Iranian Journal of Basic Medical Sciences
Vol. 11, No. 2, Summer 2008, 97 - 103
Received: May 8, 2008; Accepted: Aug 5, 2008

IJBMS

Relative Electropermeability and Electric Pulse Effectiveness in Human Breast Adenocarcinoma: An *in vitro* Study

*[1]Ameneh Sazgarnia, [1]Mohammad Hossein Bahreyni-Toosi, [2]Majid Valizadeh, [3] Fatemeh Homaei, [4]Habibollah Esmaily

Abstract

Objective(s)

This study was carried out in order to evaluate the effects of electrochemotherapy, electrical pulses and chemotherapeutic drugs on the killing of cancerous cells and their probable synergistic effects.

Materials and Methods

Electrochemotherapy treatments conducted on MCF-7 cell line derived from human breast adenocarcinoma tumor using four chemotherapeutic drugs including bleomycin, cisplatin, adriamycin and cyclophosphamide and six electrical doses. Cell survival assayed using MTT method, 72 hrs after the treatment; also the killing effects of each drug and electric dose determined. Finally, "Relative Pulse Effectiveness" and "Relative Electropermeability Effectiveness" calculated.

Results

All electrical doses decreased cell survival, significantly for bleomycin and cisplatin, however, they were only, significant in high concentration of cyclophosphamide and adriamycin. For the applied drugs, "Relative Electropermeability Effectiveness" was more than one (1.00), except for adriamycin.

Conclusion

It seems that for the diffusion of molecules into cells, application of high duration electric pulses is more efficient for high molecular weight drugs while for low molecular weight drugs, strong pulses are more effective. In intermediate molecular weight, there is no difference between increasing the pulse strength and/or duration to achieve additional electropermeability. Electropermeability effect of different electric doses and electrochemotherapy efficiency can be evaluated by "REE" and "RPE", respectively.

Keywords: Adriamycin, Bleomycin, Cisplatin, Cyclophosphamide, Electrochemotherapy, Electric pulse, MCF-7 cell line

1-Research Center of Medical Physics, Avicenna Research Institute, Mashhad University of Medical Sciences, Mashhad, Iran

* Correspondening author: Tel: +911 511 8002324 Fax: +911 511 8002320 email: sazgarniaa@mums.ac.ir

2- Zabol University of Medical Sciences, Zabol, Iran

3- Research Center of Cancer; Avicenna Research Institute; Mashhad University of Medical Science, Mashhad, Iran

4- Department of Community Medicine& Public Health, Mashhad University of Medical Sciences, Mashhad, Iran

Introduction

Restricted diffusion of some chemotherapeutic drugs into cells (1), is one of the problems to access an efficient response in cancer treatment. Increasing the administered dose can be a solution to attain an acceptable therapeutic effect. In this way, side effects of the drugs increase and normal cells suffer serious damage during treatment. Electrochemotherapy is a method in which electric pulses are applied to tumor cells together with chemotherapeutic agents such as cisplatin and bleomycin to make pores in the cell membrane (2). Increased toxicity of cisplatin and bleomycin for a number of animal and human cell lines has been demonstrated (2), while their antitumor effect in low concentration is negligible and their therapeutic effects diminish without using electric pulses (3). Therefore, the use of electrochemotherapy in clinical treatments seems to be more effective. Electrochemotherapy is based on electropermeability of tumor cells, increasing permeability in cell membrane and facilitating drug entry into the cell, decreasing blood circulation and a prolonged drug retention in tissue (3). In this way the destructive effect of the drug on tissue increases and more tumor cells are killed.

In 1994, Belehradek *et al* reported that *in vivo* tumors affected by electric pulses absorb bleomycin four times more than unaffected ones (4). In 2000, Sersa *et al* utilized electrochemotherapy with cisplatin to treat malignant skin melanoma metastases. The results indicated increased response from 22 to 48 percent resulting electric pulse application (5). In 2002, Yanai *et al* studied the effect of electrochemotherapy on YTS-1 cells derived from bladder carcinoma using adriamycin and bleomycin. Simultaneous use of electric pulses and bleomycin doubled inhibition of the growth of cells, while the application of electric pulses showed no significant effect for adriamycin (6). In 2005, Kranjc studied the effect of electrochemotherapy on the tumor cells of murine LBP sarcoma. The results indicated that electropermeability of the cells *in vitro* enhanced the effect of bleomycin (7). In one of the latest clinical studies performed on melanoma cells and dermal and hypodermal tumor nodules, electrochemotherapy has provided 75% complete response and 10% partial response in the patients (8).

In this research combined effect of chemotherapy and electropermeability was studied via separating the killing contribution of electric pulses and electropermeability on the cell death.

Materials and Methods

In this study, electrochemotherapy was conducted on MCF-7 cell line derived from human breast adenocarcinoma tumor using four chemotherapeutic drugs including bleomycin, cisplatin, adriamycin, and cyclophosphamide.

MCF-7 cell line was purchased from Pasteur Institute, Tehean, Iran, grown in RPMI-1640 medium, supplemented with 10% fetal bovine serum plus streptomycin and penicillin antibiotics. Cells were maintained in a 37 °C humidified atmosphere that contained 5% CO_2. After 2-3 days of growth and proliferation of the cells, they covered the bottom of a flask as a monolayer. Trypsin-EDTA was used to detach cells from the bottom of flask. After trypsinization, the cells were counted and suspended in PBS with a concentration of 20×10^6 cells/ml and treated as planed.

To carry out electrochemotherapy experiments, 8 electric pulses with different strengths and durations at a frequency of 1 Hz according to Table 1 were applied to the cells in the presence of one of the chemotherapeutic drugs. Concentrations of the drugs were considered as follows:
bleomycin 10 µg/ml, adriamycin 0.5 µg/ml and 2.5 µg/ml, cisplatin 0.4 µg/ml, cyclophosphamide 8 µg/ml and 16 µg/ml.

It should be noted that adriamycin and cyclophosphamide are the selective drugs in the treatment of breast cancer and the reason of their selection was to evaluate electrochemotherapy efficacy with these drugs. But, cisplatin and bleomycin are not routinely used in the treatment of breast cancer. These drugs were selected to study the chance of variations of their efficacy with electric pulses.

Table 1. Conditions of the electric doses for the cells in different groups. In all cases eight electric pulses with an interval of one second has been used.

Groups	control	1	2	3	4	5	6
Pulse strength (Volt/cm)	0	800	800	1000	1100	1500	1500
pulse duration (μSec)	0	300	400	200	100	25	100

Generating and applying the electrical pulses to the cells was performed by a square-pulse generator (electrosquare porator BTX; Model ECM) made by Genetronics Company, in a 2-mm gap cuvette. The cuvettes and cells were placed on ice before pulse application for 10 mins. In this report, the electric doses are shown in terms of a fraction with pulse strength as nominator and pulse duration as denominator.

Denominator is: $\frac{pulse-strength(volt/cm)}{pulse-duration(\mu Sec)}$

Cells incubation was carried out in RPMI 1640 containing 2% FCS and a chemotherapeutic drug for 10 mins, then the cells were washed in PBS and resuspended in the complete culture medium. Cell survival was evaluated using MTT assay 72 hrs after the treatment. Because electric pulses have managed to increase the efficacy of drug delivery into the cells and kill the cells through both direct effect on cell membrane and its rupture and also, causing permeability, we suggested two new parameters of "Relative Pulse Effectiveness (RPE)" and "Relative Electropermeability Effectiveness (REE)" to separate the share of these effects on cell death as follows:

$$RPE = \frac{\text{Cell Death\% in presence of pulse and drug}}{\text{Cell Death\% via drug without pulse}}$$

and if:

Cell Death% resulting electropermeability = (Cell Death% in the presence of pulse and drug) - [(Cell Death% by pulse + Cell Death% by drug)]

As a result:

$$REE = \frac{\text{Cell Death\% resulting electroper meability}}{\text{Cell Death\% in presence of drug without pulse}}$$

Therefore, "Relative Pulse Effectiveness" introduces the efficiency of electrochemotherapy versus chemotherapy and "Relative Electropermeability Effectiveness" just initiates electropermeability efficacy on the drug entry to the cells.

The obtained data were analyzed by SPSS version 11.5 and compared using one-way ANOVA and Tukey tests, after performing the normality test.

Results

The cell survival changes in different pulse conditions for each drug are shown in Figures 1 to 4.

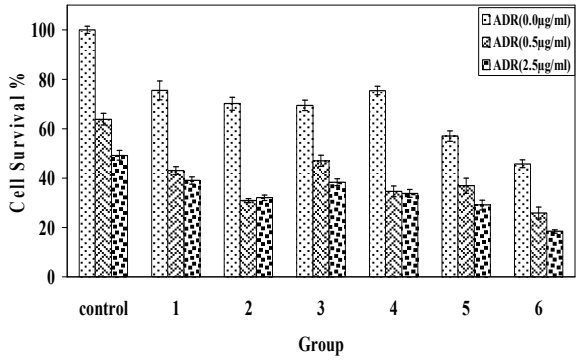

Figure 1. Cell survival of the MCF-7 cells in different electrical doses and various adriamycin concentrations 72 hrs post treatment (Each data represents the mean of four measurements ± SD).

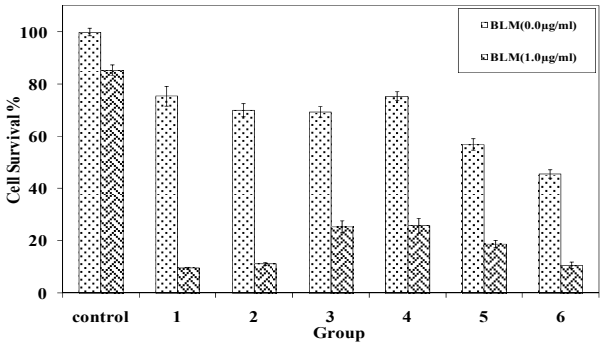

Figure 2. Cell survival of the MCF-7 cells in different electrical doses and 0.4 μg/ml of bleomycin 72 hrs post treatment (Each data represents the mean of four measurements ± SD).

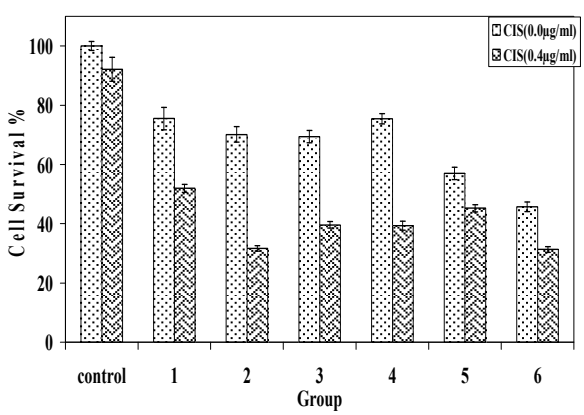

Figure 3. Cell survival of the MCF-7 cells in different electrical doses and 0.4 μg/ml of cisplatin 72 hrs post treatment (Each data represents the mean of four measurements ± SD).

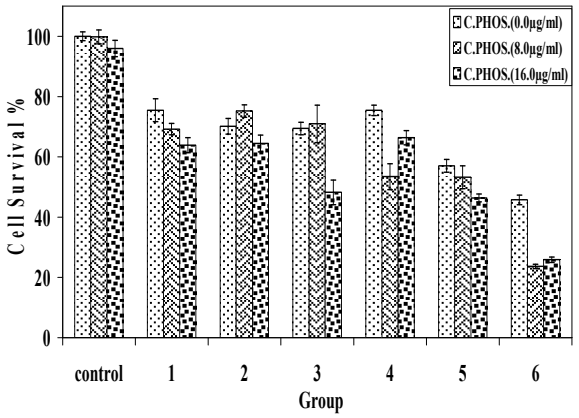

Figure 4. Cell survival of the MCF-7 cells in different electrical doses and various cyclophosphamide concentrations 72 hrs post treatment (Each data represents the mean of four measurements ± SD).

On the basis of these data, all electric doses decreased cell survival, significantly for bleomycin and cisplatin but they were only significant in high concentration of cyclophosphamide and adriamycin, The least cell survival was attained in pulses of 800/300, 800/400, 1500/100 for bleomycin and 800/400, 1500/100 for cisplatin. In both concentrations of adriamycin and cyclophosphamide, the pulses of 1500/100 provided the best response. Of course differences between two concentrations of cyclophosphamide were not significant.

The relative effects of each drug and electric dose given in Tables 2 to 5 are dependent upon bleomycin and cyclophosphamide concentrations.

Table 2. Relative Electropermeability Effectiveness, Relative Pulse Effectiveness and cell death percentage resulting permeability in the presence of adriamycin.

	REE		RPE		Cell death percentage resulting permeability	
Concentration (μg/ml)	0.5	2.5	0.5	2.5	0.5	2.5
Groups						
1	-	-	1.57	1.00	-	-
2	0.08	-	1.91	1.20	3.0	-
3	-	-	1.47	1.34	-	-
4	0.13	-	1.81	1.21	4.6	-
5	-	-	1.74	1.30	-	-
6	-	-	2.05	1.40	-	-

Table 3. Relative Electropermeability Effectiveness, Relative Pulse Effectiveness and cell death percentage resulting permeability in the presence of bleomycin.

Groups	REE	RPE	Cell death percentage resulting permeability
1	3.53	6.21	51.3
2	3.05	6.10	44.3
3	2.02	5.12	29.3
4	2.41	5.10	35.0
5	1.62	5.57	23.5
6	1.41	6.13	20.4

Table 4. Relative Electropermeability Effectiveness, Relative Pulse Effectiveness and cell death percentage resulting from permeability in the presence of cisplatin.

Groups	REE	RPE	Cell death percentage resulting from permeability
1	1.99	6.11	15.7
2	3.88	8.68	30.5
3	2.79	7.68	21.9
4	3.58	7.71	28.1
5	0.50	6.96	3.9
6	0.84	8.73	6.5

Table 5. Relative Electropermeability Effectiveness, Relative Pulse Effectiveness and cell death percentage resulting from permeability in the presence of cyclophosphamide.

		REE		RPE		Cell death percentage resulting from permeability	
Groups	Concentration (μg/ml)	8	16	8	16	8	16
1		30.45	1.85	154.00	8.87	6.09	7.52
2		-	0.41	124.05	8.74	-	1.65
3		-	4.20	145.25	12.72	-	17.09
4		44.30	4.40	168.15	11.44	8.86	17.92
5		17.95	1.61	233.95	13.18	3.59	6.56
6		109.50	3.88	381.80	18.22	21.90	15.81

Regarding the top values of "RPE" in low and high concentrations of adriamycin, electrochemotherapy by the pulses of 1500/100 increased cells death only 2.05 and 1.61 fold of chemotherapy. The highest value of "REE" in low adriamycin concentration achieved in pulses of 1100/100 that is negligible. According to the "RPE" and "REE" for bleomycin, the highest cell killing and permeability obtained using pulses of 800/300. Cell death resulting from electropermeability was 3.51 fold and electrochemotherapy was able to kill the cells 6.21 fold, relative to chemotherapy.

For cisplatin, pulses of 800/400 and 1500/100 could kill the cells. The pulses of 800/400 provided the most electropermeability (REE=3.88). According to the cyclophosphamide results, the pulses of 1500/100 induced the greatest electrochemotherapy efficiency in both concentrations and the most electropermeability in lower concentration. Also, the most electropermeability in high concentration attained using pulses of 1100/100.

Discussion

In all groups, the relative effects of pulse on cell death, as well as electropermeability decreased at high concentrations of adriamycin and cyclophosphamide.

A research similar to this work conducted by Yanai *et al* in 2002 on YTS-1 cells derived from human bladder carcinoma in which eight square electric pulses with a strength of 1000 V/cm, 100 μs duration and time interval of one second were used for electrochemical therapy. In their survey, adriamycin was used in 0.05, 0.5 and 5 μg/ml and bleomycin with 1, 10 and 100 μg/ml concentrations. After six hrs of treatment, cell survival ratio in two concentrations of 0.05 and 0.5 μg/ml of adriamycin was recorded with a significant difference against the control group, and in bleomycin, the cell survival ratio in three concentrations compared with the control group showed a significant difference (6). On the basis of the finding of present study, for the most similar electric doses compared with the above research, i.e. 1100 V/cm pulses with 100 μs duration, cell survival ratio in both concentrations of adriamycin of 0.5 and

2.5 μg/ml was significantly different in comparison with the control group. The same result observed in 1 μg/ml of bleomycin.

Kranjc *et a*l studied the effects of electrochemotherapy with bleomycin (0.28 μg/ml) on LPB cells, via eight square pulses with strength of 1200 V/cm, duration of 100 μs and a one-second interval. The results showed that application of the above electric doses to cells enhances the effect of bleomycin in killing cells with a factor of 1.5 compared with the similar group in the absence of electric pulses (7). In this study, electrical doses close to the above mentioned electric doses are 1000/200 and 1100/100 pulses. In 1 μg/ml of bleomycin, the 1000/200 and 1100/100 pulses caused 29.3 and 35 percent increase in cell death, indicating 5.12 and 5.10 fold increase in bleomycin effect, respectively. It can be stated that: if the aim of pulse application is cell killing without considering its mechanism, application of electrical doses with higher "RPE" is recommended, and where facilitation of drug entry into the cells without direct cell death is important, the pulses with more "REE" is suggested.

Serša *et al* carried out an *in vivo* study on the use of bleomycin and cisplatin in electrochemotherapy protocols on a number of animal models. They combined electrochemotherapy either with bleomycin or cisplatin with radiotherapy and demonstrated a good improvement of tumour radiation response: 1.9 fold for electrochemotherapy with bleomycin and 1.6 fold for electrochemotherapy (9). Electric pulses they applied were 1300 V/cm with 100 microseconds duration. Compare with Serša's study, in the present work, the nearest conditions are 1100 and 1500 V/cm in 100 microseconds duration that their mean relative electropermeability effectiveness is calculated 2.21 and 1.96 for electrochemotherapy with cisplatin and bleomycin respectively.

Considering the cell survival ratio in each of the applied electric doses, for adriamycin an increase in pulse duration has a higher effect on cell death induction relative to an enhancement in the strength of electric pulse. It can be concluded that the synergistic effect of electric pulse together with adriamycin is not considerable in comparison with the other three drugs. This is more pronounced for bleomycin, and increasing pulse duration is more effective than increasing their strength. For cisplatin, the effect of increased duration and electric pulse strength in increasing cell death is nearly the same. These characteristics are close to cyclophosphamide results in which increased strength compared with increasing electric pulse duration has higher outcomes in cell death. To justify the reason for this effect, it is noteworthy to regard the molecular weight of the drugs under study and their variations. Molecular weight of bleomycin is 1415.56, adriamycin 543.52, cisplatin 300.05 and cyclophosphamide 261.08 g/mole.

Considering the cell death percentage resulting electropermeability in these four drugs, the best results are related to bleomycin and cisplatin respectively which is in a good agreement with some studies that Besic reported them in a review article (10).

It seems that the application of pulses with high duration for high molecular weight drugs e.g. bleomycin is more appropriate, while for low molecular weight drugs such as cyclophosphamide more potent pulses are more effective. In intermediate molecular weights, there is no difference between increasing pulse strength and/or duration to attain more electropermeability for cisplatin and cyclophosphamide. Adriamycin has a restricted potential to cell death induction that cannot be improved via the electropermeability.

Conclusion

These findings indicate a relationship between pulse duration and diameter of electropores, as well as pulse strength and number of induced electropores in the cell membrane. In other words, longer pulse duration produces larger pores and more potent pulses create extra pores. "REE" and "RPE" can be two relative appropriate parameters to compare electropermeability effect of different electric doses and electrochemotherapy efficiency,

respectively.

Acknowledgement

The authors would like to appreciate the Research Deputy of Mashhad University of Medical Sciences (MUMS) for the financial support of this research.

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