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CELL MEMBRANE ALTERATION BY WEAK ALTERNATING ELECTRIC FIELD AT LOW FREQUENCY

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Abstract. The aim is to estimate the influence of sinusoidal (ac) field in combination with the cytostatic drug gemcitabine on the therapeutic effect of malignant cells. Electrotreatment is cell line dependent. There is threshold frequency window (1Hz) that is the most suitable for drug delivery. Hematopoietic cells are more sensitive to the electrotreatment as compared with human lymphocytes from healthy donors.

Key words: electrotreatment, drug delivery, gemcitabine.

1. INTRODUCTION

One of the multiple aspects of high electric field induced effects on the cell membrane is a transient pore formation, leading to possible entering of exogenous chemical species. If an electric field is applied to the cells, the appearance of transient pores in biological membranes can be induced. Under suitable conditions large molecules, such as DNA or drugs, can enter the cells through these pores. The process is known as electroporation. This technique has been developed for highly effective transfection [1].

In 1990 Xie *et al.*, [2] have presented transformation of E. coli cells by using low frequency, low voltage ac electric field. Theoretically such low electric field is not able to porate single biological membrane. Recently we have reported about the amplification mechanism of the field strength as well as about conditions of creating electroporation at similar circumstances [3–5].

One of the approaches for increasing the cytotoxicity of drugs used for chemotherapy, in avoiding side effects, is a concentration enhancement into the target cells and tissues. This is the main goal of electrochemotherapy. After the first application of electrical pulses for introducing cytostatic drugs, electrochemotherapy could be considered as an established method for treatment of some type skin and liver tumors [6–7].

Based on our previous experience with microorganisms, we make treatment of the malignant cell lines HL-60, SKW-3 and of the lymphocytes from healthy donors with low frequency and low voltage electric field, as well as with or without gemcitabine as cytostatic drug. The aim of this study is to increase the concentration of cytostatic drug gemcitabine into the target cells by application of ac electric field.

2. MATERIALS AND METHODS

2.1. CELL LINES

SKW-3 cell line (DSMZ No. ACC 53): human T cell leukemia, at doubling time of 30–40 h;

HL-60 (ATCC CCL 240) myeloid cell line: human acute myeloid leukemia; doubling time 25 h. All cell lines were grown as suspension culture (RPMI-1640 medium (BioWittaker, Belgium), supplemented with 10% fetal calf serum (PAA, Austria)) at 37^0C in an incubator with humid atmosphere and 5% CO_2. The passage of the cells was possible two or three times a week in order to be kept in log phase.

Isolation of human lymphocytes: fresh human peripheral blood from healthy donors was obtained from the National Centre of Blood Transfusion in Sofia. Peripheral blood lymphocytes were being prepared using Ficoll Paque gradient centrifugation. The lymphocytes were resuspended in culture medium RPMI-1640 (without phenol red) supplemented with 10% FCS, Pen Strep (1%) at a concentration of 10^7cells/ml. The lymphocytes were activated by adding 3μg/ml Phytochemagglutinin dissolved in RPMI 1640.

Gemcitabine (Gemcitabinhydrochlorid, Gemzar “Lilly”, MW 299.66, France SA, F-67640 Fegersheim, Frankreich).

2.2. VIABILITY OF CELLS

The viability of SKW-3, HL-60 cells and lymphocytes was determined by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Applichem, Germany) dye-reduction assay, as described by Mosmann [8]. The MTT test

(cytotoxic or therapeutic test) has measured the percentage of irreversibly porated cells after the application of an electric pulse of ac, with or without gemcitabine treatment. To evaluate the statistical significance of the viability reduction of cells a comparison between exposed (ac electric field and/or gemcitabine) and control probes was performed by applying the two tailed paired Student`s test t-test, with p values lower than 0.05 considered statistically significant.

2.3. ELECTROTREATMENT PROTOCOL

SKW-3, HL-60 cells and lymphocytes from healthy donors (cell density $\rho_c=10^6$ cells per volume υ = 0.1 ml) were being electroporate in 0.3 M manitol. The sample chambers (Bio Rad Laboratories, Richmond, CA) were equipped with flat parallel electrodes, with electrode distance d = 0.4 cm. The cell suspension was treated with 0.1, 1 and 10 Hz, 100V peak to peak ($E_{p\text{-}p}$) sinusoidal ac electric field (by home made generator). The applied field strength has an effective value $E_{eff}= E_{p\text{-}p}\sqrt{2}/4$. Gemcitabine at concentration 10 μM (resuspended in PBS) was added before the electrotreatment. The same volume (5 μl) of PBS without gemcitabine was added to the controls in order to keep the same electroporation conditions. Duration of the treatment was 30s. After that, 900 μl RPMI-1640, supplemented with 10% FCS, was added to each sample. The controls were treated under the same conditions but without the application of an electrical field and/or gemcitabine.

3. RESULTS

On Fig. 1 is shown the percentage of lymphocytes viability after 30s treatment with low frequencies, low electric fields in the presence or without gemcitabine. The viability of lymphocytes was being used as a control for the treatment of malignant cells. There is a small, but statistically significant ($P < 0.005$) reduction of cell viability up to 20% in the case of treatment with electric fields alone and a considerable decrease up to 50% in the case of addition of gemcitabine, especially after treatment with 1Hz alternating electric field. It is clear that the cell viability depends on the applied alternating electric field and an additional effect of gemcitabine is observed.

The cell survival fraction of SKW-3 cells is determined 24 h after treatment with ac field with or without addition of gemcitabine (Fig. 2). It was found that the cell viability depends on the field frequency by decreasing to almost 60%, in the case of 1Hz without gemcitabine and up to 80% at the same electrical conditions in combination with drug.

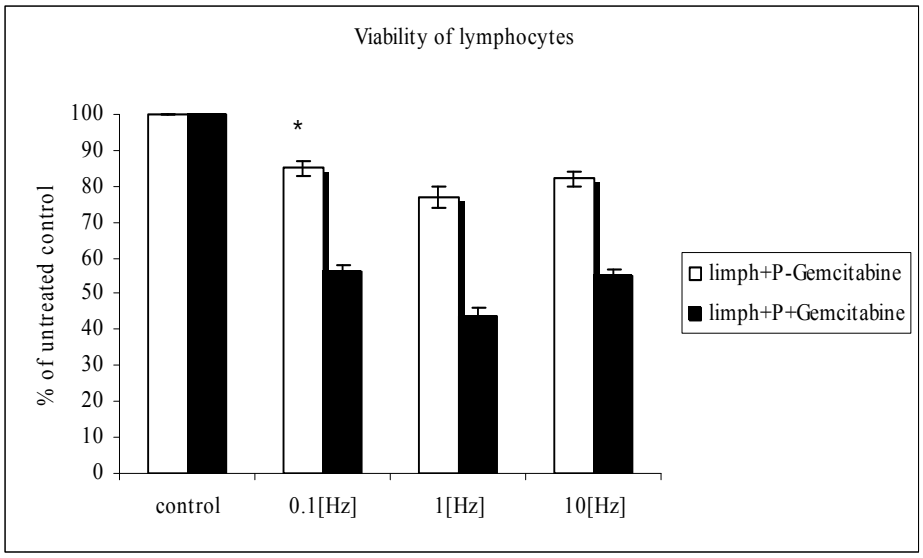

Fig. 1 – Viability of lymphocytes after electrotreatment with 100V/cm, 0.1Hz, 1Hz and 10Hz, with or without gemcitabine, time of treatment = 30s, * value of Student's t-test $P < 0.003$ and lower at other treatment conditions.

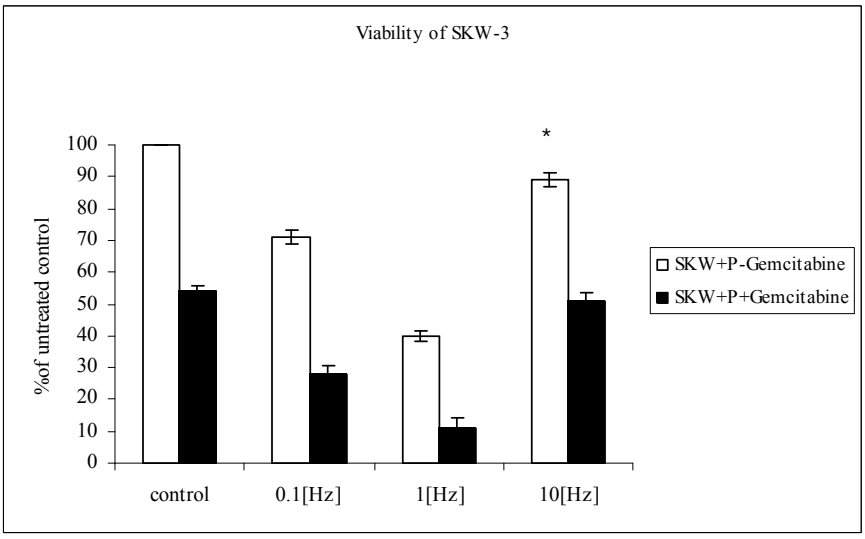

Fig. 2 – Viability of SKW-3 cell line. Electrical parameters are the same as shown in Fig. 1, with or without gemcitabine * value of Student's t-test $P < 0.05$ and significantly lower at other treatment conditions.

The viability of HL-60 cell line treated at the same parameters is shown on Fig. 3.

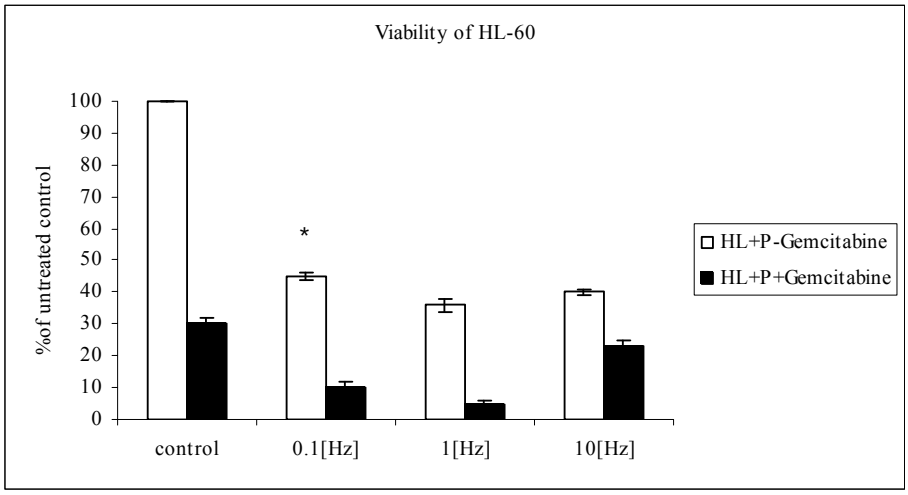

Fig. 3 – Viability of HL-60 cell line at electrical parameters as Fig. 1, with or without gemcitabine * value of Student's t-test $P < 0.0003$ and lower at other treatment conditions.

4. DISCUSSION

Chemically gemcitabine is a nucleoside analog in which the hydrogen atoms on the 2' carbon of deoxycytidine are replaced by fluorine atoms. The triphosphate analogue of gemcitabine replaces one of the building blocks of nucleic acids, in this case cytidine, during DNA replication. The process arrests tumor growth, as only one additional nucleoside can be attached to the "faulty" nucleoside, resulting in apoptosis [9]. The drug has very slow diffusion through the membrane.

The reason for the decrease in cell viability (Figs. 1–3, control plus ac field) could be the formation of free radicals. That is why the cells were resuspended in 0.3 M manitol which occurs as an antioxidant.

Our data indicate that enhanced diffusion of cytostatic drug gemcitabine across the cell membrane after application of ac electric field with low frequency and low voltage could be due to reversible electroporation. Cells were most permebilisated at 1Hz and less at 0.1 and 10Hz. A significant cell viability reduction was observed in SKW-3 and HL-60 cells compared to lymphocytes. In our study we used lymphocytes as a control because of their low degree of cell division, with low cytostatic effect of gemcitabine, nevertheless a reduced viability was recorded.

Another explanation concerning the different efficiencies of the applied electric field on lymphocytes and the two malignant cell lines could be the different cell membrane composition. One of the most important functions of the phospholipids in the cell membrane is their fluidity, which is essential for many membrane functions [10]. Reduction in the lipid content, relative to proteins, may

decrease fluidity and consequently the permeabilisation rate of the cell membrane [11], drug uptake, transport and extrusion.

Changes of the lipid composition during the malignant transformation in several experimental systems have been reported [12]. Moreover, some main characteristics of neoplastic cells may be related to the modifications in cell surface [12]. Normal mature peripheral blood lymphocytes contain more phospholipids and cholesterol per cell than chronic lymphocytic leukemia cells [13]. The structure of cholesterol allows it to reduce the freedom of movement of phospholipid acyl chains, thus rigidifying the membrane [14]. The deficiency of membrane cholesterol in the case of malignant cells can improve transformation ability due to the rigidity of cell membrane. Spugnini *et al.* [15] using electron microscopy after electrotreatment detected defects in the dynamic assembly of lipids and proteins, which generated some area with rough structures. One of the explanations of the phenomenon of cell electroporation is the capacitor model of the cell membrane. Alterations in the lipid content and the protein: lipid ratio is expected to influence the dielectric properties of the cell membrane and thus the different cell viability after treatment with ac electric fields. On the other hand, the unusual behavior and the appearance of the concentrated domains in suspensions with low conductivity such as colloidal, E. coli or DNA solutions at low alternating field strength, low frequency, have been investigated [3–5, 16]. Complete theory of the formation of such domains is not yet available. Recently, we proposed the amplification mechanism for pore creating at above field strength and conditions [4–5].

The combination of electrical parameters and differences in the cell membrane composition might explain the variations in the cell viability degree after electrotreatment.

We can claim that electric treatment is cell line dependent and malignant hematopoetic cells are more sensitive to the electrotreatment. There are some threshold frequency windows (1Hz) that is the most suitable for electroporation and electro drug delivery.

In conclusion, our results prove the beneficial effect of low frequency, low voltage ac electric fields in combination with gemcitabine on the decreased viability of malignant cell lines SKW-3 and HL-60, compared to lymphocytes. Possible application of low voltage low frequency ac electric fields in electrochemotherapy will be our future goal.

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