# We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

**5,000**
Open access books available

**125,000**
International authors and editors

**140M**
Downloads

Our authors are among the

**154**
Countries delivered to

**TOP 1%**
most cited scientists

**12.2%**
Contributors from top 500 universities


WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

## Interested in publishing with us?
## Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Chapter

# Square-Wave Electric Impulses of 10 ms and 100 V/cm of Field Force, Produced by PGen-1 Impulse Generator Device, Affect the Proliferation Patterns of Different Animal Cells

*Bratko Filipič, Lidija Gradišnik, Kristine Kovacs, Ferenc Somogyvari, Hrvoje Mazija and Toth Sandor*

## Abstract

The influence of the medium-strength electric forces (MSE) on the proliferation of adherent chicken embryo fibroblasts (CEF), VERO, MDBK, MRC-5, and HeLa; lymphoblast cells, FB1 and K562; and cell multiplications were analyzed by growth index (GI). Impulse generator device PGen-1 provided 100 V/cm square-wave impulses of 10 ms. Treatment: Samples were subjected to one or three MSE. GIs were compared with controls after 72 hours and one or three treatments: Monolayers: CEF: GI in the control is 16.76, and after one and three MSE, it is 15.81 and 7.09. Vero cells: GI in the controls is 8.39, and after one and three MSE, it is 5.39 and 5.69. MDBK cells: GI in controls is 8.39, and after one and three MSE, it is 5.39 and 5.69. MRC-5 cells: GI in controls is 5.58, and after one and three MSE, it is 4.18 and 2.60. HeLa cells: GI in controls is 13.69, and after one and three MSE, it is 10.16 and 5.37. Suspension cells: Lymphoblast FB1: GI in controls is 6.55, and after one and three MSE, it is 13.48 and 12.25. Lymphoblast K562: GI in controls is 9.07, and after one or three MSE, it is 12.37 and 13.55. To conclude: MSE in monolayer cells inhibits the GI, depending on the nature of cells. MSE enhances the multiplication of lymphoblast FB1 or K562.

**Keywords:** square-wave electric impulses, monolayer cells, lymphoblast cells, growth pattern, growth index decrease, growth index increase, Caspase-3 containing cells, percent of dead cells

## 1. Introduction

Different electric and magnetic field forces can interact with the living systems at enzymatic, cellular, or organism levels [1, 2]. Despite a numerous experimental

approaches were performed about this subject, most of the obtained data are completely different and unfortunately very often incomparable. Different basic facts are the reason for this discrepancy. Basically, the living systems *per se* can generate electric or magnetic fields and impulses. These are in the field force range from 10 to 500 mV/cm. The external electric and magnetic range can cause physiological responses at a cellular level. They are of very short forces that can be compared to this duration and are in the range of milliseconds to seconds. Usually they do not result in any important alteration in the system [3, 4]. On the contrary, the biological effects that an electric field with a field force below 1–10 mV/cm causes to a cell are likely due to interaction mechanisms occurring in the cell membrane. For the field forces that are above 1–10 mV/cm, the effects are due to interaction mechanisms occurring in the intracellular compartments. Electric field forces of 10–500 V/cm act as environmental stress factors and result only in a transitory defensive response [5]. In addition, many reports show that these interactions are field force dependent and they can provoke both the enhancement [6] and the inhibition [7] of different cellular functions. It is important that the studied types of responses show the "sensitivity windows" within the above field force range [8]. In the frame of a defined window, the system provides a definite response, while when it is outside the frame, the response disappears or suddenly turns in the opposite direction. The electric field forces that are above 500 V/cm can provoke the sustained response like in the case of electroporation. The most interesting, even the least studied, are the biological effects of medium-strength electric forces of 100 V/cm. The complication, in fact, is that these electric fields can be applied as impulses, long-lasting DC fluxes, or AC waves, and each of them can induce very different types of responses. The applied effects can be a direct ionic current or the electric field generated between the two plates of a condenser, or as a magnetic field, that can provide different interacting doses and different kinds of energies. The named forces can affect the different immune responses of animals [9, 10] or in humans [11] through the induction [12] or augmenting [13] of different immune response elements.

Exogenously added electric impulses can induce the synthesis of antiviral substances, are not interferon, interleukins, or tumor necrosis factors. Therefore, we named them "interferon-like molecules." In our experiments, we also found [14] that medium-strength square-wave impulses of direct ionic current (DC) can result in a short-term direct antiviral resistance to virus infections and in a consequence to alter the membrane properties of the target cells [15]. We therefore decided to study the changes in the expression of membrane-bound surface marker molecules that are on the surface of the immune competent cells in the human blood [16–18]. Our experiments were aimed to establish whether such exogenous electric stimulation of human leukocytes could be utilized as an immune enhancer and an antiviral protector ex vivo, preferably coupled to the dialysis process. In order to detect the potential hazards of such an application, we have to study further the effects of electrostimulation on some other parameters of the human blood.

Least but not the last, the nature and biological conditions of the target system can determine the type and extent of the response. The given conditions can induce an enhancement of cell proliferation in a suspension cell culture and the inhibition of cell proliferation in an adherent growing in monolayer cell culture [19]. Therefore, the herein presented experiments are aimed to investigate the influence and some mechanisms of the medium-strength square-wave electric impulses of the field forces of 100 V/cm, on the proliferation pattern of different animal cells growing in a monolayer or growing as the suspension culture.

## 2. Material and methods

### 2.1 The electric impulse generator device PGen-1

The electric impulse generator device PGen-1 was developed and physically realized by Dr. Sandor Toth and Dr. Ferenc Somogyvari. The PGen-1 device provides 1–300 V/cm square-wave impulses of 1–10 ms duration, with a repetition option of 1–9. It has also a continuous work option. Its repetition intervals can be set between 1 and 10 s. The device consists of two separate circuits: (1) a low-voltage circuit, running on transformed and rectified net current, being stabilized by monolithic integrated stabilizers. They work as a power source for the regulator. (2) A high-voltage circuit serves for the impulse generator itself. The outgoing voltage is regulated by a phase-splitting dimmer and is rectified and stabilized. The low-voltage settings secure the filter condenser. The analogous regulator system is composed from a stable multivibrator, governing the counter, and two synchronized mono-stable multi vibrators, generating the outgoing impulses and the visual control of the signals. The outgoing square-wave impulses are characterized in the **Appendix.** The sample chamber has a 50 ml capacity and is a polypropylene tube with a 2.5 cm in diameter, with a platinum wire electrode. It is detachable from the basic device and is autoclavable. Further details are available in the patent description of the PGen-1 device [20] (**Figure 1**).

### 2.2 The dosimetry

The adsorbed doses (AD in J/g) of the electric impulses in the samples were calculated according to Pakhomov et al. [21] by the use of their formula as follows:

$$AD = \frac{\left(E^2 \times d^2 \times W \times n\right)}{(R \times M)} \tag{1}$$

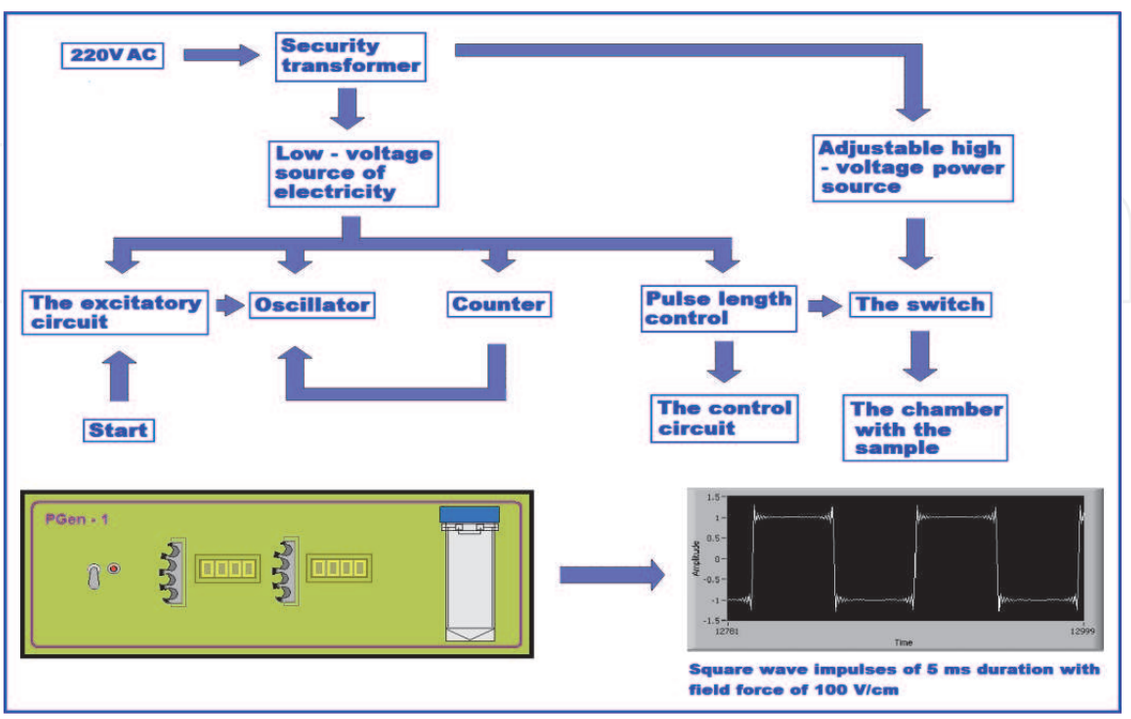



**Figure 1.**
*Electric circuits of the electric impulse generator device PGen-1.*

| Number of impulses | AD in J/g |
|---|---|
| 1 | 0.163 |
| 3 | 0.490 |

**Table 1.**
*The adsorbed dose (AD in J/g) of the electric impulses in the samples.*

In it E is the E is field in the sample (V/m), W is the pulse width ($10^{-8}$ s), n is the number of electric impulses delivered to the sample, d is the gap in the cuvette, R is the resistance of the cuvette with the sample (8–9 Ù), and M is the mass of the medium in g. The AD values in J/g of one and three impulses are shown in **Table 1**.

## 2.3 The cells used in the experiments

The following cells were used: (a) monolayer cells (chicken embryonic fibroblasts, VERO, MDBK, MRC-5, and HeLa cells) and (b) cells growing in suspension (lymphoblast cells like FB1 and K562).

## 2.4 Cell multiplication

The monolayer cell cultures were resuspended in 3 × 50 ml of medium EMEM complemented with surplus $Ca^{2+}$ and 2% of fetal calf serum (FCS). The cell lines growing in suspension were multiplied in the medium RPMI 1640 + 10% FCS. For the experiments they were resuspended in 3 × 50 ml of the medium RPMI 1640 with surplus $Ca^{2+}$ and 2% FCS.

## 2.5 The cell treatments with the electric impulses

The aliquots of 3 × 50 ml of cells suspensions with the $10^5$ cells/mL were put in the three electro-induction chambers with built-in platinum wire electrodes, for treatment. Samples were subjected to one or three square-wave impulses of 10 ms with a field force of 100 V/cm **(Figure 2)**. The untreated cells in the electro-induction chamber with built-in platinum wire electrodes represented the cell control.

## 2.6 The growth parameters determination

### *2.6.1 Growth index (GI)*

The GI was determined by the use of spectrophotometer measurements. For the experiments, there were three samples for (1) untreated cell control, (2) one impulse, and (3) three impulses. Cells were cultured in microtiter plates with EMEM medium supplemented with 10% FCS. On the next day, the medium was replaced by new medium containing 5% of FCS. The initial number of cells was determined separately. After 3 h of 5% $CO_2$ incubation at 37°C, the cells were fixed with a 0.25% solution of Glutar aldehyde, and the plates were cooled to 4°C. The cells from untreated control, cells pulsed with one impulse, and cells pulsed with three impulses were incubated for 3 days at 37°C and 5% $CO_2$. After the incubation microtiter plates with cells were fixed with a 0.25% solution of Glutar aldehyde for 20 minutes, washed with phosphate buffer saline (PBS, pH = 7.4), and stained with 4% solution of Methyl blue for 45 minutes at 37°C. Finally, the plates were thoroughly washed with tap water, air-dried, and the colour was extracted by adding of 100 μl of 0.1 M/HCl. The optical density (OD) was measured in AUTOEIA (Lab system) automatic spectrophotometer at 570/650 nm. Every

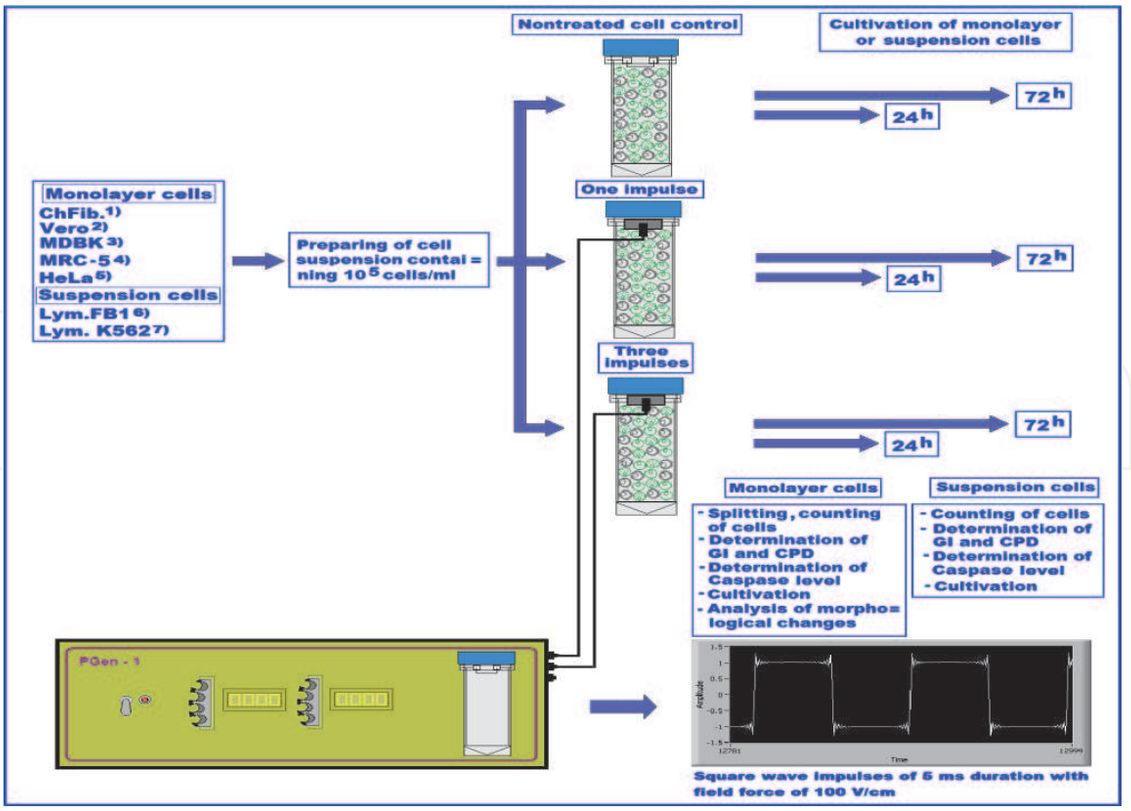



**Figure 2.**
*Scheme of the experiments. (1) chicken embryonic fibroblasts; (2) cercopithecus arthiopis kidney; (3) Madin-Darby bovine kidney epithelial cells; (4) Medical Research Council cell strain five fibroblasts derived from lung tissue; (5) Henrietta lacks cervical tumor immortal cell line; (6) Homo sapiens blood lymphoblast; (7) Homo sapiens bone marrow lymphoblast.*

sample was measured three times and the averages were calculated. The GI was calculated by the following formula: GI = absorbance after 4 days/absorbance of the initial sample [22, 23].

### *2.6.2 Percentage of GI inhibition*

From the GI of the pulsed cells by the one or three impulses and untreated control cells, the percentage of GI inhibition were calculated by the following formula: percent of GI inhibition = 100 − GI of pulsed cells with one or three impulses/GI untreated control × 100 were calculated [24, 25].

## 2.7 Viability assay

After the cell's treatment, the monolayer cells were seeded into a 6-well plates containing $3.3 \times 10^5$ cells/well. Twenty-four hours later and 72 h in the parallels, monolayer cells were detached and Trypan blue positive (=blue) cells were counted. The cell numbers were normalized to control cells as 100% [26, 27].

### *2.7.1 Procedure*

a. Bring adherent cells into suspension by the trypsin/EDTA as described previously, and resuspend them in a volume of fresh medium EMEM at least equivalent to the volume of trypsin. Centrifuge and resuspend the cells that grow in suspension in a small volume of medium. In addition, gently pipette to break up clumps.

b. Under sterile conditions take 100–200 μl of cell suspension.

c. Add an equal volume of Trypan blue (dilution factor = 2) and mix by gentle pipetting.

d. Clean the haemocytometer and the cover slips in 70% ethanol. Clean and dry them with two-site by rubbing with cotton sheets wrapped in cotton cloth.

e. Moisten the cover slip with water or exhaled breath. Slide the cover slip over the chamber back and forth using slight pressure until Newton's refraction rings appear (Newton's refraction rings are seen as rainbow-like rings under the cover slip).

f. Fill both sides of the chamber with cell suspension (approximately 5–10 μl), and view under an inverted phase contrast microscope using the 20× magnification.

g. Count the number of viable (seen as bright cells) and nonviable cells (stained blue). Ideally >100 cells should be counted in order to increase the accuracy of the cell count. Note the number of squares counted to obtain your count of >100.

h. Calculate the concentration of nonviable cells and the percentage of cells using the equation below:

$$\text{non-viable cell count (dead cells/ml)} = \frac{\text{No.of dead cells counted}}{\text{No.of large corner squares counted}} \times \text{dilution factor} \times 10.0000 \quad (2)$$

## 2.8 Caspase-3 assay

### *2.8.1 Procedure*

a. Caspase-3 activities in pulsed cells were assayed using a commercial method based on fluorochrome-labeled inhibitors of caspases (FLICA, ImmunoChemistry Technologies LLC).

b. The monolayer or suspension cells were pulsed with one or three impulses.

c. One hour after treatment, cells were gently removed from cuvettes and resuspended in a medium EMEM without foetal serum.

d. According to the manufacturer's recommendations, cells were labelled with carboxyfluorescein caspase-3 inhibitors for 1 h at 37°C under 5% $CO_2$ and protected from light as it was previously described [28].

e. Cells were washed with PBS buffer to remove the unbound reagent.

f. Cell fluorescence was determined by the use of fluorescent microscope.

g. Approximately hundreds of cells were acquired for analyses and expressed as percentage of cells showing positive fluorescence.

h. As electric fields were increased, greater numbers of cells became Caspase-3-positive with a homogeneous shift of cells into the Caspase-3-positive gate, making cell percentages with active Caspase-3 the most accurate and meaningful quantification of active Caspase-3 as an apoptosis marker.

### 2.9 Statistical evaluation

For the level of statistical significance determination ($^{*}p < 0.1$, $^{**}p < 0.05$), the T-test was used. All the data are shown as mean value ± standard deviation. The tests were performed in triplicate and each was repeated three to four times.

## 3. Results

### 3.1 The growth parameters, percentages of dead cells, and percentages of Caspase-3 positive cells of different monolayer cells

#### *3.1.1 The growth parameters and percentage of dead cells after 24 h of incubation*

The main effect of the cell's treatment with one or three impulse with the adsorbed dose (AD) of one impulse 0.163 J/g and of three impulses 0.490 J/g was the GI index inhibition expressed in percentage. The detailed results **(Table 2, Figure 3)** show the following:

Chicken fibroblasts: after one impulse 74.41% and after three impulses 57.2%
MDBK cells: after one impulse 31.35% and after three impulses 30.4%
Vero cells: after one impulse 37.2% and after three impulses 24.7%
MRC-5 cells: after one impulse 38.8% and after three impulses 41.9%
HeLa cells: after one impulse 78% and after three impulses 27%

When the percentages of dead cells after one impulse or three impulses were evaluated, the following data were obtained:

Chicken fibroblasts: after one impulse 9.2% and after three impulses 19.6% of dead cells.
MDBK cells: after one impulse 11.2% and after three impulses 12.7%.
Vero cells: after one impulse 8.6% and after three impulses 24.7%.
MRC-5 cells: after one impulse 14.7% and after three impulses 32.6%.
HeLa cells: after one impulse there were 7.4% of dead cells, and after three impulses, there were 11.3% of dead cells.

#### *3.1.2 The growth parameters and percentage of dead cells after 72 h of incubation*

After the cell's treatment with one or three impulses with the adsorbed dose (AD) of one impulse was 0.163 J/g and of three impulses with 0.490 J/g, was the growth index inhibition expressed in percentage. The results presented in **Table 2** and **Figure 4** show the following:

| | | | Monolayer cells | | | | | Suspension cells | |
|---|---|---|---|---|---|---|---|---|---|
| **Cell treatment** | **Time of treatment** | | **Chicken fibroblasts** | **MDBK[1]** | **Vero[2]** | **MRC-5[3]** | **HeLa[4]** | **FB1[5]** | **K 562[6]** |
| Untreated control | 24 h | GI[7] | 1.38 ± 0.12 | 1.19 ± 0.11 | 1.52 ± 0.13 | 1.5 ± 0.13 | 1.73 ± 0.15 | 0.94 ± 0.08 | 1.19 ± 0.11 |
| | | GI inh. % | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | % of dead cells | 4.5 | 3.9 | 5.2 | 6.2 | 5.2 | 4.2 | 6.1 |
| | | % of Casp.3 pos. cells | 2.34 | 2.02 | 2.70 | 3.22 | 2.70 | 2.18 | 3.17 |
| | | CPD[8] | 0.47 ± 0.04 | 1.37 ± 0.12 | 0.23 ± 0.02 | 0.32 ± 0.02 | −0.34 ± 0.03 | −0.1 ± 0.009 | 0.22 ± 0.02 |
| | 72 h | GI | 2.32 ± 0.2 | 2.95 ± 0.2 | 2.77 ± 0.2 | 2.00 ± 0.18 | 2.67 ± 0.24 | 2.78 ± 0.25 | 3.82 ± 0.34 |
| | | GI inh. % | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | % of dead cells | 5.2 | 3.7 | 4.9 | 6.8 | 7.3 | 5.1 | 6.7 |
| | | % of Casp.3 pos. cells | 2.02 | 1.66 | 2.20 | 3.06 | 3.28 | 2.29 | 3.01 |
| | | CPD | 1.21 ± 0.11 | 1.44 ± 0.13 | 1.09 ± 0.09 | 0.73 ± 0.06 | 0.28 ± 0.02 | 1.43 ± 0.12 | 1.9 ± 0.17 |
| 1 impulse | 24 h | GI | 0.35 ± 0.03 | 0.81 ± 0.07 | 0.96 ± 0.08 | 0.91 ± 0.08 | 0.38 ± 0.03 | 1.19 ± 0.1 | 1.9 ± 0.17 |
| | | GI inh. % | 74.4 | 31.5 | 37.2 | 38.8 | 78 | −27 | −59.1 |
| | | % of dead cells | 9.2 | 11.2 | 8.6 | 14.7 | 7.4 | 2.7 | 2.9 |
| | | % of Casp.3 pos. cells | 5.8 | 6.9 | 5.3 | 9.1 | 4.5 | 1.6 | 1.7 |
| | | CPD | −1.49 ± 0.13 | −0.40 ± 0.03 | −0.43 ± 0.03 | −0.38 ± 0.03 | −2.51 ± 0.22 | 0.40 ± 0.03 | 0.89 ± 0.08 |
| | 72 h | GI | 2.19 ± 0.19 | 2.16 ± 0.19 | 1.78 ± 0.16 | 1.5 ± 0.13 | 1.97 ± 0.17 | 5.16 ± 0.46 | 5.21 ± 0.46 |
| | | GI inh. % | 5.71 | 26.7 | 35.7 | 25.1 | 16.1 | −85.6 | −36.4 |
| | | % of dead cells | 34.7 | 12.7 | 11.4 | 38.4 | 11.2 | 3.2 | 1.8 |
| | | % of Casp.3 pos. cells | 23.5 | 8.6 | 7.7 | 26.1 | 7.6 | 2.1 | 1.2 |
| | | CPD | 1.13 ± 0.11 | 0.99 ± 0.09 | 0.45 ± 0.04 | 0.32 ± 0.02 | −0.15 ± 0.01 | 2.47 ± 0.22 | 2.33 ± 0.21 |
| 3 impulses | 24 h | GI | 0.29 ± 0.02 | 0.56 ± 0.05 | 0.88 ± 0.07 | 1.04 ± 0.09 | 0.28 ± 0.02 | 1.35 ± 0.12 | 1.97 ± 0.17 |

| | | | Monolayer cells | | | | | Suspension cells | |
|---|---|---|---|---|---|---|---|---|---|
| **Cell treatment** | **Time of treatment** | | **Chicken fibroblasts** | **MDBK[1]** | **Vero[2]** | **MRC-5[3]** | **HeLa[4]** | **FB1[5]** | **K 562[6]** |
| | | GI inh. % | 79.4 | 30.4 | 41.9 | −13.6 | 27.1 | −13.4 | −3.7 |
| | | % of dead cells | 19.6 | 12.7 | 24.7 | 32.6 | 11.3 | 1.3 | 1.7 |
| | | % of Casp.3 pos. cells | 14.5 | 9.9 | 19.3 | 25.5 | 8.2 | 1.02 | 1.3 |
| | | CPD | −1.78 ± 0.16 | −0.92 ± 0.08 | −0.55 ± 0.05 | −0.20 ± 0.01 | −2.95 ± 0.26 | 0.22 ± 0.02 | 0.94 ± 0.08 |
| | 72 h | GI | 0.98 ± 0.08 | 1.12 ± 0.11 | 1.88 ± 0.17 | 0.93 ± 0.08 | 1.05 ± 0.09 | 5.68 ± 0.51 | 5.33 ± 0.48 |
| | | GI inh. % | 57.7 | 48.3 | 31.9 | 37.7 | 46.9 | −10.1 | −2.3 |
| | | % of dead cells | 63.5 | 15 | 38.9 | 70.1 | 14.5 | 2.2 | 1.2 |
| | | % of Casp.3 pos. cells | 44.7 | 11.7 | 30.5 | 55.09 | 11.4 | 1.7 | 0.9 |
| | | CPD | −0.02 ± 0.003 | 0.04 ± 0.005 | 0.53 ± 0.004 | −0.35 ± 0.03 | −1.03 ± 0.11 | 2.33 ± 0.21 | 2.38 ± 0.21 |

[1]*Cercopithecus arthiopis kidney.*
[2]*Madin-Darby bovine kidney epithelial cells.*
[3]*Medical Research Council cell strain five fibroblasts derived from lung tissue.*
[4]*Henrietta lacks cervical tumor immortal cell line.*
[5]Homo sapiens *blood lymphoblast.*
[6]Homo sapiens *bone marrow lymphoblast.*
[7]*GI, growth index.*
[8]*CPD, cumulative population doubling.*

**Table 2.**
*The growth parameters, percentages of dead cells, and percentages of Caspase-3 positive cells.*

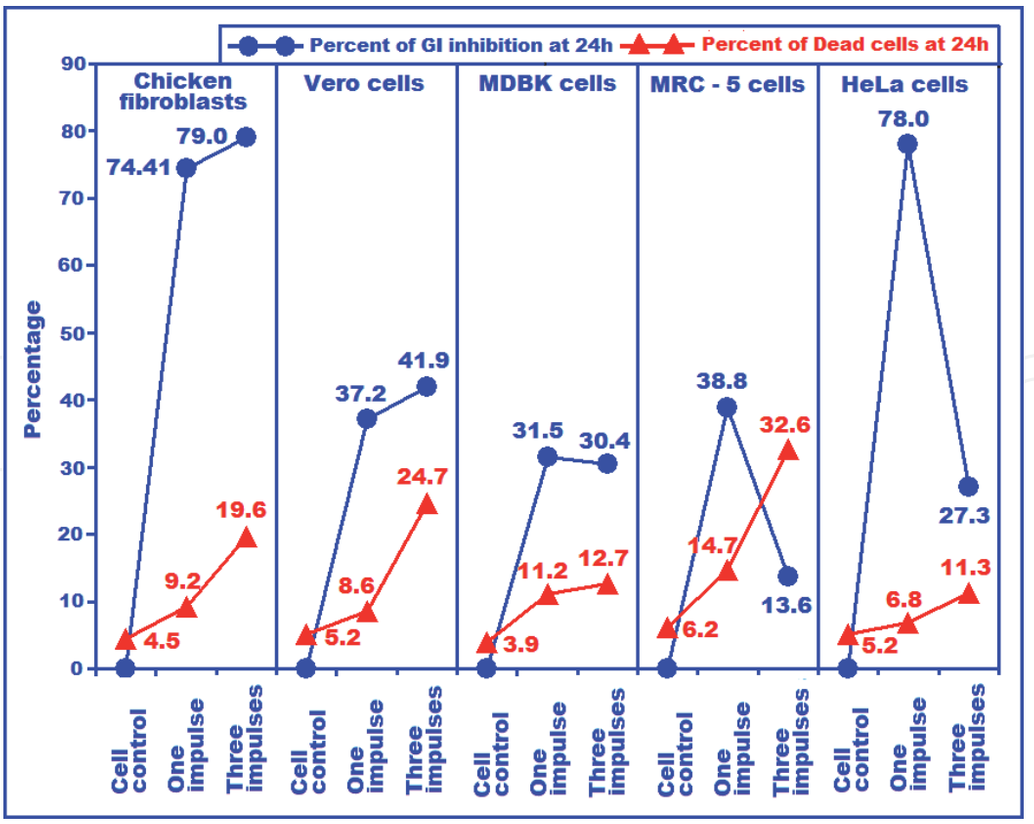



**Figure 3.**
*Growth parameters and percentage of different monolayer dead cells after 24 h of incubation.*

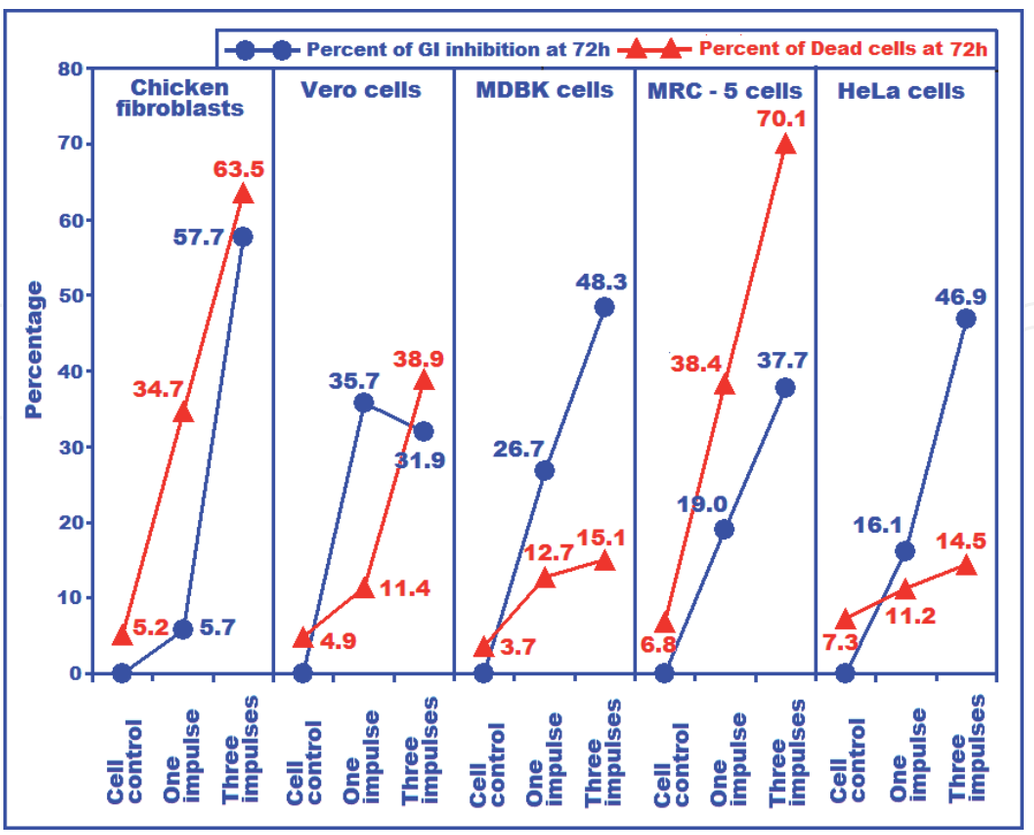



**Figure 4.**
*The Growth parameters and percentage of different monolayer dead cells after 72 h of incubation.*

Chicken fibroblasts: after one impulse 5.7% and after three impulses 57.7%.
Vero cells: after one impulse are 37.7% of GI inhibitions.
MDBK cells: after one impulse 26.7% and after three impulses 48.3%.
MRC-5 cells: after one impulse 25.1% and after three impulses this percentage is 37.7%.
HeLa cells: after one impulse it was 16.1% and after three impulses 46.9%.

The evaluation of the percentages of dead cells after one impulse or three impulses shows the following data for different monolayer cultures: In Chicken fibroblasts the percentage of dead cells after one impulse was 34.7% and after three impulses was 63.5%. In Vero cells after one impulse, there is 11.4% of dead cells and after three impulses 38.9%. In the cells MDBK, after one impulse there is 12.7% of dead cells and after three impulses 15.1%. In the case of MRC-5 cells, after one impulse there is 38.4% of dead cells and after three impulses 70.1%. In the case of HeLa cells, after one impulse there were 11.2% of dead cells, and after three impulses, there were 14.5% of dead cells.

In the evaluation of the trend of GI inhibition percentage, the following comparison was found: In most of the cells, Chicken fibroblasts, MDBK cells, Vero cells, MRC-5 cells, and HeLa cells have higher values after three impulses than after one impulse. The exception is the Vero cells, where the percentage of GI inhibition is a bit lower after three impulses. The analysis about the data of the percentage of dead cells shows the following: The highest percentage of dead cells was 63.5% after three impulses in Chicken fibroblasts. Also was higher percentage of dead cells after three impulses of 38.9. The lowest percentage of dead cells was in MDBK cells after three impulses which were 16.1%. In addition, surprisingly, in the case of MRC-5 cells, again the percentage of dead cells after three impulses is 70.1%. Moreover, the lowest percentage of dead cells was in the case of HeLa cells where it was 14.5%.

### *3.1.3 Percentage of Caspase-3 positive cells after treating of different cells growing in monolayer, with one or three impulses and cultivated for 24 h or 72 h*

During the experiments, different monolayer cells put into suspension were treated with one or three impulses with the adsorbed dose (AD) of one impulse was 0.163 J/g and of three impulses was 0.490 J/g. After 24 h or 72 h of cultivation, the cells were trypsinized, sedimented by the low speed centrifugation, and analyzed for detection of the percentage of Caspase-3-positive cells **(Figure 5)**. In the Chicken embryonic fibroblasts, the percentage of Caspase-3-positive cells after 24 h and one impulse was 23.5% and after three impulses was 14.5%. After 72 h of incubation and one impulse, the percentage of Caspase-3 positive cells was 23.2%. After three impulses, the percentage increased to 44.7%. In the case of Vero cells, after 24 h of incubation and one impulse, the percentage of Caspase-3 positive cells was 7.7%. After three impulses, the percentage increases to 19.3%. After 72 h of incubation and treatment with one impulse, the percentage of Caspase-3 positive cells was 7.5%. After three impulses, this increased to 30.5%. In the case of MDBK cells, after 24 h of incubation and treatment with one impulse, the percentage of Caspase-3 positive cells was 8.5%. After three impulses, this increased to 9.9%. After 72 h of incubation and one impulse, the percentage was 8.6%. After three impulses, this percentage increased to 11.7%. When the MRC-5 cells were analyzed, after 24 h of incubation and one impulse, the percentage of Caspase-3-positive cells was 26.1%; after three impulses, the percentage was 26.5%. After 72 h of incubation and one impulse, the percentage was 26.1%. After three impulses, the percentage highly increased to 55.9%. In the case of HeLa cells after 24 h of incubation and one

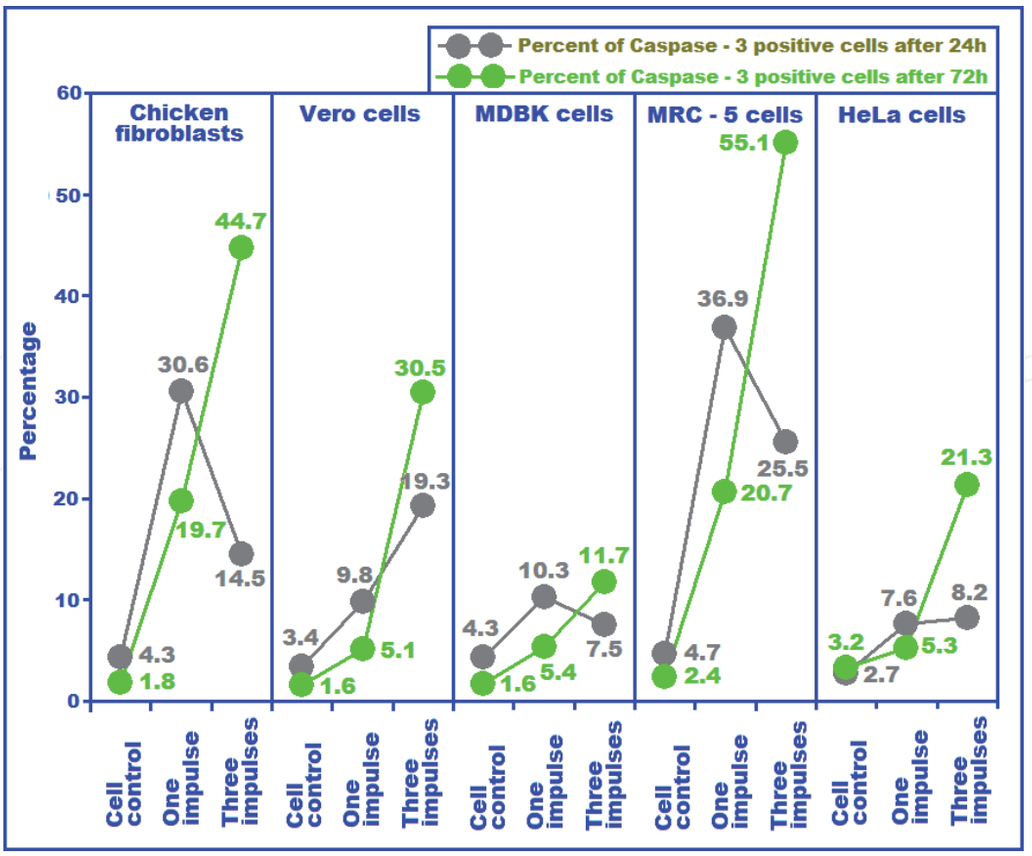



**Figure 5.**
*The percent of Caspase-3-positive cells after 24 h and 72 h of incubation of different types of monolayer cells.*

impulse, the percentage of Caspase-3-positive cells was 7.6%. After three impulses, the percentage was 8.2%. After 72 h of incubation and one impulse, the percentage of Caspase-3 positive cells was 7.6%. After three impulses, this percentage increased to 11.4%.

The comparison of the data on **Figure 5** show the following: (a) The pattern of Caspase-3-positive cells after 72 h and three impulses was the highest in MRC-5 cells 55.1% followed by chicken fibroblasts with 44.7%, Vero cells with 30.5%, MDBK cells with 11.7%, and finally in HeLa cells with 21.3%. (b) The increase of percentage of Caspase-3 positive cells after 72 h of incubation and three impulses was statistically significant in comparison to 24 h of incubation and three impulses. For Chicken fibroblasts, this was 14.5% versus 44.7%. For MRC-5 cells, this was 26.5 versus 55.9%. For Vero cells, this was 19.3% versus 30.5%. (c) For MDBK cells this was 9.9% versus 11.7% and for HeLa cells, this was 8.2% versus 21.3%. For both cell lines, the increase was not statistically significant.

## 3.2 The growth parameters, percentages of dead cells, and percentages of Caspase-3 positive cells of the in suspension growing cells

### *3.2.1 The growth parameters and percentage of dead cells after 24 h of incubation*

After the in suspension growing cell's treatment with one or three impulses having an adsorbed dose for one impulse of 0.163 J/g and for three impulses 0.490 J/g, the main effect was the GI increase expressed in percentage. In comparison, the percentage of dead cells was determined after the treatment with one or three impulses. The detailed results **(Table 2** and **Figure 6)** show the following: For the Lymphoblast cells FB1, the GI increase after one impulse was 27.1%, which fell down to 13.6% after three impulses. In comparison, the percentage of dead cells

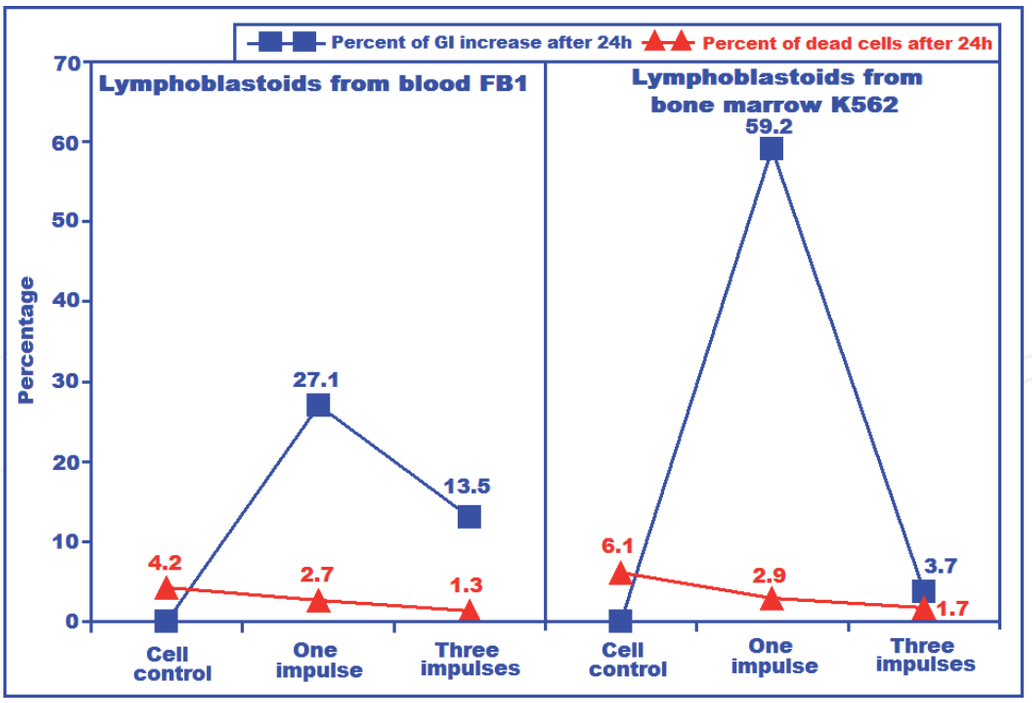



**Figure 6.**
*The Growth parameters and percentage of dead cells after 24 h of incubation and in suspension growing cells treated with one or three impulses.*

after the treatment with one impulse from 4.2% in the control fell down to 2.7%. After three impulses, this percentage fell down to 1.3%. It is unusual that GI increase also fell down after the three impulses.

For the Lymphoblast cells K562, the GI increase was even higher after one impulse, and it was 59.1%. Unexpectedly, after three impulses it fell down to 3.7%. When the percentages after one impulse to 1.7% after three impulses. In general, the GI increase in percentage was higher after one impulse than after three impulses.

### *3.2.2 The growth parameters and percentage of dead cells after 72 h of incubation*

When the cells growing in suspension were treated with one or three impulses with an AD of 0.163 J/g for one impulse and 0.490 J/g for three impulses, after 72 h of incubation **(Figure 7)**, the GI increases, which was much higher in the case of lymphoblast cells FB1 after one impulse, which was 86.5%. Again, it fell down to 10.1% after three impulses. In the case of Lymphoblast cells K562, the situation is opposite, namely, after one impulse the percentage fell down to 36.4% and after three impulses even lower to only 2.3%.

The percentage of dead cells shows in lymphoblast cells FB1 the following trend: from the control value of 5.1 to 3.2% after one impulse and to 2.2% after three impulses. In the case of Lymphoblast cells K562, from the xontrol value of 6.7 to 1.8% after one impulse to 1.2% after three impulses. Interestingly, the trend is the same in both cells.

### *3.2.3 Percentage of Caspase-3-positive cells after one or three impulses on suspension lymphoblast cells FB1 or K562 for 24 h*

When the in suspension growing Lymphoblast cells FB1 or K562 were treated with one or three impulses with an AD for one impulse of 0.163 J/g and for three impulses of 0.490 J/g, after the 24 h of incubation were analyzed for percent of Caspase-3 positive cells **(Figure 8)**. These values were compared with the

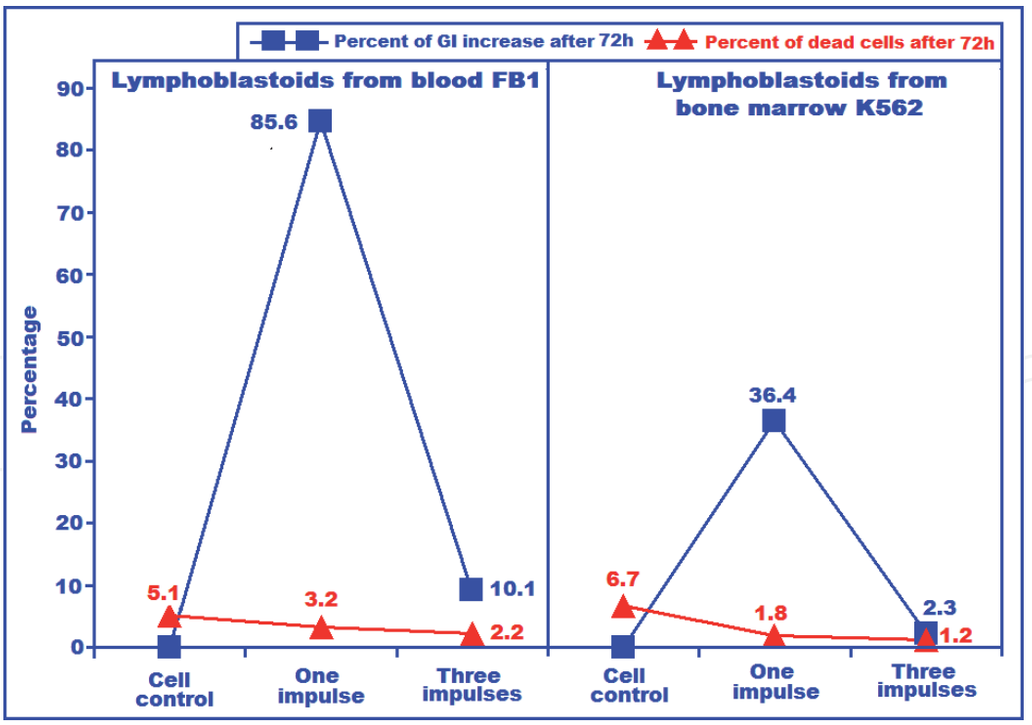



**Figure 7.**
*The Growth parameters and percentage of dead cells after 72 h of incubation and in suspension growing cells treated with one or three impulses.*

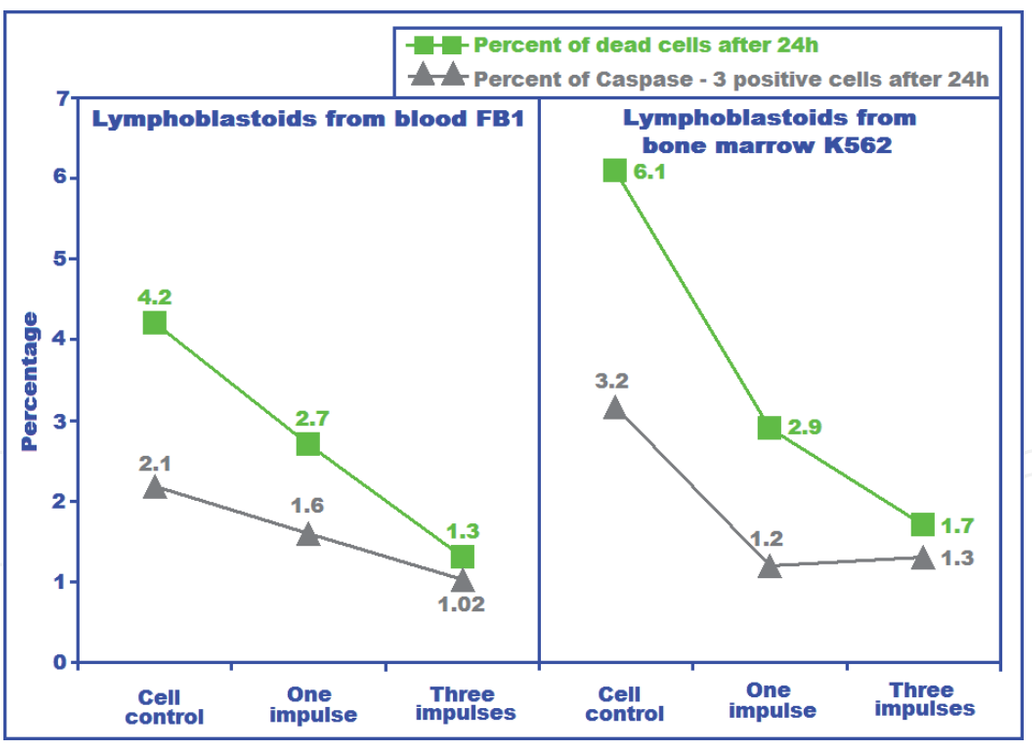



**Figure 8.**
*The Percentage of Caspase-3-positive cells after one or three impulses on in suspension growing lymphoblast cells FB1 and K562 for 24 h.*

percentage of dead cells after 24 h of incubation. In the case of lymphoblast cells FB1 in the cell control, there was 2.18% of Caspase-3 positive cells. After one impulse, this percentage was 2.1%, and after three impulses, it fell down to 1.02%.

The comparison with the percentage of dead cells after 24 h shows the following: In the cell control, there were 4.2% of dead cells. After one impulse, the percentage of dead cells fell down to 2.7%, and after three impulses, it further fell down to 1.3%.

The main conclusion is that the percentage of Caspase-3 positive cells correlates with the percentage of dead cells. In the Lymphoblast cells, K562, in the cell control, there was 3.17% of positive cells. After one impulse, this fell down to 1.2%, and after three impulses this increases a little to 1.2%.

It is interesting that the Lymphoblast cells K562 have one-third higher percentages of dead cells than Lymphoblast cells FB1. In the cell control, there were 6.7% of dead cells. After one impulse, it fell down to 1.8% and after three impulses to 1.2%.

### *3.2.4 The Percentage of Caspase-3-positive cells after one or three impulses on in suspension growing lymphoblast cells FB1 or K562 growing in suspension, after 72 h*

The in suspension growing Lymphoblast cells FB1 or K562 were treated with one or three impulses with an AD for one impulse of 0.163 J/g and for three impulses of 0.490 J/g after 72 h of incubation and were analyzed for the percentage of Caspase-3 positive cells (**Figure 9**). In comparison, the same cells were analyzed for the percentage of dead cells. In the case of lymphoblast cells FB1 in the cell control, there were 4.8% of Caspase-3 positive cells, which fell down to 2.1% after one impulse and further fell down to 1.7% after three impulses. The comparison of the percentage of dead cells shows the following: In the cell control, there were 4.6% of dead cells, which fell down to 1.2% after one impulse and further to 0.9% after three impulses.

The value for the percentage of Caspase-3-positive cells correlates with the percentage of dead cells. The values for the percentage of dead cells were higher than those for Caspase-3. When the lymphoblast cells K562 were analyzed for the percentage of Caspase-3 positive cells, it was found that in the cell control there were 4.6% of positive cells, which fell down to 1.2% after one impulse and to 0.9% after three impulses. The comparison of the percentage of dead cells shows the following: In the cell control, there were 6.7% of dead cells, which fell down to 1.8%

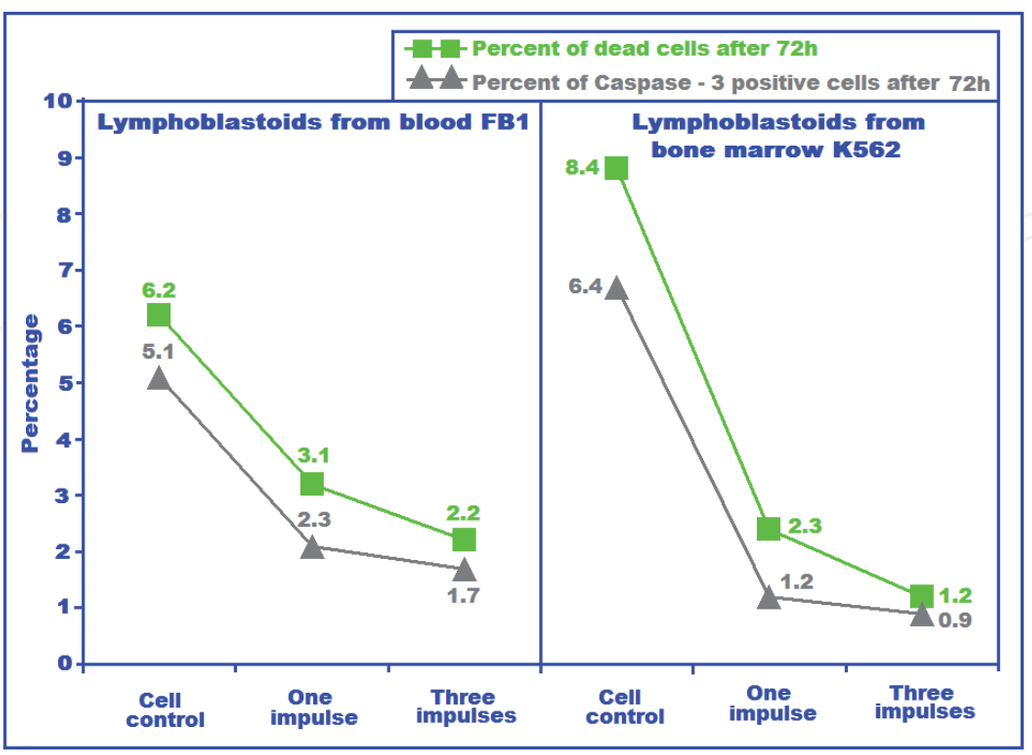



**Figure 9.**
*The Percentage of Caspase-3-positive cells after one or three impulses on suspension lymphoblast cells FB1 and K562 after 72 h of incubation.*

after one impulse and further fell to 1.2% after three impulses. In addition, in this case, the values for the percentage of Caspase-3 positive cells correlate with the percentage of dead cells.

## 4. Discussion

### 4.1 The influence of one or three square impulses with field force of 100 V/cm on different cells growing in monolayer

It was found that nanosecond pulsed electric fields (nsPEFs) can induce the direct electric fields and biological effects on the human colon carcinoma cells [29]. The main effect is the reduction of cell number after electrical impulses, together with the increase of apoptosis markers p53. The dead cells occurred through the necrosis. The direct role of Caspase-3 was not clear. In another research [30], it was found that the picosecond pulsed electric field (psPEF) could induce apoptosis in HeLa cells. The treatments with psPEF led to increased cell apoptosis and cell cycle arrest in the $G_2$/M phase. The psPEF also affected the phosphorylation levels of endoplasmic reticulum sensors and upregulated the expression of glucose-regulated protein 78 (GRP78), glucose-regulated protein 94 (GRP94), and CCAAT enhancer-binding protein (C/EBP) homologous protein (CHOP). All changes were accompanied with the elevation of intracellular $Ca^{2+}$ concentrations. The activation of Caspase-12, Caspase-9, and Caspase-3 led to the release of cytochrome *c* and the upregulation of Bax or the downregulation of Bcl-2, as observed in the HeLa cells. All these data suggest that psPEF is an efficient apoptosis-inducing agent for HeLa cells, which exerts its effects, at least partially, through the endoplasmic reticulum stress and Caspase-dependent signaling pathways. The MTT assay demonstrated that psPEF displayed strong growth inhibitory effects on HeLa cells. The explained cell death and apoptosis by nanosecond pulsed electric fields were found by Beebe et al. [31]. Despite nsPEFs having multiple cellular targets, these studies show that nsPEF affects the cell viability. The cell death depended on the presence of $Ca^{2+}$. When both events occur, cell death can arise.

In our experiments, different monolayer cells being in suspension were treated with one or three impulses having an AD for one impulse of 0.163 J/g and for three impulses of 0.490 J/g. After the treatment with one or three impulses, different monolayer cells were analyzed for the percentage of growth index inhibition, percentage of dead cells, and percentage of Caspase-3 positive cells. In the evaluation of the trend of percentage of GI inhibition, the following comparison was found: In most of the cells, chicken fibroblasts, MDBK cells, Vero cells, MRC-5 cells, and HeLa cells have higher values after three impulses than after one impulse. The exception is the Vero cells, where the percentage of GI inhibition is a bit lower after three impulses. The analysis about the data of the percentage of dead cells shows the following: The highest was the percentage of dead cells (63.5%) after three impulses in Chicken fibroblasts. Also the percentage of dead cells (38.9%) was higher after three impulses. The lowest was the percentage of dead cells in MDBK cells after three impulses (16.1%). Surprisingly, in the case of MRC-5 cells, again the percentage of dead cells after three impulses is 70.1%. And the lowest was the percentage of dead cells in the case of HeLa where it was 14.5%.

The comparison of the data about the percentage of Caspase-3 positive cells shows the following: (a) The pattern of Caspase-3 positive cells after 24 h and three impulses was the highest in MRC-5 cells with 55.9%, followed by Chicken fibroblasts with 44.7%, Vero cells with 30.5%, MDBK cells with 11.7%, and finally in HeLa cells with 11.4%. (b) The increase of percentage of Caspase-3 positive cells

after 72 h of incubation and three impulses was statistically significant in comparison to 24 h of incubation and three impulses. For chicken fibroblasts, the percentage was 14.5% versus 44.7%. For MRC-5 cells, the percentage was 26.5% versus 55.9%. For Vero cells, the percentage was 19.3% versus 30.5%. For MDBK cells, the percentage was 9.9% versus 11.7%, and for HeLa cells, the percentage was 8.2% versus 11.4%. For both cell lines, the increase was not statistically significant.

It is important that our data are in quite good agreement with the data of Zhang [32] even he worked on human A375 melanoma cells only. He found that the nsPEFs failed to induce apoptosis of A375 melanoma cells, though it induced necrosis. However, the viability and migration of A375 melanoma cells were significantly inhibited by nsPEFs. It also suppressed the proliferation of A375 melanoma cells by restricting the cells in G0 phase.

### 4.2 The influence of one or three square impulses with a field force of 100 V/cm on the cells that grow in suspensions

The Ren et al. [33] in their experiments studied the influence of nanosecond pulsed electric fields on the activation of intrinsic Caspase-dependent and Caspase-independent cell death in Jurkat cells in vitro. In other world, is the cell dead in Jurkat cells Caspase-dependent or Caspase-independent. Experiments with U937 Lymphoid cell line cultures treated with pulsed electromagnetic field (PEMF) revealed that PEMF decreased the apoptosis of cells [34]. In order to discriminate and clarify mechanisms responsible for cell death after PEMF, cell culture was stimulated with death inducer Puromycin. Puromycin treatment combined with simultaneous single PEMF exposition reduced the death cell rate. Three times PEMF exposition increased the cell viability by about 25%. These data were in agreement with Grassi et al. [35], who found that 50 Hz EMF exposure enhanced proliferation and inhibited Puromycin induced cell death in human Neuroblastoma and rat neuroendocrine cells.

In our experiments, cells growing in suspension, like Lymphoid cells FB1 or K562, were treated with one or three impulses having an AD for one impulse of 0.163 J/g and for three impulses of 0.490 J/g. After the treatment with one or three impulses, different cells growing in suspension were analyzed for the percentage of GI increase, percentage of dead cells, and percentage of Caspase-3 positive cell decrease.

The pattern of the percentage of GI increase after one or three impulses after 24 h at lymphoblast FB1 shows a GI increase after one impulse of 27.1%, which fell down to 13.6% after cell treatment with three impulses. At lymphoblast K562, the picture is opposite: after one impulse the GI increase was 59.1% versus 3.7% after three impulses. The pattern after 72 h at lymphoblast cells FB1 shows 85.6% after one impulse versus 10.1% after three impulses. In lymphoblast cells K562, the picture is opposite: after one impulse 36.4% and after three impulses 2.3% only.

The percentage of dead cells after one or three impulses after 24 h, at Lymphoblast cells FB1, shows the decrease after one impulse from 2.7 to 1.3% after three impulses. The same trend was found in the Lymphoblast cells K562. The decrease of percentage of dead cells was from 2.9% after one impulse to 1.7% after three impulses. After 72 h, we found the same trend: at FB1 cells, the percentage was 3.2% after one impulse versus 2.2% after three impulses. In K562 cells, the percentage was from 1.8% after one impulse to 1.2% after three impulses.

When the pattern of the changes of the Caspase-3 positive cells after one or three impulses was analyzed, the following picture was found after 24 h: in the lymphoblast cells FB1 after one impulse 2.7% which fell to 1.3% after three impulses. It is similar in the picture in the lymphoblast cells K562: from 1.2% after one impulse to

1.3% after three impulses. Therefore, the percentage of Caspase-3 positive cells is independent from the number of impulses.

The percentage of Caspase-3 positive cells after 72 h in the lymphoblast cells FB1 was 2.1% after one impulse and fell down to 1.7% after three impulses. In the case of Lymphoblast cells K562, the percentage was from 1.2% after one impulse to 0.9% after three impulses.

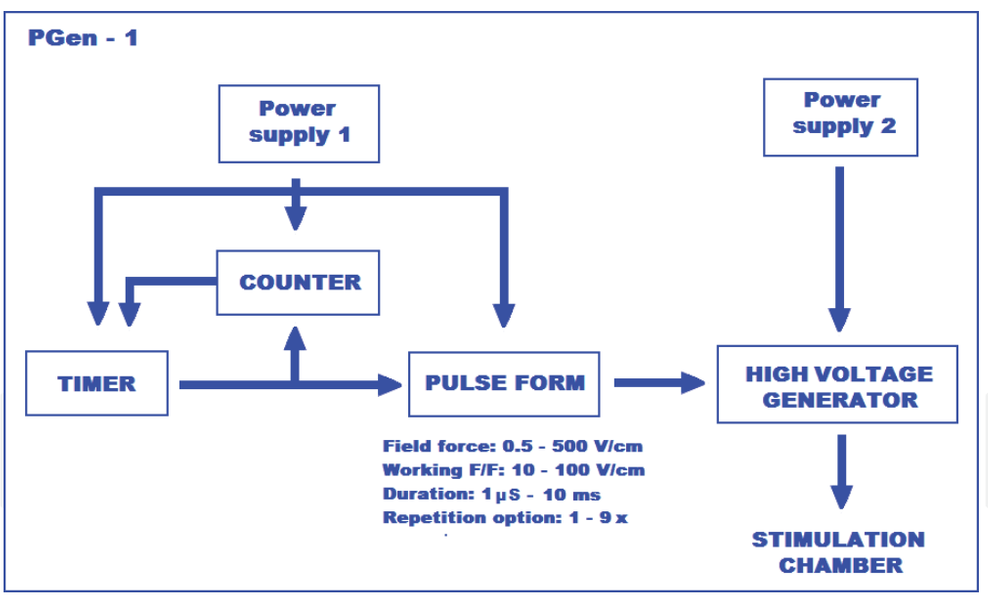
## Acknowledgements

This research was supported by Ivan Čermak, Crodux plin D.o.o., 10000 Zagreb, Croatia, and it was performed via the Project: "Oncolytic Newcastle disease virus in the veterinary medicine' of the Croatian Institute for Experimental and Translational Medicine, Koledinečka 03, 10040 Zagreb, Croatia. Authors are indebted to Tomaž Velnar for English suggestions.

## Conflict of interests

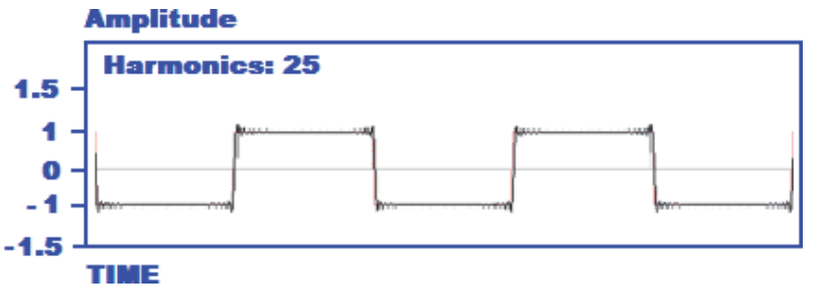
All authors declared no conflicts of interest.

## Appendix

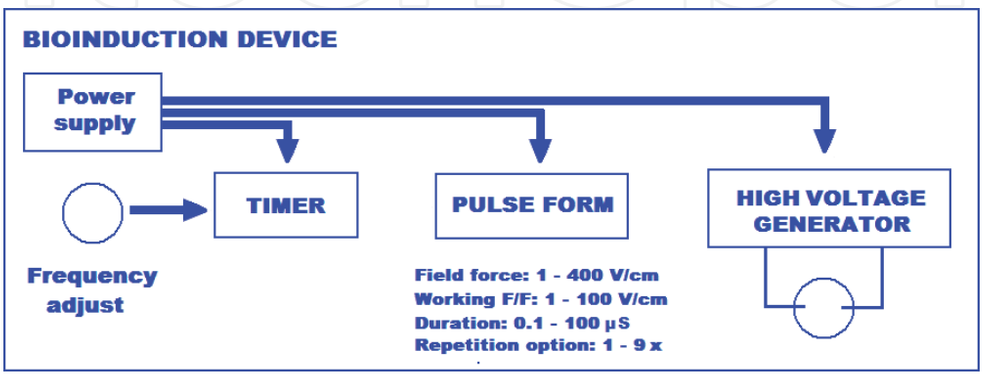


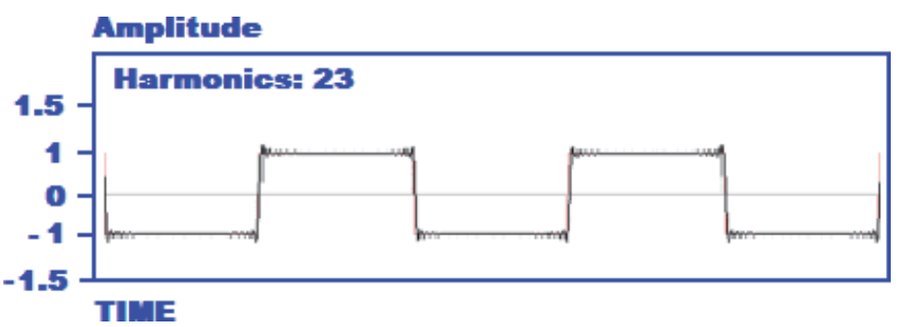

The PGen-1 device generates the characteristic square-wave impulse. This can be mathematically described as Gibbs phenomenon, which is the result of an instantaneous change to a system with a finite number of samples. A pure sine wave has only a single fundamental frequency and no higher-order harmonics. A square wave has an infinite number of frequency components. A sine wave can be transformed into a square wave by adding harmonics to its fundamental. As such, a number of harmonics will produce a perfect square wave with a number of harmonics of 25. Such use of harmonics to build a square wave is demonstrated in the figure below:

*DOI: http://dx.doi.org/10.5772/intechopen.90506*




The picture of such a square wave is very similarly with this produced on the bioinduction device described by Pretnar et al. [14] with the number of harmonics of 23.



## Author details

Bratko Filipič[1*], Lidija Gradišnik[2], Kristine Kovacs[3], Ferenc Somogyvari[4], Hrvoje Mazija[1] and Toth Sandor[5]

1 CIETO (Croatian Institute for Experimental and Translational Oncology), Zagreb, Croatia

2 Medical Faculty of Maribor, Institute of Biomedical Sciences, Maribor, Slovenia

3 Sylvester Comprehensive Cancer Center, Coral Glades, University of Miami, USA

4 Department of Medical Microbiology and Immunobiology, University of Szeged, Szeged, Hungary

5 TEWA Consultant, Szeged, Hungary

*Address all correspondence to: bratko.filipic@gmail.com

## References

[1] Frey HA. On the nature of electromagnetic fields interactions with biological systems. FASEB Journal. 1993; **7**:272-281. DOI: 10.1096/fasebj.7.2.8440406

[2] Meulenberg WJC, Todorović V, Čemažar M. Differential cellular effects of electroporation and electrochemotherapy in monolayers of human microvascular endothelial cells. PLoS One. 2012;**7**(12):e52713. DOI: 10.1371/journal.pone.0052713

[3] Bolognani L, Del Monte V, Francia F, Costato M. Low-frequency electromagnetic pulsed field stimulation of yeast. Electromagnetic Biology and Medicine. 2009;**11**(1):1-10. DOI: 10.3109/15368379209012847

[4] Im C-H, Jun CS, Sekihara K. Recent advances in biomagnetism and its applications. Biomedical Engineering Letters. 2017;**7**:183. DOI: 10.1007/s13534-017-0042-3

[5] Cui Y, Liu X, Yang T, Mei YA, Hu C. Exposure to extremely low-frequency electromagnetic fields inhibits T-type calcium channels via AA/LTE4 signaling pathway. Cell Calcium. 2014;**55**(1):48-58. DOI: 10.1016/j.ceca.2013.11.002

[6] Araújo QFO, Alice Z, Coelho ZAM, Isabel CP, Margarit CPI, Vaz-Junior AC, et al. Electrical stimulation of *Saccharomyces cerevisiae* cultures. Brazilian Journal of Microbiology. 2004; **35**(1–2):97-103. DOI: 10.1590/S1517-83822004000100016

[7] Holan V, Machala O, Dao T, Minowada J. Effect of direct current on T cell activity: Modulation of interleukin-2 production. Immunobiology. 1994;**190**:368-371. DOI: 10.1016/S0171-2985(11)80608-X

[8] Berg H. Problems of weak electromagnetic field effects in cell biology. Bioelectrochemistry and Bioenergetics. 1999;**48**(2):355-360. DOI: 10.1016/s0302-4598(99)00012-4

[9] Huang J, Ye Z, Hu X, Lu L, Luo Z. Electrical stimulation induces calcium-dependent release of NGF from cultured Schwann cells. Glia. 2010;**58**(5):622-631. DOI: 10.1002/glia.20951.w

[10] Velizarov S. Electric and magnetic fields in microbial biotechnology: Possibilities, limitations, and perspectives. Electromagnetic Biology and Medicine. 1999;**18**(2):185-212. DOI: 10.3109/15368379909012912

[11] Funk WHR, Monsees KT. Effects of electromagnetic fields on cells: Physiological and therapeutical approaches and molecular mechanisms of interaction. Cells, Tissues, Organs. 2006;**182**(2):59-78. DOI: 10.1159/000093061

[12] Petrini M, Polidori R, Ambrogi F, Vaglini F, Zanioli P, Ronca G, et al. Effect of different low-frequency electromagnetic fields on lymphocyte activation: At which cellular level? Electromaganetic Biology and Medicine. 1990;**9**:159-166. DOI: 10.3109/15368379009119803

[13] Katsuki S, Li Y, Miyakawa D, Yamada YR, Onishi N, Lim S. Response of mammalian cells to non-thermal intense narrowband pulsed electric fields. In: 11th European Conference of Antennas and Propagation (EUCAP). 2017. DOI: 10.23919/EuCAP.2017.7928345

[14] Pretnar G, Filipic B, Golob A, Skodic A, Toth S, Mecs I, et al. Electroinduction of interferon-like proteins. Bioelectrochemistry and Bioenergetics. 1991;**25**:183-193. DOI: 10.1016/0302-4598(91)87002-X

[15] Filipič B, Kovacs K, Somogyvari F, Ihan A, Ocosovsky I, Koren S, et al. The effects of medium-strength electric

impulses on human blood. Bioelectrochemistry. 2000;**52**(1):29-36. DOI: 10.1016/S0302-4598(00)00079-9

[16] Gal G, Toth M, Toth S, Kiss E. Alpha and gamma interferon production of dialysed chronic uraemic and kidney transplant patients. Abstracts EDTA Brussels Belgium VI. 1985;**25-29**:49-49

[17] Filipič B, Koren S, Kovacs K, Somogyvari F, Toth S. Naprava in postopek za elektrostimulacijo celic (Device and method for electrostimulation of cells) (in Slovenian). Slovenian Patent No. SI 20111 A, 600–214/98-MZT-6 z dne: 03.07. 2000. (P-9800214. 1998)

[18] Kovacs K, Somogyvari F, Filipič B, Mecs I, Toth S. Induction of antiviral response by electric pulses. Electromagnetic Biology and Medicine. 2000;**19**(3):279-287. DOI: 10.1081/JBC-100102119

[19] Filipic B, Gradišnik L, Somogyvari F, Toth S, Mazija H. The biological activity of medium-strength square-wave electric impulses on the proliferation patterns of different animal cells. ALTEX Proceedings. 2019; **8**(1):52-52. DOI: 10.1314/RG.2.2.1938040326

[20] Filipič B, Koren S, Kovacs K, Somogyvari F, Toth S. Naprava in postopek za elektrostimulacijo celic—Device and method for electro stimulation of cells (in Slovenian). Slovenian Patent No. SI 20111A, 600–214/98-MZT-6 z dne: 03.07. 2000 (P-9800214. 1998)

[21] Pakhomov AG, Phinney A, Ashmore J, Walker K III, Kolb FJ, Kono S, et al. Characterization of the cytotoxic effect of high-intensity, 10-ns duration of electrical pulses. IEEE Transactions on Plasma Science. 2004;**32**(4):1579-1586. DOI: 10.1109/TPS.2004.831773

[22] Rink H, Vornhagen R, Koch HR. Rat lens epithelial cells in vitro. I. Observation on aging, differentiation and culture alternations. In Vitro. 1980; **16**(1):15-19. DOI: 10.1007/bf02618195

[23] Filipič B, Golob A, Toth S, Mecs I, Beladi I, Likar M. Interactions between human and porcine interferons. Acta Virologica. 1991;**35**:19-26

[24] Filipič B, Schauer P, Suhar A. Changes of the intracellular levels of some hydrolases during the spontaneous transformation of rat embryonal fibroblasts (REF) (Wistar strain) cell line. Farm. Vest. 1984;**35**(3):239-243

[25] Ito M. Microassay for studying anticellular effects of human interferons. Journal of Interferon Research. 1984;**4**(4):603-608. DOI: 10.1089/jir.1984.4.603

[26] Singh A, Kakkar P, Pant AB. Comparative evaluation of cytotoxic effects of MTAD and sodium hypoclorite using LDH and Trypan blue assays: An in vitro study. Saudi Endodontic Journal. 2018;**8**(3):189-195. DOI: 10.4103/sej.sej_75_17

[27] Jauregui OH, Hayner TN, Driscoli LM, Williams-Holland R, Lipsky MH, Galleti MP. Trypan blue dye uptake and lactate dehydrogenase in adult hepatocytes—Freshly isolated cells, cell suspensions and primary monolayer cultures. In Vitro. 1981; **17**(12):1100-1109. DOI: 10.1007/bf02618612

[28] Ford WE, Ren W, Blackmore PF, Schoenbach KH, Beebe SJ. Nanosecond pulsed electric fields stimulate apoptosis without release of pro-apoptotic factors from mitochondria in B16f10 melanoma. Archives of Biochemistry and Biophysics. 2010;**497**:82-89. DOI: 10.1016/j.abb.2010.03.008

[29] Hall HE, Schoenbach HK, Beebe JS. Nanosecond pulsed electric fields (nsPEF) induce direct electric field effects and biological effects on human

colon carcinoma cells. DNA and Cell Biology. 2005;**24**(5):283-291. DOI: 10.1089/dna.2005.24.283

[30] Chen W-J, Xiong Z-A, Zhang M, Yao C-G, Zhao Z-Y, Hua Y-Y, et al. Picosecund pulsed electric fields induce apoptosis in HeLa cells via the endoplasmic reticulum stress and caspase-dependent signaling pathways. International Journal of Oncology. 2013; **42**:963-970. DOI: 10.3892/ijo.2013.1774

[31] Beebe JS, Sain MN, Ren W. Induction of cell death mechanisms and apoptosis by nanosecond pulsed electric fields (nsPEFs). Cell. 2013;**2**:136-162. DOI: 10.33390/cells1010136

[32] Zhang B. Effect of low-field high-frequency nsPEFs on the biological behaviors of human A375 melanoma cells. IEEE Transactions on Biomedical Engineering. DOI: 10.1109/TBME. 2017.2784546 TBME-01034-2017.R1

[33] Ren W, Sain MN, Beebe J. Nanosecond pulsed electric fields (nsPEFs) activate intrinsic caspase-dependent and caspase-independent cell death in Jurkat cells. Biochemical and Biophysical Research Communication. 2012;**421**:808-812. DOI: 10.1016/j. bbrc.2012.04.09

[34] Kaszuba-Zwoinska J, Wojcik K, Bereta M, Zombier A, Pierzchalski P, Rokita E, et al. Pulsating electromagnetic field stimulation prevents cell death of puromycin treated U937 cell line. Journal of Physiology and Pharmacology. 2012; **61**(2):201-205. DOI: 10.1515/cmble-2015-0006

[35] Grassi C, D'Ascenzo M, Torsello A, Martinotti G, Wolf F, Cittadini A, et al. Effects of 50 Hz electromagnetic fields on voltage-gated $Ca^{2+}$ channels and their role in modulation of neuroendocrine cell proliferation and death. Cell Calcium. 2004;*35*(4):307-315. DOI: 10.1016/j.ceca.2003.09.001