

Full Length Research Paper

Influence of electroporation on matrix metalloproteinases (MMPs) pattern activity in malignant glioma cells propagated *ex vivo*

Monira M. Rageh^{1*}, Anwar A. Elsayed¹, Amr A. Abd-Elghany¹, Mohammed S. Mohammed²

¹Department of Biophysics, Faculty of Science, Cairo University, Egypt.

²Department of Cancer Biology, National Cancer Institute (NCI), Cairo University, Egypt.

Accepted 18 June, 2007

Cells exposed to short and intense electric pulses become permeable to a number of various ionic molecules. This phenomenon was termed electroporation or electropermeabilization and is widely used for *in vitro* drug delivery into cells and gene transfection. The present work is a trial to study the effect of single exponential pulsed electric field (PEF) using intensity range 0.3 to 5.7 KV /cm and 1 m sec duration on human malignant glioma cells (U251MG). Under electrical conditions maintaining cell viability, no permeabilization can be detected for macromolecules such as DNA for pulse durations shorter than 1m sec. The aim of this study was to find optimum values of field strengths: First, to maintain cell viability for gene transfection and electrochemotherapy for further investigations. The above range of field strengths was used because a strong increase in the electric field intensity may increase transport of macromolecules, but as it may induce a loss in cell viability, indicated by the morphological observations in this study; it is associated with a decrease in plasmid expression. Second, to investigate the influence of PEF on metastatic biomolecules secreted in the cultured media such as matrix metalloproteinases (MMPs). MMPs enzyme activity was reduced at electric field intensities (0.3 - 1.15 KV/cm) by a factor of (25 - 100%). It is recommended to use the field intensity 0.85 KV/cm to put the glioma cells under biochemical stress during the electroporation protocol using single exponential pulse and 1 ms duration.

Key words: Malignant glioma cells, electroporation, MMPs.

INTRODUCTION

Exposure of suspended cells to an exponentially decaying external electric field pulse of high intensity (kV/cm) and short duration (from 100 μ s to several milliseconds) leads to a reversible electric breakdown of the membrane (Zimmermann et al., 1974). The breakdown of the membrane firstly is associated with a temporary increase of the membrane permeability. These permeability changes may be rapidly reversible or irreversible depending on the intensity and the width of the electrical pulses, as well as the composition of the suspending medium. After the rapid increase of the membrane permeability, many de-

layed effects of the electrical stimulation were observed. These slower secondary effects included membrane fusions, membrane bleb formation, endocytotic reactions, and reorganization of the cytoskeletal network and in severe cases lysis of the cells. Global membrane rupture and cell death were mainly due to these secondary effects. Experiments showed that electrical stimulation introduced pores of limited sizes in the plasma membrane (Neumann et al., 1989). These pores could be resealed without losing the cytoplasmic macromolecular contents, and most cells survived after pore resealing. Electroporation possessed many applications in molecular biology, genetic engineering, drug delivery, and biotechnology (Tsong, 1996). The exponential decay electroporators generally places considerable stress on the

*Corresponding author. E-mail: monirarageh@yahoo.com.

cells being treated because discharging the entire capacitor into the cuvette often generates significant heat. Exponential electroporators are also effectively single pulse devices. The pulse duration parameter was shown to be crucial for the penetration of macromolecules into Chinese hamster ovary cells under conditions where cell viability was preserved (Role and Teissie, 1998). No permeabilization could be detected for molecules with molecular mass higher than 10 kDa for pulse durations shorter than 1 ms. Increasing the electric field intensity E may increase transport of macromolecule, but as it may induce a loss in cell viability, it is associated with a decrease in plasmid expression. Optimum conditions for membrane electropermeabilization were obtained at high pulse duration T values and moderate values of the electric field intensity E to preserve the viability (Rols and Teissie, 1990a). There was much evidence that matrix metalloproteinases (MMPs) played an important role in extracellular matrix remodeling in physiologic situations, such as fetal tissue development and post-natal repair, and that excessive breakdown of extra-cellular matrix by MMPs occurred in many pathologic conditions including periodontitis, dermal photo aging, rheumatoid arthritis and chronic ulceration. In addition, controlled degradation of the extracellular matrix by MMPs was thought to play an important role in tumor invasion and metastasis (Kahari and Saarialho-Kere, 1997, 1999; Nagase and Woessner, 1999; Shapiro, 1998). MMPs are continually growing family of enzymes which currently consists of at least 23 well characterized members (McDonnell et al., 2002), and according to structure and substrate specificity they can be divided into subgroups of collagenases, stromelysins, gelatinases, membrane-type MMPs (MT-MMPs) and other MMPs. Latent progelatinase A (66 kDa) can be detected in the conditioned medium of four highly invasive glioma cell lines (UWR2, UWR3, U251MG and SNB19) (Yamamoto, 1996). Matrilysin (MMP-7) is one of the stromelysins, which is the smallest MMPs (Latent MW 28 kDa and the active MW 19 kDa) as it lacks the hemopexin domain. The aim of this study was to find an optimum value of field strength corresponding to the lowest enzyme activity of MMPs secreted by U251MG cells to put the cells under biochemical stress during the electroporation protocol using single exponential pulse and 1m sec duration.

MATERIALS AND METHODS

Cell culture (Canatella et al., 2001)

1. Human malignant glioma cells (U251 MG) derived from malignant astrocytic tumors were grown as a monolayer in a sterile/ γ -irradiated tissue culture treated flask (25 cm³) at a humidified 37°C, 5% CO₂ incubator (HERA Cell, Germany) using medium designed at Roswell Park Memorial Institute (RPMI-1640) with 2 mM-glutamine, 10% (v/v) heat inactivated (1/2 h at 56°C) fetal calf serum (FCS) and 1% antibiotics (100 μ g/ml penicillin, 100 μ g/ml streptomycin) purchased from Sigma Chemical company, St. Louis, MO, USA.

2. After 3 days the cells were examined under the inverted light microscope Olympus, 1x 70 (Tokyo, Japan) with magnification 100x and the cells had taken a spindle shape.

Application of the electrical pulse to the cells

The required number of flasks containing monolayer of exponentially growing cells was treated with 1 mL trypsin EDTA (sigma co.) until the cells can be brought into suspension. The trypsin EDTA was removed by pouring off the supernatant after centrifugation at 105 x g (1200 rpm, Labofuge Ae, Germany). The pellet was washed in phosphate buffer saline (PBS), at ambient temperature. The cells were recentrifuged and resuspended in PBS at ambient temperature to $1.5-2 \times 10^6$ cells/mL. Electroporation was performed at room temperature by introducing 400 μ L of cell suspension into the cuvette of electroporator 1000 (Cambridge, UK) which has parallel plate aluminum electrodes and 2 mm gap width. The cells were electroporated using single exponential pulse, exponential decay time constant 1 ms and field strengths (0.3 - 5.7 kV/cm). All the above steps were performed under sterile conditions inside a laminar flow hood. After electroporation the cells were transferred back into RPMI-1640 culture medium and incubated for 3 and 6 days.

Morphological observation

The cells exposed to different values of field strengths and harvested 3 days were investigated under the inverted light microscope Olympus, 1 x 70, (Tokyo, Japan), with magnification 100x using a camera connected to the microscope to observe the change in cell morphology and viability (Rols and Teissie, 1998).

Gelatin zymography

The enzymatic activity and molecular weight of electrophoretically separated forms of collagenolytic enzymes were determined in the conditioned medium of the cells with and without pulsed electric field treatment using SDS-PAGE. A 10 % polyacrylamide gel was prepared as described by Laemmli (1970) with modification where 0.5% of gelatin type B which acts as an enzymatic substrate was added to the resolving gel. The gel was pre-electrophoresed for 30 minutes to remove undesirable materials in the gel (unreacted monomers and ammonium persulphate). Equal volumes of samples (the conditioned medium of the cells treated with and without pulsed electric field range 0.3 KV/cm to 1.65 KV/cm) and loading buffer (0.125 M Tris HCl, pH 6.6; 4% SDS; 20% glycerol; 0.005% bromophenol blue) were mixed. 20 μ L of each sample mixture was applied in each well. The current used in the electrophoresis procedure was 10 mA till the samples reach the end of stacking gel and the current was raised to 20 mA when the samples were in the resolving gel for 3 h. The temperature of electrophoresis did not exceed 37°C throughout the whole procedure. After the electrophoresis was completed, the gel was immersed in washing buffer (50 mM Tris HCl; pH 7.5, 5 mM Ca Cl₂, 1 mM Zn Cl₂, 2.5% Triton X-100) for 30 min at room temperature. The washing buffer was removed and an adequate volume of incubation buffer (its constituent is the same as washing buffer except the concentration of triton X - 100 is 1%) was added to immerse the gel and was incubated at 37°C overnight. Then the incubation buffer was removed. The gel was stained using Coomassie brilliant blue G-250 solution (0.125% Coomassie brilliant blue G-250, 50% methanol, 10% glacial acetic acid and 40% distilled water) for half hour while shaking at room temperature. Then the staining buffer was removed and the gel was immersed in a destained solution (I) (50% methanol, 10% glacial acetic acid and 40 % distilled water) for 5

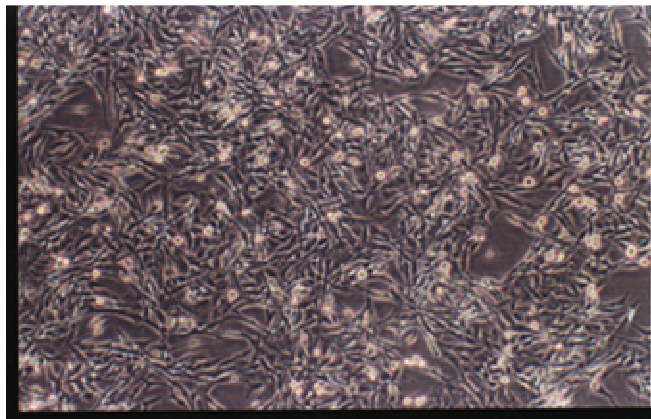


Figure 1. The human malignant glioma cells (U251MG) growing *in vitro*. (1): Control (untreated) cells.

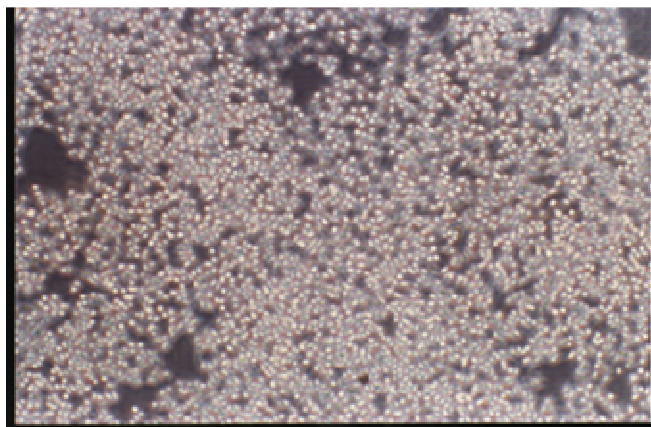


Figure 2. The human malignant glioma cells (U251MG) growing *in vitro*. Cells treated with electric pulse of intensity 0.3 kV/cm.

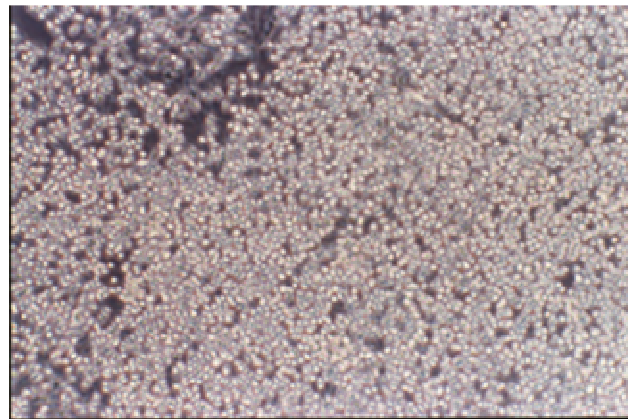


Figure 3. The human malignant glioma cells (U251MG) growing *in vitro*. Cells treated with electric pulse of intensity 0.7 kV/cm.

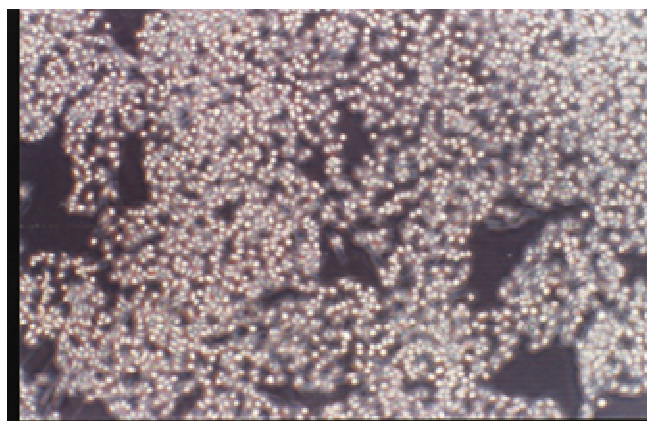


Figure 4. The human malignant glioma cells (U251MG) growing *in vitro*. Cells treated with electric pulse of intensity 1.15 kV/cm.

min at room temperature. The destained solution (I) was removed and an adequate volume of destained solution (II) (5% methanol, 7% glacial acetic acid and 88% distilled water) was added to the gel until the zones of MMPs clearly appeared. Gelatinolytic activity was quantified by densitometric measurement of the intensity of the bands seen on the gels (Yamamoto et al., 1996).

RESULTS

Effect of pulsed electric field on cell morphology

The ability of pulsed electric field to produce morphological changes and cell viability was observed using inverted light microscope. The pictures obtained showed global changes in membrane morphology where the control group of U251MG cells had spindle shape as shown in (Figure 1). After the application of single exponential pulse using different field intensities from 0.3 kV/cm to 5.7 kV/cm, the cells became rounded and some of them fused together (Figures 2 - 10). Cell viability observations

showed significant decrease in the number of cells at field intensities up to 1.3 kV/cm (Figures 5 - 9), the maximal response was observed at 5.7 kV/cm where few cells can be seen as shown (Figure 10).

Gelatin zymography

Proteases are capable of gelatinolysis generating zones of lysis, which appeared as white bands against a dark blue background. In contrast, all non-gelatinolytic proteins appeared as blue stained bands. Zymograms showing gelatinases expression in conditioned media of the human malignant glioma cells (U251MG) harvested for 3 and 6 days are shown in Figures 11 and 16 respectively. The control sample in lane 2 has two major bands with gelatinolytic activity migrating at relative molecular weights of 66 kDa and 19 kDa. The gelatinolytic activities at 66 and 19 kDa corresponding possibly to active MMP-2 and MMP-7 respectively.

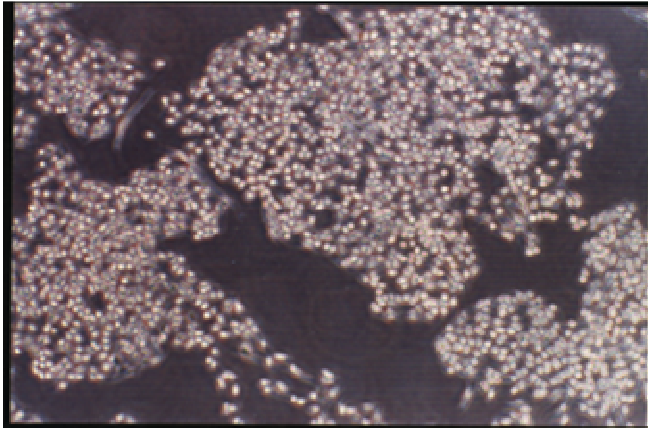


Figure 5. The human malignant glioma cells (U251MG) growing *in vitro*. Cells treated with electric pulse of intensity 1.3kV/cm.

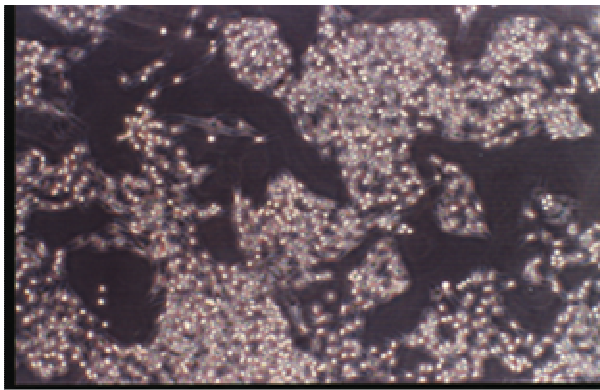


Figure 6. The human malignant glioma cells (U251MG) growing *in vitro*. Cells treated with electric pulse of intensity 1.65 kV/cm.

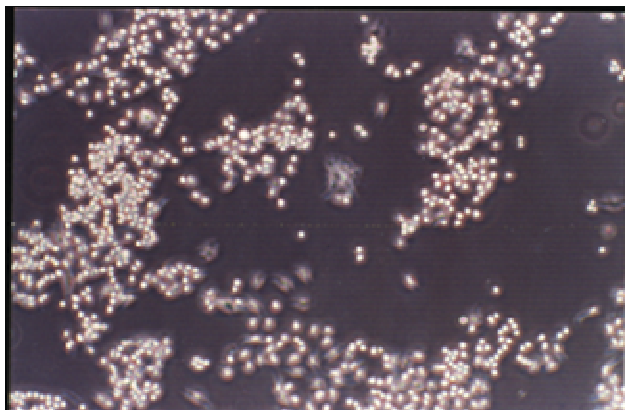


Figure 7. The human malignant glioma cells (U251MG) growing *in vitro*. Cells treated with electric pulse of intensity 2.1 kV/cm.

Figure 11 showed the intensity of the gelatinolytic bands corresponding to active MMP-2 (66 kDa) in the condition media of the U251MG cells harvested for 3

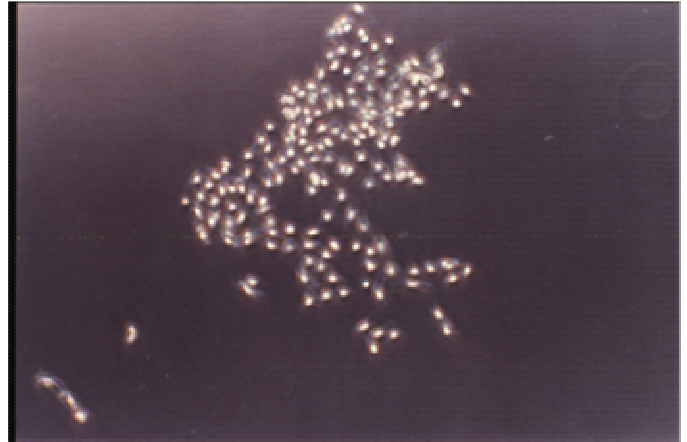


Figure 8. The human malignant glioma cells (U251MG) growing *in vitro*. Cells treated with electric pulse of intensity 3.6 kV/cm.

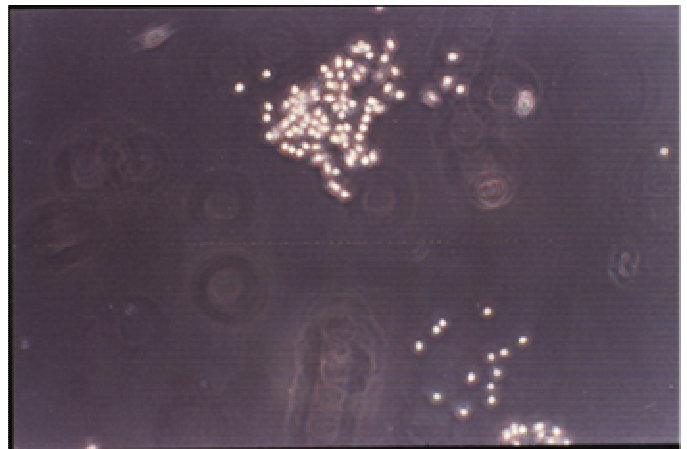


Figure 9. The human malignant glioma cells (U251MG) growing *in vitro*. Cells treated with electric pulse of intensity 5.2 kV/cm.

days due to the effect of electric pulse at different intensities (0.3, 0.55, 0.75, 0.85, 1.15, 1.3 and 1.65 kV/cm) showed minimum activity (less than the control) of enzymes at electric field intensities 0.3 to 1.15 kV/cm, but the activity of the enzymes was increased at field intensities 1.3 and 1.65 kV/cm. Bands corresponding to active MMP-7 migrating at 19 kDa, appeared only at electric field intensities 1.3 kV/cm and 1.65 kV/cm. Moreover extra bands appeared at electric field intensities 1.3 and 1.65 kV/cm and migrated at relative molecular weights 240 kDa and 92 kDa respectively.

Figure 16 showed the intensity of the gelatinolytic sharp and wide bands corresponding to active MMP-2 and MMP-7 in the conditioned media of cells treated with electric pulse at different intensities (0.3, 0.55, 0.75, 0.85, 1.15, 1.3 and 1.65 kV/cm) and harvested for 6 days. The activity of MMP-2 and MMP-7 in all the treated samples were weak compared to control (untreated) samples. Densitometric scanning as a semi-quantitative method of

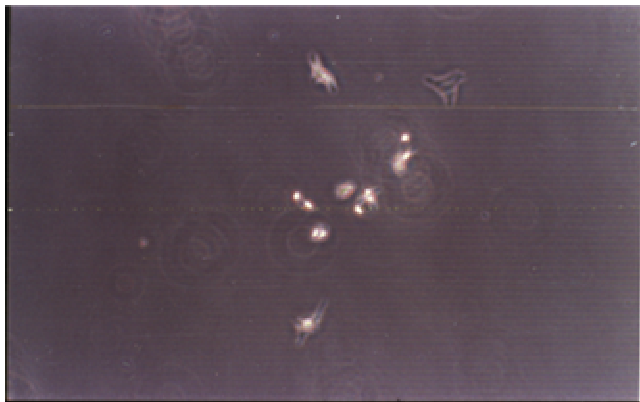


Figure 10. The human malignant glioma cells (U251MG) growing *in vitro*. Cells treated with electric pulse of intensity 5.7 kV/cm.

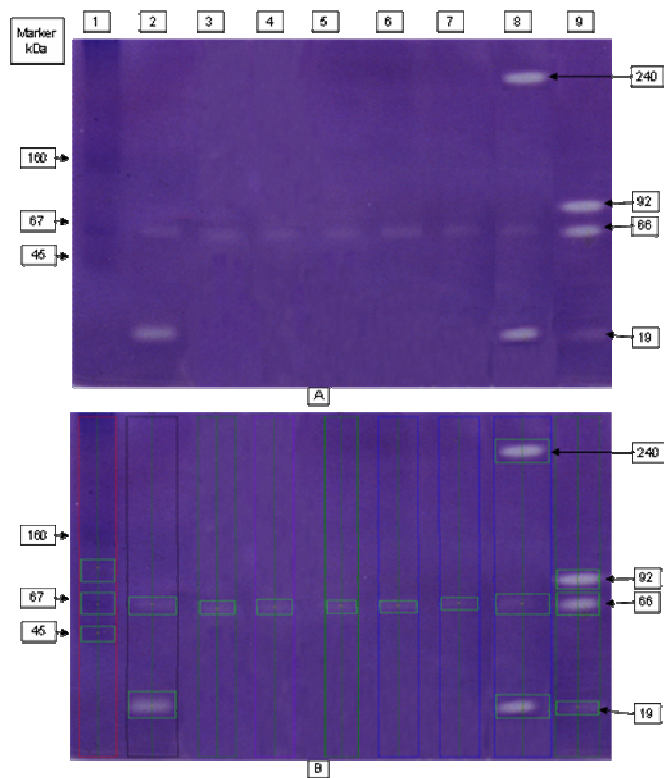
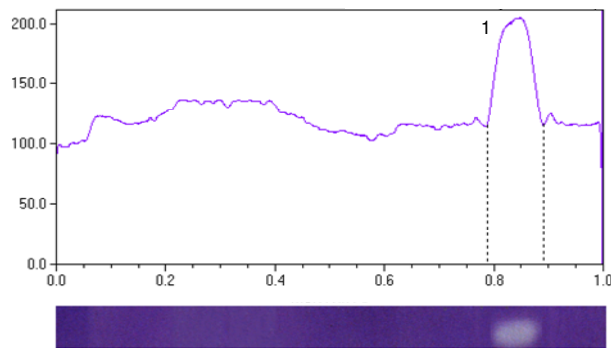


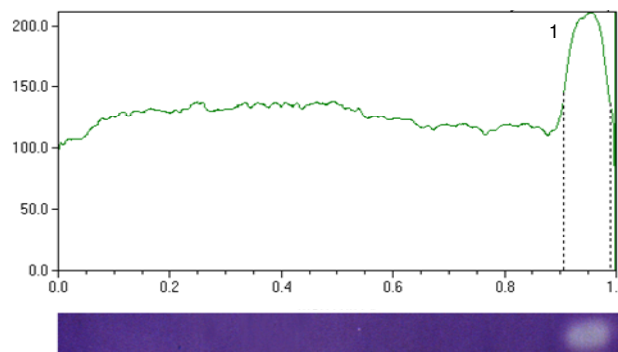
Figure 11. [A] SDS-PAGE (10%) Zymography of matrix metalloproteinases (MMPs) in the conditioned media (CM) of U251MG cells harvested at 3 days and treated with single exponential pulse using different intensities as well as in the control sample. [B] Computer scan of the same bands Lane 1: protein marker; Lane 2: control (untreated); Lane 3: 0.3 kV/cm; Lane 4: 0.55 kV/cm; Lane 5: 0.75 kV/cm; Lane 6: 0.85 kV/cm; Lane 7: 1.15 kV/cm; Lane 8: 1.3 kV/cm; Lane 9: 1.65 kV/cm.

analysis was setup to quantify the intensity of the above mentioned MMPs bands (Figures 12 - 15 and 17, 18) and could give values for the activity of protease enzymes in treated samples relative to the control (untreated) sam-



Electric field intensity (kV/cm)	Control	0.3 kV/cm	0.55 kV/cm	0.75 kV/cm	0.85 kV/cm	1.15 kV/cm	1.3 kV/cm	1.65 kV/cm
Intensity of the peak	0	0	0	0	0	0	980424	0

Figure 12. Densitometric scanning of an extra band at 240 kDa in Figure 11. Table illustrates the intensity of the peaks.



Electric field intensity (kV/cm)	Control	0.3 kV/cm	0.55 kV/cm	0.75 kV/cm	0.85 kV/cm	1.15 kV/cm	1.3 kV/cm	1.65 kV/cm
Intensity of the peak	0	0	0	0	0	0	0	719015

Figure 13. Densitometric scanning of an extra band at 92 kDa in Figure 11. Table illustrates the intensity of the peaks.

ple.

DISCUSSION

Electric fields have been employed in several different types of cancer therapy. Some of these involve radio frequency or microwave devices that heat the tumor greater than 43°C to kill the cells via hyperthermia (Nuccitelli et al., 2006). Others use static or slowly varying pulsed electric fields that accumulate the charges along the plasma membrane and, as a result, the potential difference across the membrane will change from the resting value. The plasma membrane behaves as a leaky dielec-

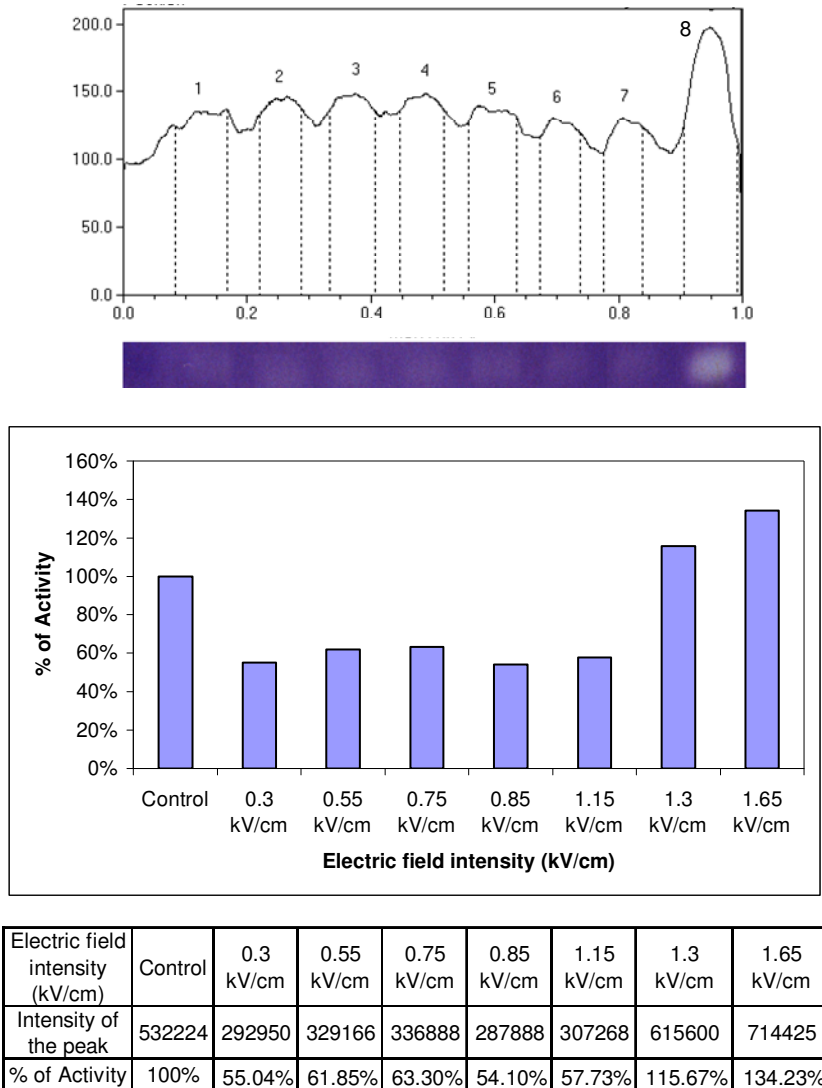


Figure 14. Densitometric scanning of MMP-2 (66 kDa) in Figure 11. Table illustrates the intensity of the peaks and the % of change from control considering the control as 100 %.

tric, allowing for transfer of molecules across the cell membrane. The pore size is a function of the duration of the electric field pulse. Traditional electroporation utilizes pulses of microseconds to milliseconds in duration and few hundred volts to several kilovolts per centimeter. This generates pores large enough to transfer large molecules across the membrane (Frey et al., 2006). If the cell is exposed to greater electric field, the electroporated state is irreversible and cell death will follow. It is suggested that death from irreversible permeabilization occurs less frequently *in vivo* than *in vitro*. It is thought that the efflux of important molecules from the cell is greater *in vitro* than *in vivo*, because the extra cellular space in tissue is quite small compared to the extra cellular medium in cell cultures (Gothelf et al., 2003). According to morphological observations in the present work, morphological changes

of the cell membrane from spindle shape (Figure 1) to rounded one (Figures 2 - 10) were observed. These changes may be due to the presence of a temperature gradient across cell membrane due to non uniform heating, water activity of the cytoplasm will be different from that of the external medium effects. Thermal osmosis effects would cause cell swelling (Tsong, 1996).

The exponential decay electroporators generally places considerable stress on the cells being treated because discharging the entire capacitor into the cuvette often generates significant heat. Exponential electroporators are also effectively single pulse devices. The dependence of viability on field strength was explained by Canatella et al. (2001). The study of Canatella and his colleagues indicated that above a quasi-threshold, viability decreases with increasing field strengths. In this study

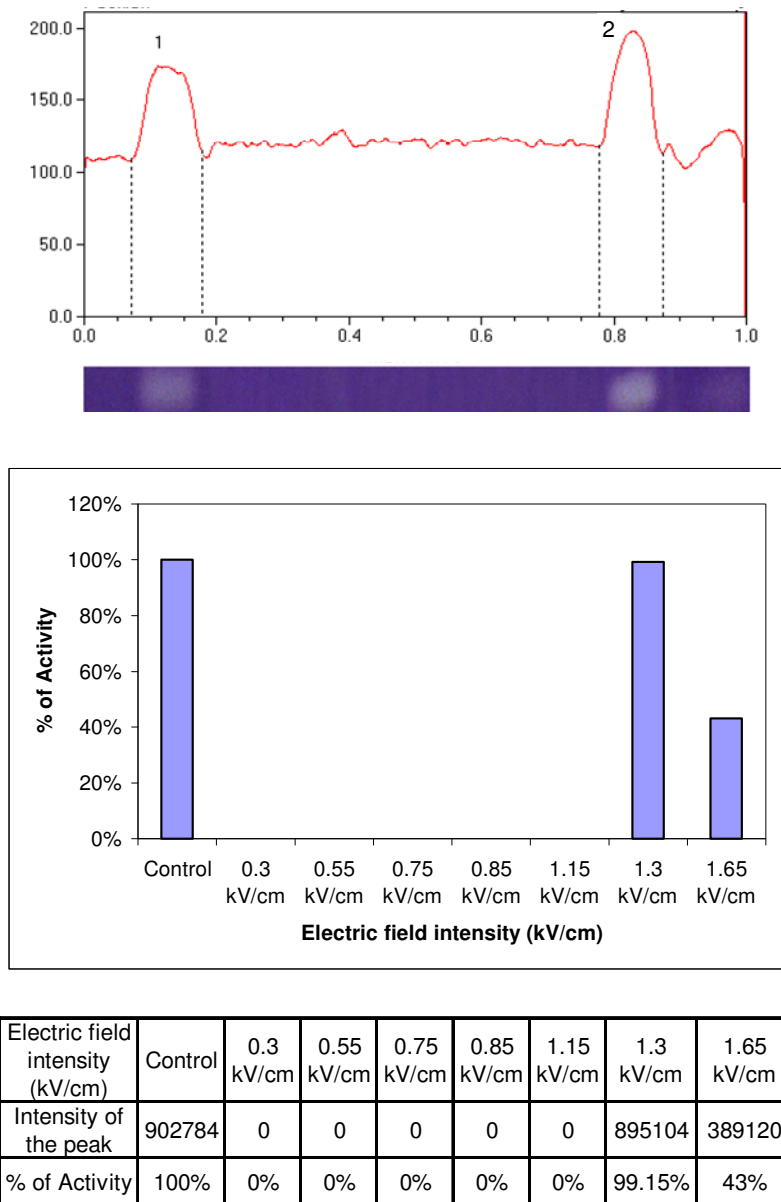


Figure 15. Densitometric scanning of MMP-7 (19 kDa) bands in Figure 11. Table illustrates the intensity of the peaks and the % of change from control considering the control as 100 %.

cell viability were affected greatly by the exposure of U251MG cells to pulsed electric field starting at 1.3 kV/cm. The marked decrease in cell density at high electrical field strengths may be due to cellular stress caused by electroporation. This may lead to cell lysis (Tsong, 1996; Rols and Teissie, 1998; Hofmann et al., 1999).

Tumor cells can release a variety of degradative enzymes, and enhanced proteolytic activity that has been associated with tumor growth, angiogenesis, invasiveness and metastasis such as matrix metalloproteinases MMPs (Hewitt et al., 1991). MMPs activities were detected in various brain tumor extracts as well as in the conditioned media highly invasive glioma cell lines by

several investigators (Rao et al., 1993; Yamamoto et al., 1996). In this study, the results showed the presence of several gelatinolytic enzymes in the conditioned media of the human glioma cells (U251MG) which were harvested for 3 and 6 days. The sharp bands at 66 and 19 kDa showed that the gelatinolytic activity increased at electric field intensities 1.3 and 1.65 kV/cm (Figure 11). The activity of MMP-2 was increased by 34% at 1.65 kV/cm (Figure 11). However the bands corresponding to activate MMP-7 (19 kDa) appeared only in the control samples and in that treated with 1.3 and 1.65 kV/cm (Figure 11), indicating that the electric field inhibited the activity of MMP-7 at other field values. Extra bands at

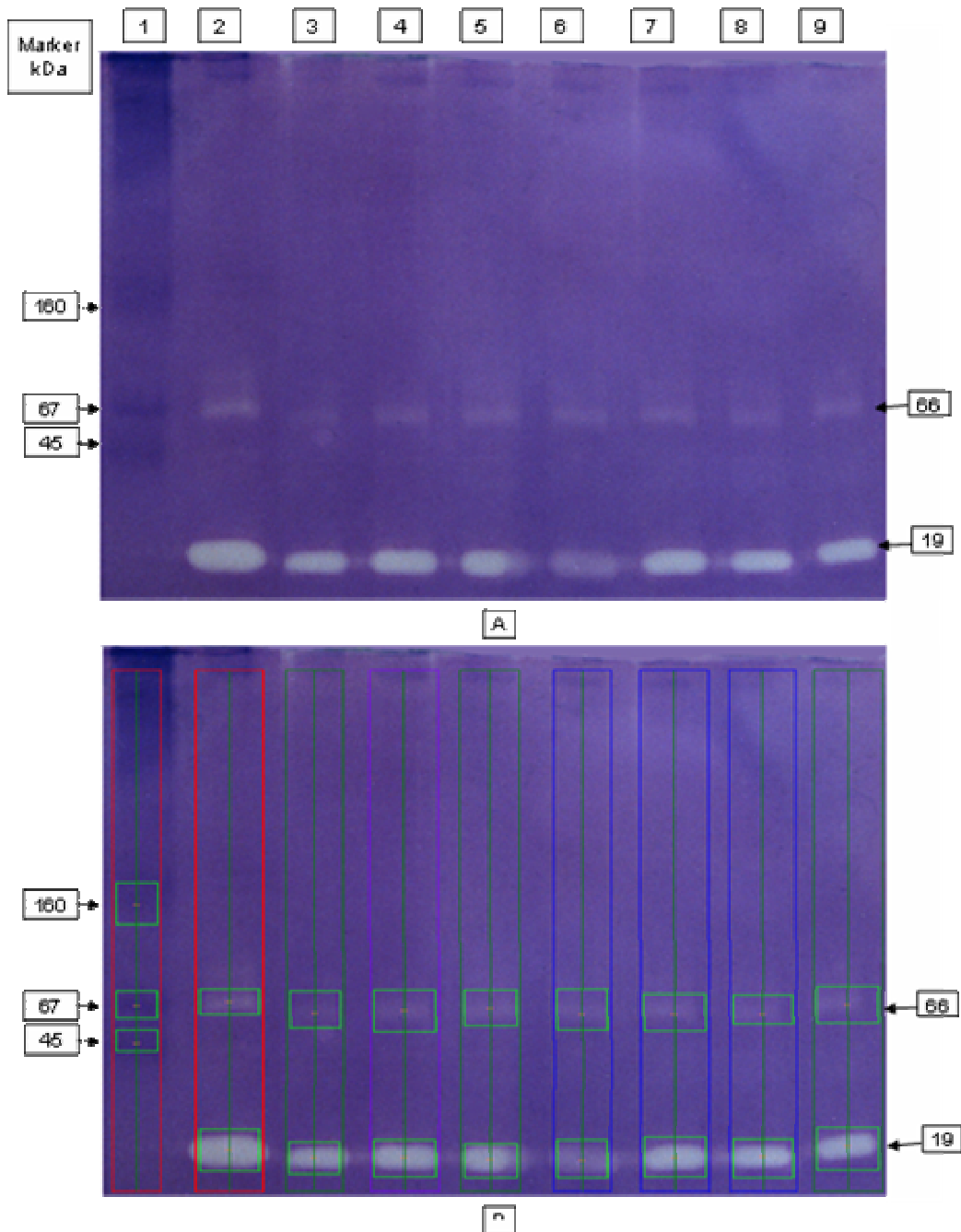
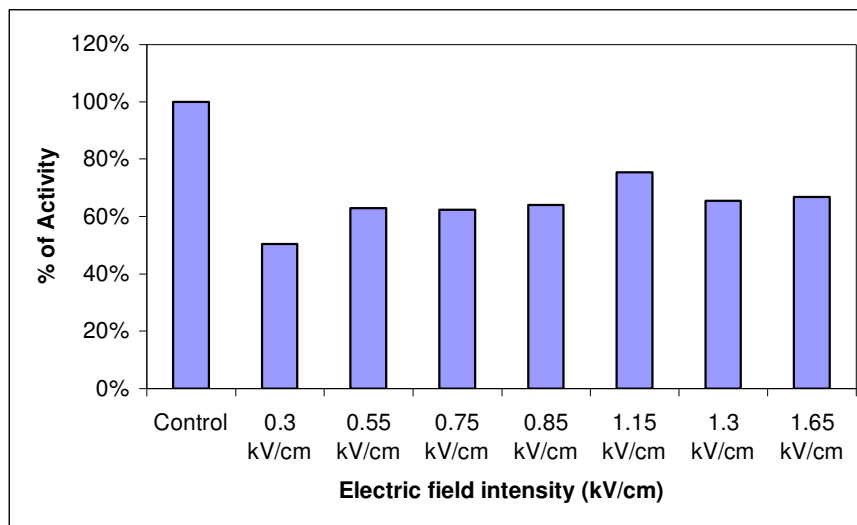
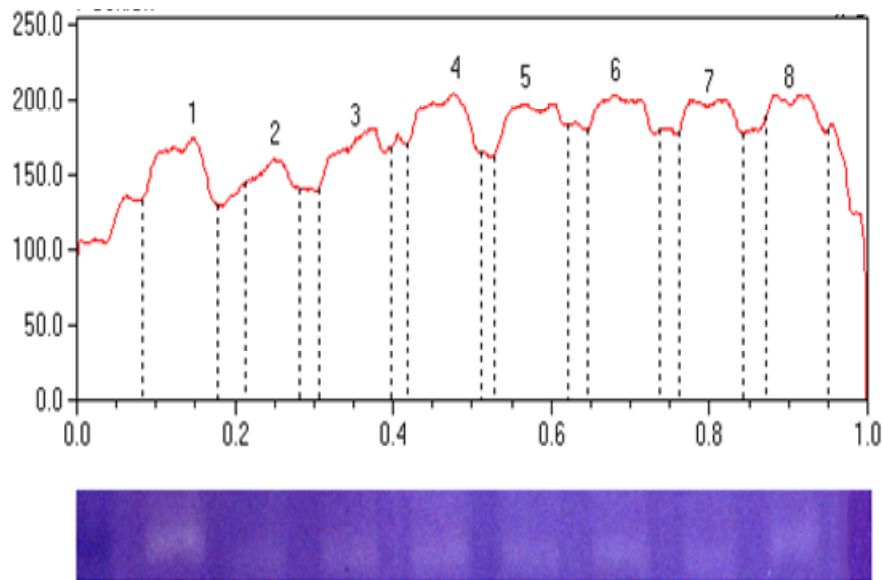


Figure 16. [A] SDS-PAGE (10%) Zymography of matrix metalloproteinases (MMPs) in the conditioned media (CM) of U251MG cells harvested at 6 days and treated with single exponential pulse using different intensities as well as in the control sample.[B] Computer scan of the same bands. Lane 1: protein marker; Lane 2: control (untreated); Lane 3: 0.3 kV/cm; Lane 4: 0.55 kV/cm; Lane 5: 0.75 kV/cm; Lane 6: 0.85 kV/cm; Lane 7: 1.15 kV/cm; Lane 8: 1.3 kV/cm; Lane 9: 1.65 kV/cm.

240 and 92 kDa (Figure 11) were observed for field strengths 1.3 and 1.65 kV/cm respectively. This may be due to cellular stress caused by pulsed electric field, which may lead to cell lysis and other types of proteases

produced by the cells. The obtained results also demonstrated that at 6 days after treatment with PEF the tumor cells regained its ability to produce activated MMP-2 and MMP-7 (Figure 16). Samples treated with 0.85 kV/cm



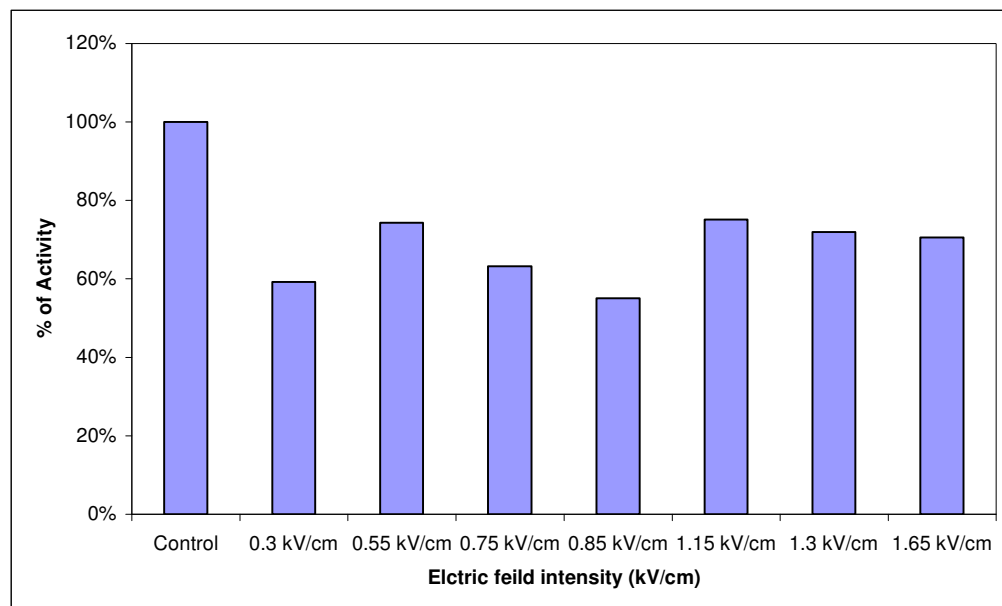
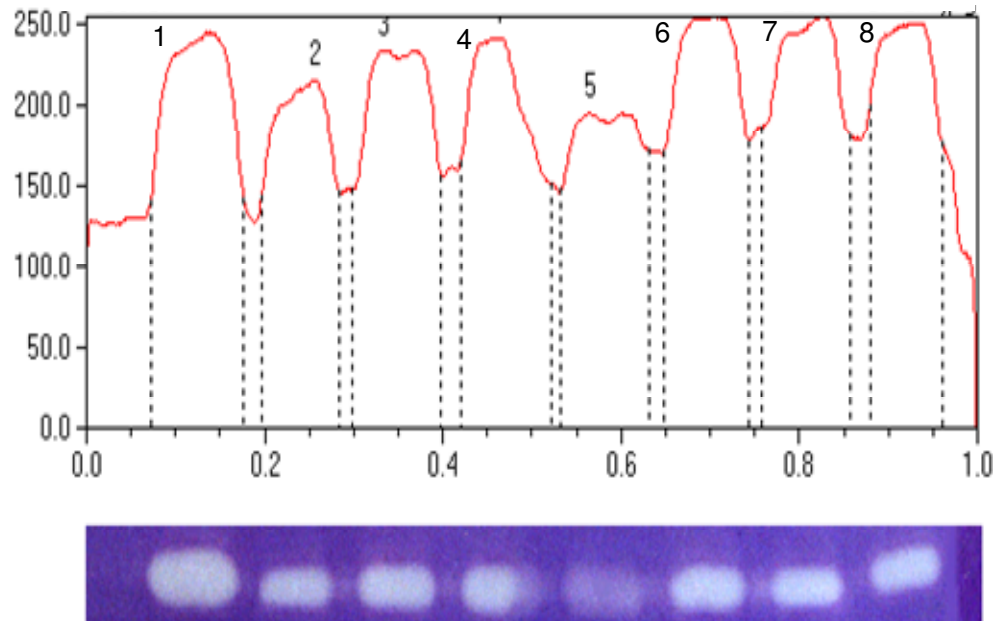
Electric field intensity (kV/cm)	Control	0.3 kV/cm	0.55 kV/cm	0.75 kV/cm	0.85 kV/cm	1.15 kV/cm	1.3 kV/cm	1.65 kV/cm
Intensity of the peak	373752	188384	235264	232960	239506	282048	244644	249516
% of Activity	100%	50.40%	62.95%	62.33%	64.08%	75.46%	65.46%	66.76%

Figure 17. Densitometric scanning of MMP-2 (66 kDa) bands in Figure 16. Table illustrates the intensity of the peaks and the % of change from control considering the control as 100 %.

exhibited the lowest activity for MMP-7 (19 kDa) whereas, samples treated with 0.3 kV/cm exhibited the lowest activity for the other protease MMP-2, 66 kDa.

In conclusion, it was found that both 0.3 and 0.85 kV/cm represented suitable values for putting the cells

under biochemical stress during gene or drug delivery by electroporation technique since PEF at these values could exhibit marked depression in the activity of the biomolecules operating in tumor angiogenesis and metastasis. The electric pulse intensity 0.85 kV/cm was chos-



Electric field intensity (kV/cm)	Control	0.3 kV/cm	0.55 kV/cm	0.75 kV/cm	0.85 kV/cm	1.15 kV/cm	1.3 kV/cm	1.65 kV/cm
Intensity of the peak	606600	359476	450560	383438	333424	455560	436032	427808
% of Activity	100%	59.26%	74.28%	63.21%	54.97%	75.10%	71.88%	70.53%

Figure 18. Densitometric scanning of MMP-7 (19 kDa) bands in Figure 16. Table illustrates the intensity of the peaks and the % of change from control considering the control as 100 %

en as an optimum value for the therapeutic target for cancer management, since as shown by Canatella et al.

(2001) uptake increases with increasing field strength and in some case may reach constant value at high field

strength.

REFERENCES

- Canatella PJ, Karr JF, Petros JA, Prausnitz MR (2001). Quantitative study of electroporation-mediated molecular uptake and cell viability, *Biophys. J.* 80: 755-764.
- Frey W, White JA, Price, OR, Blackmore FP, Joshi PR, Nuccitelli R, Beebe SJ, Schoenbach HK, Kolb FJ (2006). Plasma membrane voltage changes during nanosecond pulsed electric field exposure, *Biophys. J.* 90: 3608-3615.
- Gothelf A, Mir ML, Gehl J (2003). Electrochemotherapy: results of cancer treatment using enhanced delivery of bleomycin by electroporation, *Cancer Treat. Rev.* 29: 371-387.
- Hewitt RE, Leach IH, Powe DG, Clark IM, Cawston TE, Turner DR (1991). Distribution of collagenase and tissue inhibitor of metalloproteinases TIMP in colorectal tumors, *Int. J. Cancer* 49: 666-672.
- Hofmann F, Ohnimus H, Scheller C, Strupp W, Zimmermann U, Jassoy C (1999). Electric field pulses can induce apoptosis, *J. Membrane Biol.* 169: 103-109.
- Kahari VM, Saaialho-Kere U (1997). Matrix metalloproteinases in skin, *Exp. Dermatol.* 6: 199-213.
- Kahari VM, Saaialho-Kere U (1999). Matrix metalloproteinases and their inhibitors in tumor growth and invasion, *Ann. Med.* 31: 34-45.
- Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T₄, *Nature* 227: 680-685.
- McDonnell S, O'Connor A, Murray D, Lynch CC (2002). Matrix metalloproteinases In: Genazzani AR eds. Hormone replacement therapy and cancer, The Parthenon publishing group ACRC press company.
- Nagase H, Woessner JF (1999). Matrix metalloproteinases, *Biol. Chem.* 274: 21491-21494.
- Neumann E, Sowers, AE, Jordan CA (1989). Electroporation and electrofusion in cell biology, plenum press, London.
- Nuccitelli R, Pilquett U, Chen X, Ford W, Swanson RJ, Beebe JS, Kolb FJ, Schoenbach HK (2006). Nanosecond pulsed electric fields cause melanomas to self destruct, *Biochem. Biophys. Res. Comm.*, 343: 351-360.
- Rao JS, Steck PA, Mohanam S, Stetler-Stevenson WG, Liotta LA, Sawaya R (1993). Elevated levels of M, 92000 type IV collagenase in human brain, *Cancer Res.* 53: 2208-2211.
- Rols MP, Teissié J (1990a). Electroporation of mammalian cells to macromolecules: control by pulse duration, *Biophys. J.* 58: 1089-1098.
- Rols MP, Teissié J (1998). Electroporation of mammalian cells to macromolecules: control by pulse duration, *Biophys. J.* 75: 1415-1423.
- Shapiro SD (1998). Matrix metalloproteinases degradation of extracellular matrix: biological consequences, *Curr. Opin. Cell Biol.* 10: 602-608.
- Tsong TY (1996). Electrically stimulated membrane breakdown, In: Lynch PT, Davey MR eds. Electrical manipulation of cells. Chapter 2 p. 15.
- Yamamoto M, Mohanam S, Sawaya R, Fuller GN, Seiki M, Sato H, Gokuslan ZL, Liotta LA, Nicolson GL, Rao JS (1996). Differential expression of membrane type matrix metalloproteinases and its correlation with gelatinase A activation in human malignant brain tumors in vivo and in vitro, *Cancer Res.* 56: 384-392.
- Zimmermann U, Pilwat G, Riemann F (1974). Dielectric breakdown of cell Membranes, *Biophys. J.* 14: 881-889.