Effects of low intensity static electromagnetic radiofrequency fields on leiomyosarcoma and smooth muscle cell lines

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Abstract

In this study we investigated the effects of low intensity static radiofrequency electromagnetic field (EMF) causing no thermal effects, on leiomyosarcoma cells (LSC), isolated from tumors of fifteen Wistar rats induced via a 3,4-benzopyrene injection. Electromagnetic resonance frequencies measurements and exposure of cells to static EMF were performed by a device called multi channel dynamic exciter 100 V1 (MCDE). The LSC were exposed to electromagnetic resonance radiofrequencies (ERF) between 10 kHz to 120 kHz, for 45 min. During a 24h period, after the exposure of the LSC to ERF, there was no inhibition of cells proliferation. In contrast, at the end of a 48h incubation period, LSC proliferation dramatically decreased by more than 98% ($P<0.001$). At that time, the survived LSC were only 2% of the total cell population exposed to ERF, and under the same culture conditions showed significant decrease of proliferation. These cells were exposed once again to ERF for 45 min (totally 4 sessions of exposure, of 45 min duration each) and tested using a flow cytometer. Experiments as above were repeated five times. It was found that 45% of these double exposed to ERF, LSC (EMF cells) were apoptotic and only a small percentage 2%, underwent mitosis. In order to determinate their metastatic potential, these EMF cells were also counted and tested by an aggregometer for their ability to aggregate platelets and found to maintain this ability, since they showed no difference in platelet aggregation ability compared to the LSC not exposed to ERF (control cells). *In conclusion*, exposure of LSC to specific ERF, decreases their proliferation rate and induces cell apoptosis. Also, the LSC that survived after exposed to ERF, had a lower proliferation rate compared to the LSC controls ($P<0.05$) but did not loose their potential for metastases (platelet aggregation ability). The non-malignant SMC were not affected by the EMF exposure ($P<0.4$).The specific ERF generated from the MCDE electronic device, used in this study, is safe for humans and animals, according to the international safety standards.

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Introduction

Electromagnetic radiofrequency fields have been widely studied for their effects on biological species including humans [1]. Radiofrequencies (RF) are usually found in earth environment, coming from cosmic radiation and emitted by communication systems and other technologies. Humans and other species are therefore well adapted to radiofrequencies and is well documented that these fields are harmless at radiation levels below guidelines [2]. Thus, using radio waves at intensities and frequencies below safety limits seems safe for humans and animals. There are however, quite a few data on the effects of electromagnetic fields (EMF) on various kinds of malignant cells in experimental animals and in humans, some of them referring to the application of electromagnetic resonance principles [3, 4]. The main concepts expressed so far referring to the effects of EMF on malignancies are: the intensity, frequency and duration of application of the electromagnetic waves. The EMF may act as co-carcinogen combined with the primary carcinogen, especially in experimental animals but can also exert anticarcinogenic effects, inhibiting the proliferation of malignant cells in vitro as well as decreasing the size of the experimental tumors in vivo [1, 5-7]. Studies of the EMF pro-carcinogenic effects in experimental animals are not numerous and seems to have many uncertainties [1, 5]. On the contrary, studies on the EMF anticancer effects are abundant and their methodology is well documented [8]. It has been also shown, that the cytostatic effects of the EMF on cancer cells are not related to their thermal effects but are temperature-independent [9-11]. Furthermore, malignant cells secrete ag-

onists, inducing platelet aggregation, a procedure facilitating metastasis [12]. Effects of EMF at RF, on such a procedure have not been studied so far.

The aim of the present study was to investigate the effects exerted by low intensity static RF EMF on: a) the proliferation and the viability of the leiomyosarcoma cell (LSC) lines, b) the cell cycle of LSC and c) the platelet's aggregation induced by LSC cells and smooth muscle cells (SMC).

Materials and methods

In the present study cell cultures of LSC, originating from tumors developed in Wistar rats were used, since sarcoma cells are cells with increased metabolic activity [13] and resistant to chemotherapy and irradiation [14].

Production of LSC and SMC

The malignant cells were isolated from selected leiomyosarcoma (LMS) tumors of Wistar rats. Fifteen (7 male and 8 female) Wistar rats, of the fifth generation of a certain couple, 60 days old, were subcutaneously injected in their right scapula by 1 ml of 3,4-benzopyrene solution (B[a]P) in tricapryline in a dose of 10.08 mg/ml. The rat LMS tumors B[a]P induced, have the following characteristics: a) sensitivity to antioxidant substances and free radical scavengers indicating that their proliferation is related to the release of reactive oxygen species [15-17], b) their proliferation can be inhibited via the administration of cycloxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) inhibitors [18] and c) ability to induce LMS tumors when inoculated into Wistar rats. After 110 days (maximum 135 days) of B[a]P injection, all animals developed malignant tumors at the site of injection. All tumors were histologically identified as LMS. The tumors were surgically removed and cut under aseptic conditions into pieces of 0.5 cm^3 in volume. Each piece was placed immediately in cold Ringer's solution and sliced down again to smaller pieces (1 mm^3). All smaller pieces were inserted for 5 min into 5 ml of cold Dulbecco's modified Eagles medium (DMEM) solution which contained small quantities of trypsin and then these pieces were incubated in the same DMEM solution at 37Æ C for 4 h, with gentle mixing every 15 min. After incubation the DMEM solutions containing the tumor pieces were centrifuged at 900 rpm for 10 min and the supernatant was rejected. The remaining cells were resuspended in DMEM+10% fetal bovine serum (FBS) solution and seeded in plastic coated dishes of 52 mm in diameter. Tumor cells after had been subcultured, were examined histologically and were characterized as LSC.

In order to verify if these LSC were active and able to induce the same type of malignancy in rats, 4×10^6 of them were suspended in Hanks salt solution and inoculated into every Wistar rat as follows: the animals were anaesthetized with midazolame and ketamine, and a surgical opening was made in their outer skin layer at the middle of their back. The tissue underneath was traumatized by lancing with a sharp blade in order to bring fresh blood to the surface. LSC were then aseptically infused into the operated area and the incision was immediately sutured. The animals developed medium-sized malignant tumors approximately 12 cm^3 in volume, within the first 10 days after inoculation.

Smooth muscle cells (SMC) were isolated from the aorta of Wistar rats and cultured again by the methods described above.

Induction of tumors on Wistar rats was performed according to the European Community Directives and International Legacy for animal protection and according to the European Union Directive for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (86/609/EEC, article 9, ¨1 and 2).

Electromagnetic field equipment

Electromagnetic resonance frequencies measurement and exposure of cells to static EMF were performed by a device called Multi Channel Dynamic Exciter 100 V1 (MCDE) invented by K. Havelas [19]. The MCDE has been certified by the Greek National Centre for Scientific Research "Dimocritos" and the Greek Atomic Energy Agency for its safe use in humans and animals. This device is consisted of two basic parts: a) an EMF generator of various intensities. The range intensities for the electrical field were 1.1 to 1.11 ± 0.01 V/m and for the magnetic field were 0.0027 to 0.0029 ± 0.00005 A/m. The radiofrequencies generating from the MCDE system range from 10 Hz to 1 MHz. The power density of the electromagnetic field was 100 mV/cm^3 approximately. b) an electron paramagnetic resonance spectrometer with specific characteristics. This spectrometer was a scanner-recorder of the ERF emitted from biological systems (targets), such as localized tumours in experimental animal, or cultured cancer cells, exposed to EMF. It is well known that the normal function of any biological system produces electromagnetic waves of specific frequencies caused by cell electromagnetic properties (electric membrane potential, transport of ions charges etc). The specific ERF emitted from an EMF excited biological system, was recorded as an electromagnetic resonance fingerprint of this system. This was due to the electromagnetic resonance taking part between ERF produced by the generator and ERF emitted from the biological system.

The MCDE was supported by a sophisticated software easy to use, to be submitted for accreditation (copyright). First, it was necessary to record the electromagnetic resonance fingerprints emitted from the biological systems (targets). Then, by selecting the specific MCDE programs of the software, the appropriate ERF for the exposure of the target were calculated and then emitted to the target.

Estimation of LSC and SMC electromagnetic resonance frequencies

Electromagnetic resonance fingerprints of LSC and SMC were recorded before and after their exposure to EMF (10Hz-1MHz). The ERF of LSC electromagnetic resonance fingerprints were modulated using the proper software and after that, the cancer cells were exposed to these modulated electromagnetic fingerprints for 45 min. The cell proliferation and cell morphology were checked every 24 h. A total of 492 different frequencies emitted from LSC were recorded (Fig. 1).

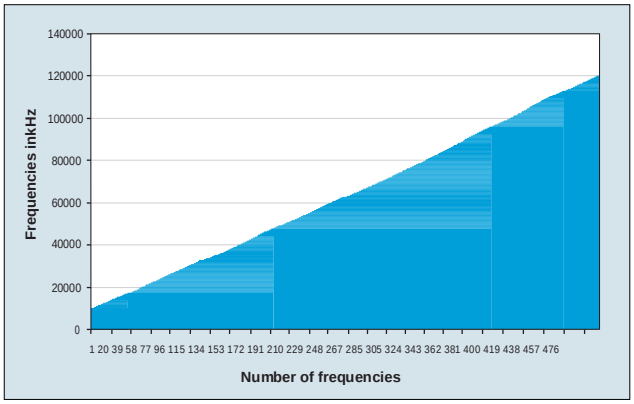


Figure 1. The fingerprint spectrum of 492 frequencies emitted from LMS cells of Wistar rat after an accurate registration by the resonating system of the MCDE apparatus

Method of LSC and SMC exposure to EMF

a. LSC treatment

Twelve Petri dishes with 10 ml growth medium were seeded with $1x10^5$ LSC (zero time). The LSC cultures were incubated in 37Æ C at 95% O_2 + 5% CO_2 atmosphere for 48 h. Then the medium was replaced by 10 ml of freshly prepared DMEM. At 72 h from zero time, six LSC cultures (EMF cells) were placed into a Faraday apparatus at room temperature (RT) and exposed to modulated electromagnetic fingerprints (from 10 to 120 kHz and intensities from 1.1 to 1.11 ± 0.01 V/m for the electric field and 0.0027 to 0.0029±0.00005 A/m for the magnetic field), for 45 min. A total of 492 frequencies were used for LSC exposure to EMF. The used frequencies were derived from a transformation of the 492 frequencies recorded as the LSC electromagnetic fingerprints (Fig. 1) by a specific algorithm [Σf(r) + f(em) = c, f(r) = registered frequency, f(em) = emitted frequency, c = a constant]. The other six LSC cultures (control cells) remained at RT for the same time as EMF cells but without being exposed to EMF (sham exposure). The control and EMF cells were incubated once again at the same conditions as before, for about seven hours. At 79 h and 96 h from zero time, the cells of each culture were counted, subcultured at about 1×10^5 cells per plate and incubated at the same as above conditions. At 120 h from zero time, the EMF cells were re-exposed to EMF as before and 24 h later, both EMF and control cells in each plate were counted and examined under the microscope. Then, to examine their proliferation rate in relation to time, both groups (EMF and control cells) were subcultured and incubated at the same described conditions, in order to estimate the time till cell confluence. The cells were then preserved in liquid nitrogen.

b. SMC treatment

The SMC isolated from Wistar rat aorta, were subcultured in the same conditions as described above for LSC and exposed to EMF in the same manner and frequencies as LSC.

These cell experiments (LSC and SMC treatment) were performed five times, as described above and results were statistically evaluated.

LSC manipulations

The preserved LSC in liquid nitrogen EMF and the control cells were defrost and cultured again till cell confluence. Twelve plates were then seeded with the same number of LSC and incubated for 24 h. The EMF cells were again exposed to EMF for 45 min after 24 h and 48 h. Six hours after the last session, cell samples from each plate were taken for testing their cell cycle phases by a flow cytometer Becton Dickinson, FACScan system (Becton Dickinson, USA, California).

Estimation of platelet's aggregation ability of LSC

The potential for metastasis of the LSC was determined from their ability to aggregate platelets, before and after their exposure to EMF, using the aggregometer CA-500, (Cronolog Co, USA), and the Cronolog kit for platelets aggregation. The Cronolog kit was used to verify the normal platelet responses. A total of 120 tests were performed on blood samples taken from six healthy volunteers who abstained from drugs and alcohol for ten days before the tests. The tests were performed in platelet rich plasma (PRP) isolated from the blood samples and prepared according to the manual of the apparatus. The platelet aggregation tests were performed after the suspension of either 5×10^5 EMF cells or of the same number of control cells, in PRP.

Statistics

Student's t-test was used for the statistical evaluation of the results and $P<0.05$ was considered statistically significant.

Results

Results were obtained from five repetitions of cell culture experiments and were as following:

Cell proliferation rate

Our results were as follows: a) Twenty-four hours after the first and second session of exposure to the EMF, the proliferation rate of the LSC was slightly decreased, in comparison to the control cells proliferation ($P<0.05$). Also the microscopic examination showed that the highest percentage of LSC was under stress, showing round-shaped cells with abortive pseudopodia and formation of nuclear membrane blebs. b) The multiplication rate of the EMF exposed LSC after 48 h of incubation, was dramatically decreased at a percentage higher than 98%, ($P< 0.0001$ compared to the control cells) and most of the exposed malignant cells were found either dead (mainly apoptotic) or extremely stressed: round shaped cells, formation of bleeds in the outer cell membrane, absence of pseudopodia (Fig. 2). c) The survived LSC after one session of EMF exposure, showed a great difficulty in proliferating according to the time needed till cell confluence (six days of incubation) as compared to the time needed for control cells confluence (three days), (Fig. 3). Also, the survived LSC developed a remarkable resistance to EMF, showing only 20% decrease of cell proliferation rate, as compared to the much higher decrease in proliferation rate of **98%,** shown after their first EMF exposure.

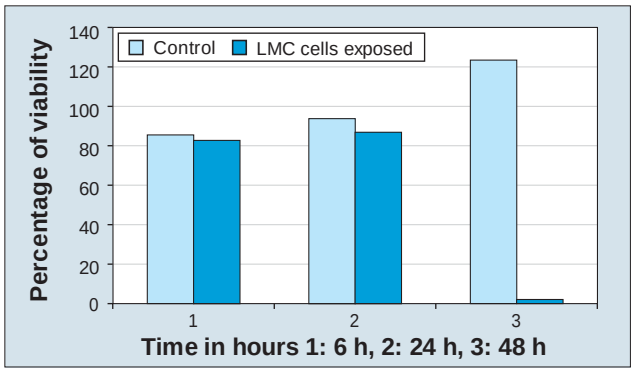


Figure 2. The viability of the EMF exposed LSC after 48 h of incubation was dramatically decreased at a percentage higher than 98%, (P<0.0001 compared to the control cells)

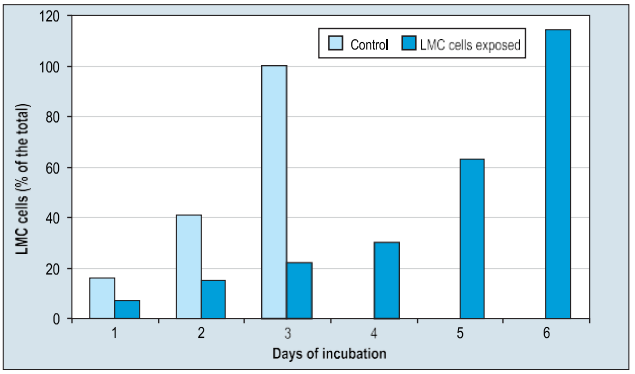


Figure 3. The survived LSC after one session of EMF exposure, showed a significant difficulty in proliferating according to the time needed till cell confluence (six days of incubation) in with comparison to the time needed till control cells confluence (three days)

Table1. *Flow cytometry for non exposed to EMF sarcoma cells*

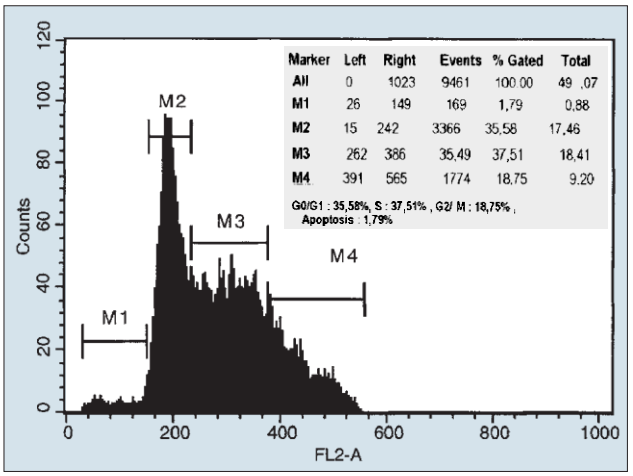
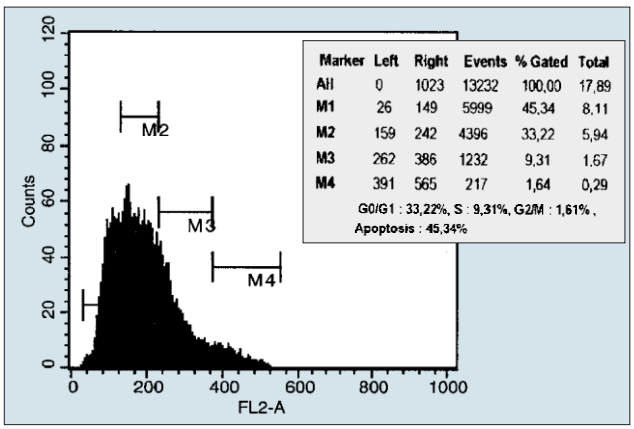


Table 2. *Flow cytometry for exposed to EMF sarcoma cells*



LSC distribution in the cell cycle phases

Flow cytometry revealed that after the fourth cell exposure to EMF, 33% of the LSC were found to be in G0/G1, 9% in S phase, 2% in mitosis and 45% were undergoing apoptosis, while the control cells were found to be: 36% in G0/G1, 38% in S phase, 19% in mitosis and 2% undergoing apoptosis (Tables 1 and 2).

SMC viability after exposure to EMF

The SMC seem not to be affected by EMF exposure at 24 and 48 h (Fig. 4). The exposed SMC had a proliferation rate 10% lower than the non exposed SMC (control). This difference was not statistically significant (P<0.4).

Estimation of LMS cells "potential for metastasis" by platelet aggregation

The aggregation ability of the control as well as of the exposed to EMF cells, was 78%, and almost equal to the ability to aggregate by adenosine diphosphate (82%). According to the above, LSC exposure to EMF did not affect significantly the "potential for metastases" of LSC (Fig. 5).

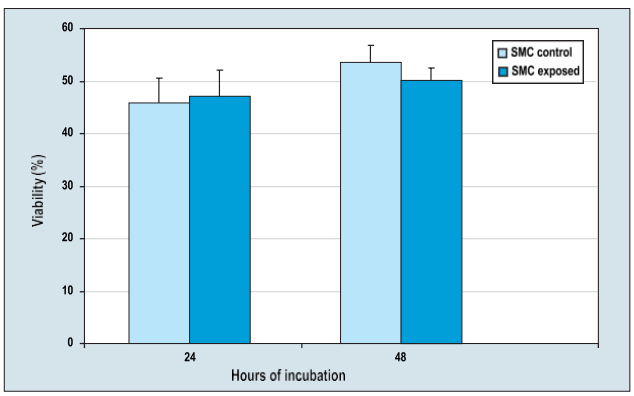


Figure 4. Viability of SMC not exposed and exposed to EMF. The non exposed SMC have a rate of growth 10% larger from the exposed ones [P<0.4]

Estimation of cell electromagnetic resonance – frequencies

Electromagnetic resonance frequencies of the: a) unexposed to EMF LSC were ranging between 10.5 to 120.5 kHz, b) exposed to EMF LSC, between 10 to 120 kHz and c) SMC between 10 to 120 kHz. Spectrum analysis of the above estima-

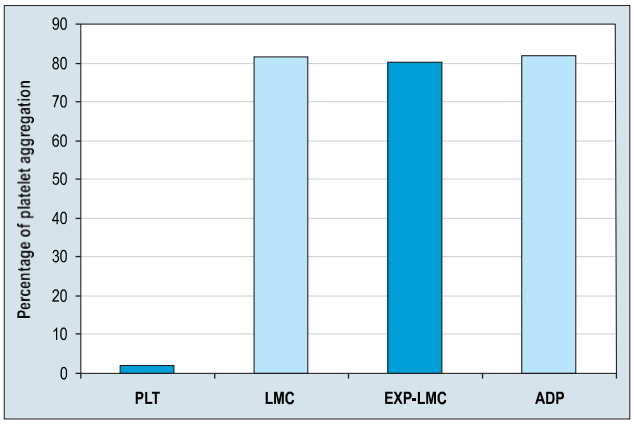


Figure 5. LSC exposure to EMF did not affect significantly the capacity of LSC to aggregate human platelets. PLT: resting platelets, LMC: platelet aggregation LMC induced, EXP-LMC: platelet aggregation induced by exposed LMC, ADP: control of platelet aggregation by ADP as agonist

tions revealed that the electromagnetic resonance frequencies of LSC exposed to EMF showed significant differences as compared to those of the unexposed LSC (control cells) presenting an almost 70% similarity to the electromagnetic resonance frequencies recorded from the SMC (data not shown).

Discussion

Our findings indicate that high growth inhibition of LSC of more than 95% can be accomplished by applying a radiofrequency EMF according to electromagnetic resonance principles. The results of this study reinforce other authors findings on the proliferation inhibition and on the significant morphological alterations of human melanoma cell lines exposed to low power electromagnetic waves of 50-80 GHz [20] and also on the apoptotic effects of static EMF of 1.95 GHz on human epidermoid cancer cells [21].

In the present study antiproliferative and apoptotic effects have been achieved by exposing LSC to a static EMF of low energy waves and frequencies between 10 to 120 kHz. This field is far lower than the permitted upper limits of exposure (According to Greek Govern Journal 1105, issue 2, Sept 6, 2000, the upper limits for fields of 10 Hz till 10 MHz are: density of the electric field: f/500 mA/m^2, SAR: 0.08 W/kg, SAR of head and neck: 2 W/kg, SAR of Limbs: 4 W/kg and there is not limit for Power density), being thus, safe to use in animals and humans [19]. Effects, as above, of RF on malignant cells (LSC) may be attributed to energy transfer. It has been shown that the average specific absorption rate of RF in living organisms can be substantially higher at resonant frequencies [22]. EMF may also enhance electron spin of free radicals, leading possibly to their neutralization, especially those produced by the activation of arachidonic cascades [18, 21, 23-26]. It is also known that the EMF induce free radicals production that may act as activators of signal transduction pathways [12, 24, 25]. According to the above, it is possible that the effects of these EMF, could be similar to the effects of antioxidants and free radical scavengers on the LSC lines.

The high percentage of the exposed LSC found in apoptosis (45%) in comparison to that of the unexposed to EMF control cells (2%), could be due to the effect of the EMF on cellular membranes activating signal transduction pathways, leading to genes expression of cell apoptosis [7, 9, 21, 27] or to inactivation of anti-apoptotic genes [20]. The low percentage of cells found in synthesis and in mitosis (9% and 2% respectively) compared to that ofthe control cells (38% and 19% respectively), indicates that radiofrequency of the EMF can act as cell cycle inhibitor, similarly to the effect of magnetic field on DNA synthesis [28, 29, 9]. There is also evidence that exposure to the EMF may reduce the immunoreactive p53 expression in tumor bearing mice [24], which has been found increased in the BaP-induced LMS in Wistar rats [14]. The lower sensitivity of LSC after their fourth exposure to ERF, compared to the higher sensitivity after their first exposure, indicates that LSC may have developed some type of resistance to EMF.

Our finding, that the electromagnetic resonance fingerprints of LSC changed after their exposure to EMF and resembled those of the SMC, may indicate that some differentiation in the LSC function has taken place. This differentiation is supported by our -yet unpublished- data indicating that the EMF treated LSC, after being inoculated to Wistar rats, remarkably abolish tumor induction and development, in contrast to inoculated but unexposed to EMF LSC, that develop tumors in a 100% of the experimental animals. There are also references indicating that the EMF, specific or generalized, is able to induce morphological differentiation in both cancer and non cancer cells [30,31]. Nevertheless, the LSC exposed to the EMF seem to retain their potential for metastasis as they can still aggregate platelets.

In conclusion, we consider essential that this low intensity EMF as studied for the first time in this paper, is carefully designed on the basis of the emitted electromagnetic frequencies from the target cells, in order to be effective. Our data, concerning the changes of LSC electromagnetic resonance fingerprints after repeated exposures to EMF, indicate that, in order to affect cells functions, it is crucial to make regular readjustments of the electromagnetic resonance fingerprints, in order to differentiate the LSC phenotype to a phenotype with an electromagmetic resonance fingerprinting as close as possible to that of the normal cells (SMC). In this case, the LSC should loose their malignant phenotype, so that their inoculation to Wistar rats could not cause malignant disease. Experiments, in our lab, seem to confirm this hypothesis.

It is also of interest that the intensity of the electric field we have used was 75 times lower and the intensity of the magnetic field we have used was more than 1800 times lower than the related average intensities of the international safety standards according to the National Centre for Physical Sciences of Greece **("Dimokritos", Athens, Greece)** [19]. Due to the above, the use of the electronic device applied in this study for the treatment of cancer cells, seems to be **safe**. Unpub- lished data of ours, from the follow up of tumor-bearing ani- mals and cancer patients treated with EMF by the above method, confirm the above.

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