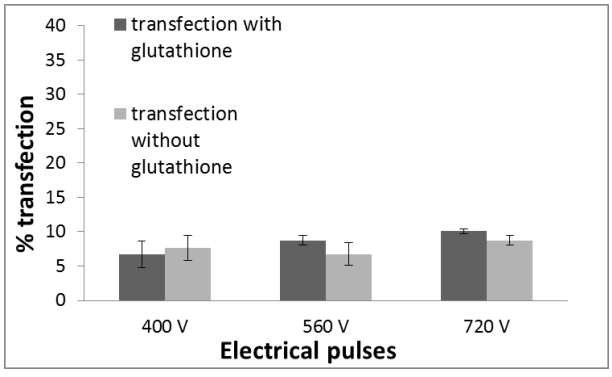
The efficiency of transfection and cell survival was observed with a fluorescent microscope (Zeiss 200, Axiovert, West Germany). Images were then captured by using MetaMorph Imaging System, Visitron, West Germany. The percentage of transfection was observed using plasmid DNA (GFP), and the percentage of survival was determined as a number of cells present in non-treated samples and number of cells in samples exposed to electric pulses. For determination of cell number cells were stained ith a Hoechst 33342, which is a flourescent dye, that binds to the minor groove of the double stranded DNA. Cells in negative control were not exposed to electric pulses.

3 Results

To determine effects of different antioxidants on gene electrotransfer and viability of electroporated cells we performed in vitro experiments on CHO cells. We have used GFP coding plasmid for determining transfection of cells and Hoechst dye for determining viability of cells.

In Fig 1 we can see effects of glutathione on gene electrotransfer with high voltage pulses of µs (Fig 1a) and ms (Fig 1b) duratio . As we can see, the percentage of transfection increases from around 6% at 1kV/cm (400V) to about 11% at 1.8 kV/cm (720V). Fig 1b also shows transfection with glutathione, but with longer ms pulses. The transfection percentages were higher than with µs pulses and they ranged from 8% at 0.4 kV/cm (160V) to around 14,5% at 0.8kV/cm (320V).

In Fig 2 we present effects of vitamin E on gene. As we can see from Fig 2a 2a, transfection ranges from around 11% at 1kV/cm (400V) to around 17% at 1,8 kV/cm (720V). Fig2b, similarly as Fig. 2a shows effect of vitamin E on gene electrotransfer, but with longer ms pulses. Transfection increases from around 21% at 0.4 kV/cm (160V) to approximatly 28% at 0.8kV/cm (320V).



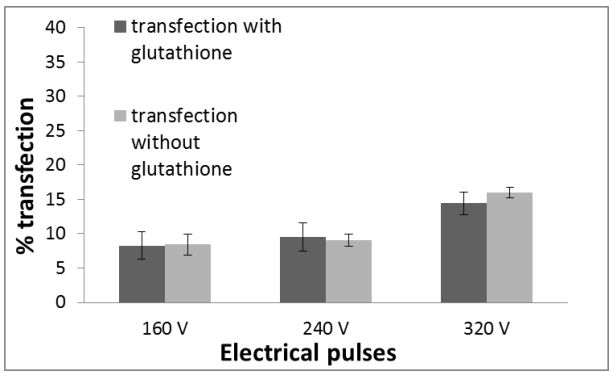
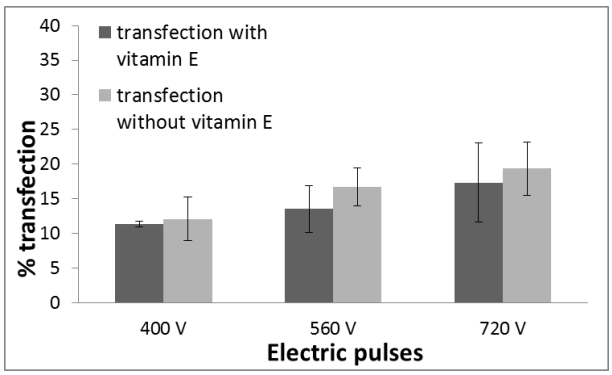


Figure 1: Effect of glutathione on gene electrotransfer efficency. A) with µs pulses of 8 × 200µs, and B) with ms pulses of 8 × 5ms, results are presented as a mean of three independent experiments ± standard error.



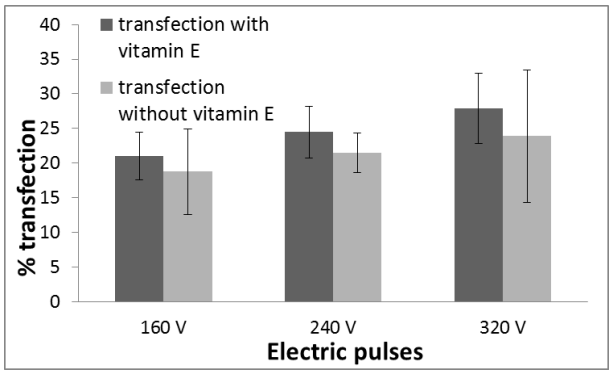
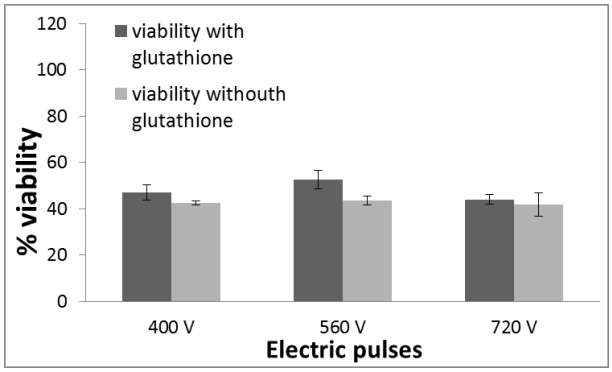


Figure 2: Effect of vitamine E on gene electrotransfer efficency. A) with µs pulses of 8 × 200µs, and B) with ms pulses of 8 × 5ms, results are presented as a mean of three independent experiments ± standard error

In Fig 3 we present the effect of glutation on cell viability with µs pulses (Fig 3a) and ms pulses (Fig 3b). As we can see in Fug 3a, viability in all three parameters did not change much. Viability at 1 kV/cm (400V) was around 47% and at 1.8 kV/cm (720V) around 44%. In Fig 3b we can see that viability of cells was around the same as in Fig 3a. The lowest viability we got was with ms pulses at 0.8 kV/cm (320V) and it was around 40%. Interestingly we got also the highest viability with ms pulses, namely around 55% at 160V.
Fig 4 also shows viability rates, but with vitamine E. In Fig 4a (µs pulses) we can clearly see a drop in viability which drops from 85% at 1 kV/cm (400V) to around 51% at 1.8 kV/cm (720V). In Fig 4b (ms pulses) the drop continues and it drops from 42% at 0.4 kV/cm (160V) to 37% at 0.8 kV/cm (320V).



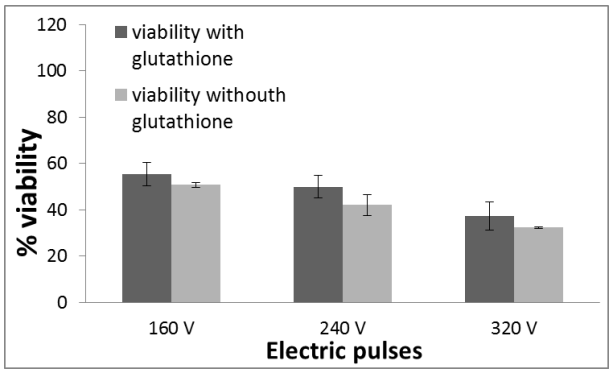
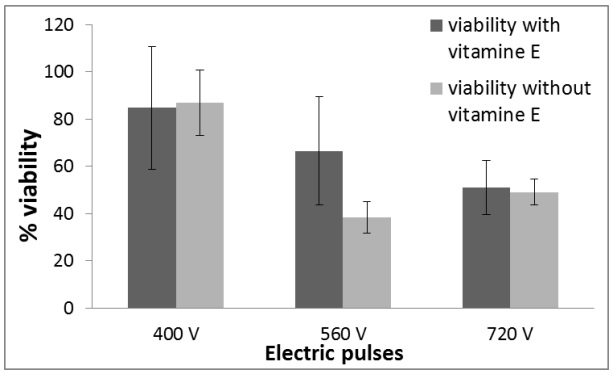


Figure 3: Effect of glutathione on cell viability. A) with µs pulses of 8 × 200µs, and B) with ms pulses of 8 × 5ms, results are presented as a mean of three independent experiments ± standard error.



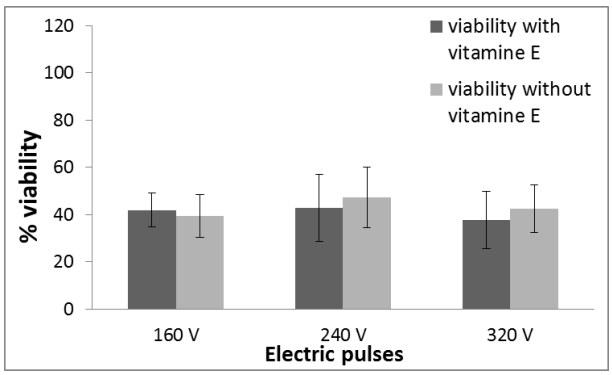


Figure 4: Effect of vitamine E on cell viability. A) with µs pulses of 8 × 200µs,

and B) with ms pulses of 8 × 5ms, results are presented as a mean of three independent experiments ± standard error.

4 Discussion & Conclusions

The aim of our study was to test the effect of two antioxidants: glutathion and vitamin E, in vitro on plated CHO cells on gene electrotransfer efficiency and cell viability after electroporation.

Gene electrotransfer is a promising method of gene delivery where cell survival is an important factor, therefore we rested the effect of two antioxidants on percentage transfection and cell viability.

As expected percentage of transfection increased when higher voltages or longer pulses, were used (Fig. 1). Percentage of transfected cells in experiments with gluthamine and vitamine E were different. Results in experiments with glutathione showed that the percentage of transfected cells increased with milisecond pulses, but not as much as with vitamine E. Percentage of transfected cells by using glutathion with µs pulses was lower (6.5% with µs and 14,5% with ms pulses) in comparison with 12% and 24%, respectively in experiments with vitamin E.

We expected, that we would improve viability of cells with both antioxidants but our results show that there was not much difference between experiements with or without added antioxidants. The viability of cells with both antioxidants decreased in accoradance with longer ms pulses. We also obtained that percentage of viability in experiments with glutathione didn't decrease for longer pulses as we suspected it would, but rather stayed around the same level in all parameters. However, in experiments with added vitamin E, we clearly obtained decrease in viability form 85% at µs pulses to around 37% for longer ms pulses.

Our results did not support our hypothesis, that the presence of added antioxidants would improve cell survival after electroporation.

This can primarily be attributed to the fact that after electroporation we added to the cells FBS (fetal bovine serum), which has many growth factors, and as well as some of the antioxidants, with which could have improved survival. To better understand the impact of vitamin E and glutathione the experiments without added FBS should be performed.

Better transfection, which was obtained in experiments with vitamin E can be attributed to the fact that we used freshly isolated plasmids, whereas in the experiments with added glutathione the plasmid was not freshly isolated. This could significantly affect the proportion of transfected cells. Transfection results for different pulsing protocols are consistent with the results of other studies (1,6,8), namely that the percentage of transfection increases with length of pulses, which we have also shown.

To summarize, we have showed that glutathione and vitamin E didn't have effect on cell viability after electroporation, but further investigations are needed to understand all underlying mechanisms.

5 Literature

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