Gene Ther Mol Biol Vol 14, 42-48, 2012

The effects of antioxidative additives on electroporation efficacy

Short Technical Report
***with preliminary results**

Ľubomíra Tóthová[1] Janka Bábíčková[1], Peter Celec[1,2,3]

[1] Institute of Molecular Biomedicine, Comenius University, Bratislava, Slovakia
[2] Department of Molecular Biology, Comenius University, Bratislava, Slovakia
[3] Institute of Pathophysiology, Comenius University, Bratislava, Slovakia

***Correspondence:** Ľubomíra Tóthová, MSc, PhD, Institute of Molecular Biomedicine, Comenius University, Sasinkova 4, 811 08 Bratislava, Slovak Republic
Phone: +421 2 59357371, Fax: +421 2 59357631, Email: tothova.lubomira@gmail.com

Keywords: ascorbic acid; α-tocopherol; electropermeabilization; gene delivery; melatonin

Received: 15 May 2012; Revised: 18 June 2012
Accepted: 21 June 2012; electronically published: 22 June 2012

Summary

Gene transfer into eukaryotic or prokaryotic cells is an emerging field in scientific research, however with transfection success being low. Many approaches to increase this efficiency rate are being tried, one of which is electroporation. The aim of this experiment was to analyze the possible enhancing effects of antioxidants on gene transfer mediated by *in vitro* and *in vivo* electroporation. *E. coli* DH5α cells were electroporated with plasmid pBR322. The antioxidants, melatonin and a combination of vitamin C and E were added directly to the electroporation buffer or the cells were pre-treated by growth in a medium containing these antioxidants. VEGF plasmid in the presence or absence of the antioxidants was administered. Vitamins C and E, and melatonin improved the electroporation efficacy *in vitro*, if they were applied directly into the electroporation buffer. A significant decrease in electroporation efficiency appeared if the cells were pre-treated with melatonin compared with the control group. The presence of antioxidant vitamins in electroporated muscles increased VEGF expression twofold. The results show that antioxidants are able to increase the efficiency of electroporation-mediated gene transfer, however further studies regarding dosing and timing should be performed.

I. Introduction

Electroporation (or electropermeabilization) is a widely used method for improving the efficiency of gene transfer into prokaryotic and eukaryotic cells. Short pulses of high voltage electric field induce differences in cell membrane potential. Under this condition membrane becomes permeabilized for the transfer of large molecules such as DNA through the membrane. Despite the fact that many details about the process, especially the movement of DNA and its interaction with the membrane, are unknown, electroporation is used for DNA delivery both, *in vitro* and *in vivo* (Escoffre et al, 2009). The current electroporation protocols are the result of try-and-see experiments, even those used in clinical trials for gene therapy or DNA vaccination (Bodles-Brakhop et al, 2009). Many electroporation conditions that may influence the efficacy, such as duration of electric impulses and their number, cell concentrations, temperature, DNA concentration etc. were studied (Teissie et al, 2005; Sukhorukov et al, 2005). A wide range of different electrodes can be used for both *in vitro* and *in vivo* trails, depending on cell or tissue types to be electroporated (Raptis and Firth, 2008). Electromagnetic pulses seem to be another way how to increase efficiency of gene transfer. Some of the studies report DNA to be transferred into the cells after conjugation to metallic nanoparticles which in subsequent electric field penetrates the membrane into the cell and release the genetic material (Stride et al, 2009). Nevertheless, the electromagnetic pulses have some other potentially unwanted effects. Apart from cell transfection, it might temporarily activate/inactivate other gene transcription and translation as is NFκB (Natarajan et al, 2006) or up-regulation of mRNA levels of heat shock protein hsp70 in p53 deficient mice (Czyz et al, 2004). Parameters of electric pulses have been also studied as a crucial part of electropermeabilization protocols. Some authors preferred rather repeated pulses than single pulse, or fusion of one high-voltage pulse with a series of low-voltage pulses were used (Molnar et al, 2004; Andre et al, 2008). Nevertheless, one of the least studied factors potentially limiting the efficiency of electrotransfer of DNA is the toxicity of electric pulses (Beebe et al, 2003). Apoptosis induced by electric pulses might have applications in the treatment of tumors, but can also have a negative impact on the survival of cells after DNA delivery. The underlying mechanism is unclear, but seems to involve the generation of reactive oxygen species (ROS) (Benov et al, 1994). Gabriel and Tessie (1994) reported, that generation of ROS is associated with the permeabilized part of the cell membrane, and occurs only when the electoropermeabilization of membrane is reversible (Gabriel and Teissie, 1994). Recently, their results have been confirmed by Markelc et al. (2011). In addition, it has been reported that the antioxidant tempol increases gene electrotransfer (Markelc et al, 2011).

Based on previous findings, regulation of oxidative stress in cells and tissues may play a crucial role in electroporation-mediated gene delivery efficiency. The aim of our study was to test the effects of antioxidants on gene transfer efficiency in *in vitro* and *in vivo* electroporation. In this study, we also tried to confirm our hypothesis that the addition of different antioxidants could lead to higher survival rate of cells after electroporation *in vitro* and *in vivo*. Both vitamin E (hydrophobic molecule) and C (hydrophilic molecule) are generally used as effective antioxidants, but their synergistic potential is often omitted (Celec et al, 2003). Additionally, as proven before, vitamin C alone in doses up to 6mM had no effect on cell survival after or before the electroporation (Markelc and Tevz, 2011). Therefore, we decided to use their combination and explore the effects of these additives on electroporation efficiency.

II. Methods

Bacteria and plasmids

Bacterial strain *E. coli* DH5α was grown in Luria-Bertani medium at 37°C. Super optimal broth (SOB) medium was used for preparation and electroporation of bacterial cells and cultivation of recombinant strains (Hanahan, 1983). Electrocompetent bacterial cells were prepared as described by Ausubel et al. (Ausubel et al, 2002). Plasmid CA-VEGF was a kind gift from prof. Yoshikazu Yonemitsu, Chiba University, Japan. CA-VEGF plasmid contains the human cDNA for vascular endothelial growth factor (VEGF) under the CMV promoter (Lipinski, 2011). Plasmid pBR322 has the ampicilin and tetracycline resistance genes, and was purchased from Fermentas (Lithuania). For plasmid isolation QIAprep spin miniprep kit (Qiagen, Germany) was used.

In vitro experiment

E. coli DH5α cells were electroporated using a cuvette and microplate electroporator (Gene PulserXcell Electroporation System, Biorad, USA). Pre-set bacterial protocol was used for electroporation (voltage-2500V, capacitance-25μF and resistance-200Ω). Plasmid pBR322 was used for the DNA transfer and provided antibiotic resistance for surviving transfected cells. Melatonin (1mM, Sigma-Aldrich, Germany) and a combination of vitamin C and E (1mM, Sigma-Aldrich, Germany) were added directly to the electroporation buffer (cold sterile, 10% glycerol) or the cells were pre-treated by growth in a medium containing these antioxidants for 3 hours. All measurements were done in eightplicates. Cell survival was measured spectrophotometrically at 660 nm on Saphire II instrument (Tecan, Austria) after cell cultivation in microplates and on agar plates. Electroporation efficiency index was calculated as the ratio of cells cultivated under selective and non-selective conditions.

In vivo experiment

Fifteen C57BL/6 mice (5 males in each group, 3 months of age) used in *in vivo* preliminary experiment were purchased from AnLab (Czech Republic). Animals had *ad libitum* access to food pellets and water. Animals were divided into three groups as follows: CTRL, VEGF and VIT. Plasmid CA-VEGF (3 μg in a volume of 50 μl) factor (VEGF) was injected by needle electrodes directly into the gastrocnemius muscle of mice anaesthetized with isoflurane. Before injection, the plasmid DNA was dissolved either in deionized water (VEGF group) or in a solution containing vitamin C and E (1 mM, VIT group). Control group (CTRL) received the same amount of deionized water, pH 7.0. Immediately following the injection, 2 x 3 electric pulses with changing polarity after 2 pulses with voltage 100 V, were applied to the muscle using needle electrodes (5mm apart, 0.5 mm diameter, gold plated straight genetrode, model 508, pulse duration 20ms, frequency 50Hz, interpulse pause 100ms) connected to ECM 830 *in vivo* electroporator (Harvard Apparatus, USA). Three days after electroporation, the mice were sacrificed and muscle tissue samples were taken for RNA isolation and biochemical analyses. This experiment was approved by Ethics Committee of the Institute of Molecular Biomedicine, Comenius University in Bratislava, Slovakia.

Biochemical analyses

Total RNA was isolated with TriReagent kit (MRC, USA) according to the protocol of the manufacturer. Purity and concentration of RNA was assessed using spectrophotometer (Nanodrop, Thermo Fisher Scientic, USA). VEGF expression was analyzed using real time RT PCR (QuantiTect SYBR green RT-PCR kit, Qiagen, Germany) with specific primers. Peptidylprolylisomerase A gene was used as a housekeeping gene. Delta delta Ct method was used for relative quantification of expression of selected genes. The following PCR program (50°C 10 min, 95°C 5 min, 95°C 10 seconds and 60°C 30s repeated 40 times) on Realplex 4S Mastercycler (Eppendorf, Germany) was used.

Thiobarbituric acid reactive substances, advanced oxidation protein products, advanced glycation end products, total antioxidant capacity and the ferric reducing ability of the tissue were measured to assess oxidative damage of the tissue and antioxidant status by established spectrophotometrical and spectrofluorometrical methods as described elsewhere (Celec et al, 2011). All measurements were done on Saphire II instrument (Tecan, Austria).

Statistical analysis

For statistical analysis Microsoft Office Excel 2007 and XL Statitics 5.1 were used. Differences between groups were evaluated using One-way ANOVA and post hoc Bonferroni modified t-test. P-values less than 0.05 were considered significant. Data are presented as mean + standard deviation.

III. Results

In *in vitro* experiment, direct application of melatonin and combination of antioxidants vitamin C and E increased plasmid electroporation efficiency, both by 17%, after measurement of optical density (OD) in cultivated microplates. Due to variability, only the effect of melatonin was significant (**Fig. 1A**, T=2.72, p=0.05). If colony forming units (CFU) on agar plates were enumerated, both additives increased the electroporation efficiency significantly (**Fig. 1B**, T=2.72, p=0.05 for melatonin; T=2.56, p=0.05 for vitamins).

In *in vitro* experiments, pre-treatment of cells 3 hours prior to electroporation with melatonin resulted in a significant decrease of electroporation efficiency (**Fig. 1C**, by 15.3% according to OD measurements after cultivation in microplates, T=2.3, p=0.05; Fig. 1D, by 77.5% according to enumeration of CFU after cultivation on agar plates, T=5.9, p=0.002). On the other hand, pre-treatment with vitamin C and E increased electroporation efficiency by 18% in microplates (**Fig. 1C**, T=2.5, p=0.04 for cultivation in microplates), as well as on agar plates (**Fig. 1D**, T=2.3, p=0.05).

In vivo application of plasmid DNA in a solution containing vitamin C and E resulted in a 50% reduction of transgene expression in comparison to plasmid DNA in deionized water if no electroporation was used. When electroporation was used administration of vitamins tended to induce a 50% increase in VEGF expression. The differences were not significant due to high variability in real time RT PCR (**Fig. 2A, B**). Expression of housekeeping gene did not differ between groups (data not shown). No significant differences were found in any analyzed oxidative stress markers at day 3 and 7 after electroporation (data not shown).

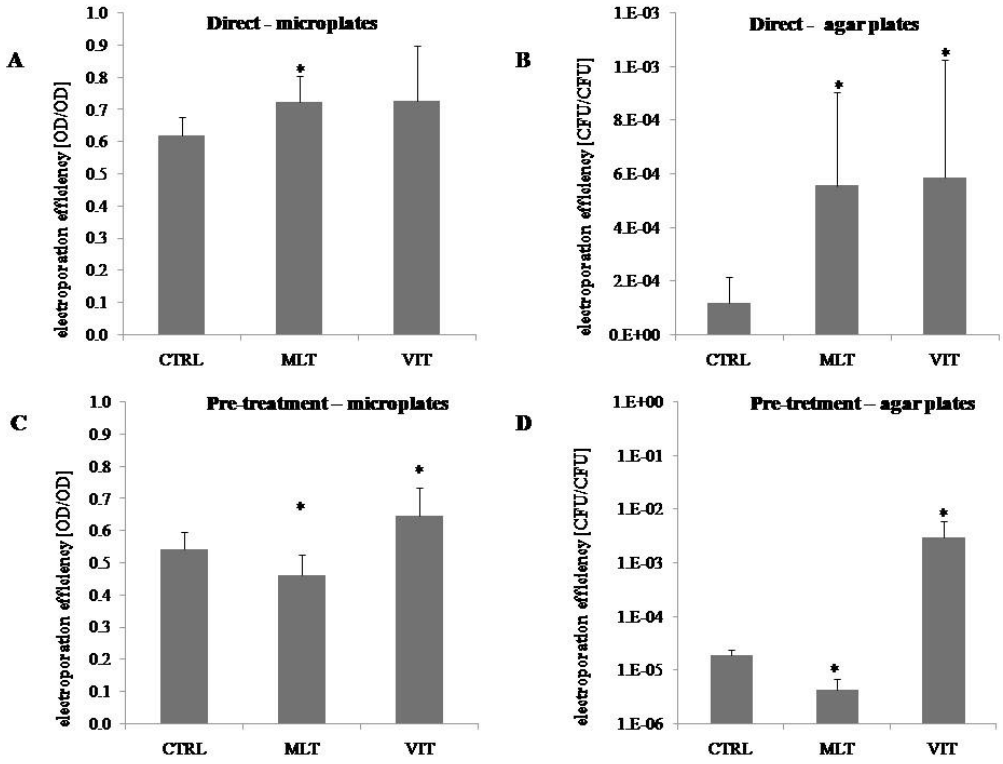

Figure 1: Electroporation efficiency following different treatments with additives *in vitro*. The antioxidants were added directly to the electroporation buffer (A – cultivation in microplates, B – cultivation on Agar plates) or the cells were pre-treated with antioxidants for three hours (C – cultivation in microplates, D – cultivation on Agar plates). * denotes $p<0.05$.

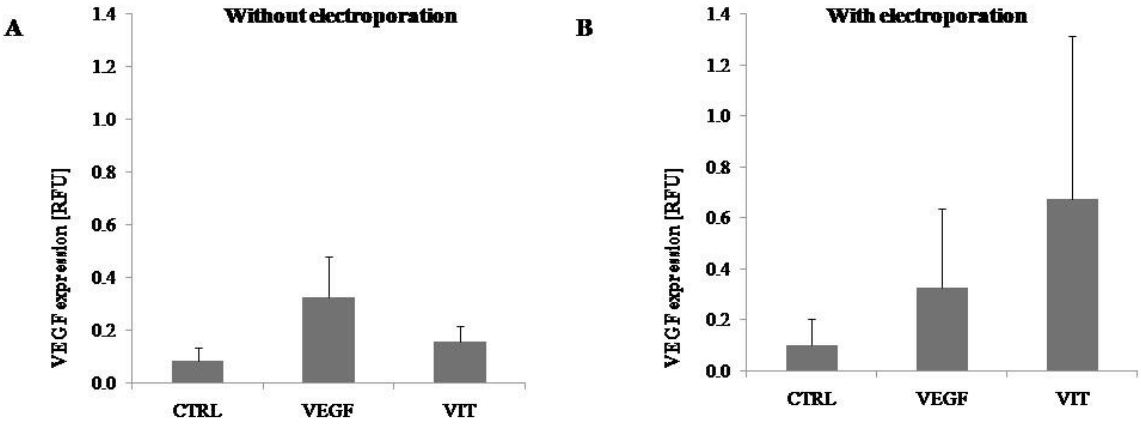

Figure 2: Analysis of VEGF gene expression by real time RT PCR in gastrocnemius muscle three days after electroporation *in vivo* (A – without electroporation, B – with electroporation). * denotes $p<0.05$.

IV. Discussion

Antioxidants in our experiments increased *in vitro* electroporation efficiency if applied directly to the electroporation buffer. Melatonin and vitamins C and E act as scavengers of free radicals that are produced during electroporation (Lipinski, 2011). In our experiment, the pre-treatment with vitamins increased electroporation efficiency.

These results are in accordance with previously published data (Gabriel and Teissie, 1994). Markelc et al. used high concentrations of vitamin C and tempol just prior to electroporation in order to increase the survival rate of the muscle cells.

Moreover, they showed that even 6mM concentration of vitamin C resulted in more than 70% survival of cells. Use of 1mM of vitamin C had less than

10% effect on cell survival. In addition, tempol increased the viability of cells after electroporation, while vitamin C had no effect. In our study, direct application of vitamin C and E during electroporation improved efficacy and transfection of plasmids into bacterial cells on agar also in lower concentrations of antioxidants, when compared to Markelc study (Markelc et al., 2012). Similar tendency was observed with direct application of vitamin C and E in microplates. This could be due to the synergistic effect of vitamin C as hydrophilic antioxidant and vitamin E as hydrophobic antioxidant (Celec et al., 2003). The pre-treatment of bacterial cells with melatonin decreased electroporation efficacy. The negative effect of melatonin might be related to the previously described antibacterial properties of melatonin (Tekbas et al, 2008).

Although the *in vivo* variability of transgene expression was too high to show significant differences, the tendencies between groups were comparable to the *in vitro* data. Vitamins seem to inhibit the gene transfer without electroporation, potentially due to low pH of the ascorbic acid. If electric pulses are applied, the antioxidant vitamins improve DNA delivery as shown by an increased transgene expression. Similar results were reported for the synthetic antioxidant tempol in *in vivo* (Markelc et al., 2012). Although electroporation efficacy was increased only in the presence of high concentrations of tempol, these results indicate that gene transfer efficiency mediated by electroporation could be improved by the addition of different ROS scavengers (Qian et al, 2006; Sabri et al, 1998).

Nevertheless, the idea of cell protection mediated enhanced gene transfer is not knew. Dimethylsulfoxide as cell membrane stabilizing agent, used mainly for cryoprotection, showed to increase electroporation efficiency 8-fold in *in vitro* experiments (Melkonyan et al, 1996).The lack of effects on oxidative stress markers could be explained by a very subtle and time-limited oxidative stress response induced by electroporation while samples were taken 3 days after electroporation. These results should be reproduced on eukaryotic cell cultures *in vitro* and in other tissues *in vivo*, as the conditions favorable for electroporation-mediated DNA delivery vary considerably between tissue types. Further studies should concentrate on whether such antioxidant approach would be efficient similarly when using alternating or direct currents, and whether frequencies used would further influence the efficacy result (Zhan et al, 2012). Such optimized electroporation method would be potentially applicable in gene transfer while studying brain development (Kanjhan et al, 2008) in neonatal pups, where high voltage usage are linked with high mortality and low efficiency rate of transfer (Ding et al, 2012), therefore new protective approaches are needed. Yet, another application would be therapeutical. For example, Machado-Aranda et al found out reduced lung injury after gene delivery of the Na^+/K^+ ATPase pump in mice (Machado-Aranda et al, 2012). Although short-termly, the results were promising for the treatment of acute lung injury, the voltages used were too high (200V/cm) and long-term mortality was not assessed. On the other hand, electroporation seems to be effective system to deliver nucleic acid into cancer cells (Heller et al, 2011). Cancer cells are prone to oxidative stress damage, since their energetic metabolism for antioxidant enzymes is directed to anaerobic metabolism (Daye et al, 2012) and endogenous antioxidant systems down-regulated through hypoxia-inducible factor (Goda et al, 2012). Electroporation protocols should bear this in mind, and the efficacy of gene delivery into such environment could be increased by adding antioxidant supplements that act as protections. Nevertheless, further studies are needed in order to prove this hypothesis.

In conclusion, our preliminary results show that a use of antioxidant vitamins during electroporation looks like a promising tool to improve the efficiency of electroporation-mediated gene transfer, both, *in vitro* and *in vivo*. In the future, studies evaluating the dose, timing together with endogenous antioxidant combination enhancement (glutathione, superoxide dismutase) should be performed and standardized.

Acknowledgement

This study was funded by the Slovak Research and Development Agency (SRDA) – VMSP-II-0027-09. The authors would like to thank Prof. Yoshikazu Yonemitsu (Department of Gene Therapy, Chiba University, Japan) for providing us with CA-VEGF plasmid.

References

Andre FM, Gehl J, Sersa G, Preat V, Hojman P, Eriksen J, Golzio M, Cemazar M, Pavselj N, Rols MP, Miklavcic D, Neumann E, Teissie J, Mir LM. (**2008**) "Efficiency of high- and low-voltage pulse combinations for gene electrotransfer in muscle, liver, tumor, and skin". **Hum Gene Ther**.19, 1261-71.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. **(2002)**Short Protocols in Molecular Biology. New York: Wiley. p. 1512.

Beebe SJ, Fox PM, Rec LJ, Willis LK, Schoenbach KH. (**2003**) "Nanosecond, high-intensity pulsed electric fields induce apoptosis in human cells". **Faseb Journal**.17, 1493.

Benov LC, Antonov PA, Ribarov SR. (**1994**) "Oxidative damage of the membrane-lipids after electroporation". **General Physiology and Biophysics**.13, 85-97.

Bodles-Brakhop AM, Heller R, Draghia-Akli R. (**2009**) "Electroporation for the Delivery of DNA-based Vaccines and Immunotherapeutics: Current Clinical Developments". **Molecular Therapy**.17, 585-92.

Celec P, Jani P, Smrekova L, Mrlian A, Kudela M, Hodosy J, Boor P, Kristova V, Jakubovsky J, Jezova D, Halcak L, Bozek P, Slamova J, Ulicna O, Hojsik D, Jurkovicova I. (**2003**) "Effects of anabolic steroids and antioxidant vitamins on ethanol-induced tissue injury". **Life Sci**.74, 419-34

Celec P, Hodosy J, Behuliak M, Palffy R, Gardlik R, Halcak L, Mucska I. (**2011**) "Oxidative and carbonyl stress in patients with obstructive sleep apnea treated with continuous positive airway pressure". **Sleep Breath**.

Czyz J, Guan K, Zeng Q, Nikolova T, Meister A, Schonborn F, Schuderer J, Kuster N, Wobus AM. (**2004**) "High frequency electromagnetic fields (GSM signals) affect gene expression levels in tumor suppressor p53-deficient embryonic stem cells". **Bioelectromagnetics**.25, 296-307.

Daye D, Wellen KE. (**2012**) "Metabolic reprogramming in cancer: Unraveling the role of glutamine in tumorigenesis". **Semin Cell Dev Biol**.23, 362-9.

Ding XF, Zhao YQ, Hu ZY, Lin K, Wang F, Liu SH, Wu Y, Wu LY, Zhao T, Huang X, Zhu LL, Fan WH, Fan M. (**2012**) "Efficient gene transfer into neonatal mouse brain using electroporation". **Neurochem Res**.37, 1392-8.

Escoffre JM, Portet T, Wasungu L, Teissie J, Dean D, Rols MP. (**2009**) "What is (Still not) Known of the Mechanism by Which Electroporation Mediates Gene Transfer and Expression in Cells and Tissues". **Molecular Biotechnology**.41, 286-95.

Gabriel B, Teissie J. (**1994**) "Generation of reactive-oxygen species induced by electropermeabilization of Chinese hamster ovary cells and their consequence on cell viability". **Eur J Biochem**.223, 25-33.

Goda N, Kanai M. (**2012**) "Hypoxia-inducible factors and their roles in energy metabolism". **Int J Hematol**.95, 457-63.

Hanahan D. (**1983**) "Studies on transformation of Escherichia coli with plasmids". **J Mol Biol**.166, 557-80.

Heller R, Shirley S, Guo S, Donate A, Heller L. (**2011**) "Electroporation based gene therapy--from the bench to the bedside". **Conf Proc IEEE Eng Med Biol Soc**.2011, 736-8.

Kanjhan R, Vaney DI. (**2008**) "Semi-loose seal Neurobiotin electroporation for combined structural and functional analysis of neurons". **Pflugers Arch**.457, 561-8.

Lipinski B. (**2011**) "Hydroxyl radical and its scavengers in health and disease". **Oxid Med Cell Longev**.2011, 809696.

Machado-Aranda DA, Suresh MV, Yu B, Raghavendran K. (**2012**) "Electroporation-mediated in vivo gene delivery of the Na+/K+-ATPase pump reduced lung injury in a mouse model of lung contusion". **J Trauma Acute Care Surg**.72, 32-9; discussion 9-40.

Markelc B, Tevz G, Cemazar M, Kranjc S, Lavrencak J, Zegura B, Teissie J, Sersa G. (**2011**) "Muscle gene electrotransfer is increased by the antioxidant tempol in mice". **Gene Ther**.

Melkonyan H, Sorg C, Klempt M. (**1996**) "Electroporation efficiency in mammalian cells is increased by dimethyl sulfoxide (DMSO)". **Nucleic Acids Res**.24, 4356-7.

Molnar MJ, Gilbert R, Lu Y, Liu AB, Guo A, Larochelle N, Orlopp K, Lochmuller H, Petrof BJ, Nalbantoglu J, Karpati G. (**2004**) "Factors influencing the efficacy, longevity, and safety of electroporation-assisted plasmid-based gene transfer into mouse muscles". **Mol Ther**.10, 447-55.

Natarajan M, Nayak BK, Galindo C, Mathur SP, Roldan FN, Meltz ML. (**2006**) "Nuclear translocation and DNA-binding activity of NFKB (NF-kappaB) after exposure of human monocytes to pulsed ultra-wideband electromagnetic fields (1 kV/cm) fails to transactivate kappaB-dependent gene expression". **Radiat Res**.165, 645-54.

Qian HS, Liu P, Huw LY, Orme A, Halks-Miller M, Hill SM, Jin F, Kretschmer P, Blasko E, Cashion L, Szymanski P, Vergona R, Harkins R, Yu J, Sessa WC, Dole WP, Rubanyi GM, Kauser K. (**2006**) "Effective treatment of vascular endothelial growth factor refractory hindlimb ischemia by a mutant endothelial nitric oxide synthase gene". **Gene Ther**.13, 1342-50.

Raptis L, Firth KL. (**2008**) "Electrode assemblies used for electroporation of cultured cells". **Methods Mol Biol**.423, 61-76.

Sabri N, Pelissier B, Teissie J. (**1998**) "Ascorbate increases electrotransformation efficiency of intact maize cells". **Anal Biochem**.264, 284-6.

Stride E, Porter C, Prieto AG, Pankhurst Q. (**2009**) "Enhancement of microbubble mediated gene delivery by simultaneous exposure to ultrasonic and magnetic fields". **Ultrasound Med Biol**.35, 861-8.

Sukhorukov VL, Reuss R, Zimmermann D, Held C, Muller KJ, Kiesel M, Gessner P, Steinbach A, Schenk WA, Bamberg E, Zimmermann U. (**2005**) "Surviving high-intensity field pulses: strategies for improving robustness and performance of electrotransfection and electrofusion". **J Membr Biol**.206, 187-201.

Teissie J, Golzio M, Rols MP. (**2005**) "Mechanisms of cell membrane electropermeabilization: a minireview of our present (lack of ?) knowledge". **Biochim Biophys Acta**.1724, 270-80.

Tekbas OF, Ogur R, Korkmaz A, Kilic A, Reiter RJ. (**2008**) "Melatonin as an antibiotic: new insights into the actions of this ubiquitous molecule". **Journal of Pineal Research**.44, 222-6.

Zhan Y, Cao Z, Bao N, Li J, Wang J, Geng T, Lin H, Lu C. (**2012**) "Low-frequency ac electroporation shows strong frequency dependence and yields comparable transfection results to dc electroporation". **J Control Release**.160, 570-6.