EFFECTS OF LOW INTENSITY RADIOFREQUENCY STATIC ELECTRO-MAGNETIC FIELDS (EMFs), ON SARCOMA CELL LINES.

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Abstract

In this study we investigated the effects of low intensity static electromagnetic field (EMF) causing no thermal effects, on sarcoma cells, isolated from tumors of Wistar rats. The tumors were developed via 3, 4-benzopyrene injection in the rats. The cancer cells were exposed to EMF using frequencies between 10 kHz to 120 kHz of the radiowave spectrum for 45 minutes. During a 24-hours period after cancer cell exposure to EMF, no inhibition of cell proliferation appeared. In contrast, at the end of 48 hours incubation time, the cancer cell proliferation was dramatically decreased in ratio > 95%. Also, the survived sarcoma cells after the exposure to EMF (2% of the total cell population exposed to EMF) showed a significant decrease to proliferate under the same culture conditions. These cells were then exposed once again to EMF for 45 minutes (totally 4 sessions of exposure) and tested using a flow cytometer. It was found that a great percentage (45%) of these cells, double exposed to EMF, was apoptotic and only a small percentage of them was found under mitosis (2 %). Additionally, the cells were counted and tested using an aggregometer for their ability to aggregate the platelets (an indicator of their metastatic potential) and they didn't show any difference in comparison to the sarcoma cells not exposed to EMF (control cells).

Key Words: Sarcoma cells, Static electromagnetic fields, radiofrequency waves , resonance, platelet aggregation, metastasis.

Abbreviations : Electromagnetic fields : EMF , Smooth Muscle cells : SMC, Malignant sarcoma cells : MC, SC, Platelets Reach Plasma : PRP , 3,4-benzopyrene : B[a]P.: DMEM , Fetal Bovine serum : FBS , Radio – frequencies : RF, Electron Paramagnetic Resonance : EPR , Electron Spin Resonance : ESR.

Introduction

There is a lot of data dealing with the effects of electromagnetic fields (EMFs) on cells, experimental animals and humans, some of them referred to application of electromagnetic resonance principles(1, 2). Dealing with malignancy , the following main concepts have been expressed, so far, depending on the intensity, frequency and duration of application of the electromagnetic waves : The EMFs may act as co-carcinogens in combination with the initiating carcinogen , especially in experimental animals and the EMFs can exert anticarcinogenic effects, inhibiting the proliferation of malignant cells in vitro as well as decreasing the size of the experimental tumors in vivo (3,4,5,6). The studies on EMFs pro-carcinogenic effects in experimental animals are however, not numerous and it seems that the described methods have a lot of uncertainty (3,4). In comparison, the studies on EMF anticancer effects are abundant and their methodology is well documented (7). It has been also shown, that the cytostatic effects of the EMFs on cancer cells are not related to their thermal effects but are exerted via temperature-independent actions(8,9,10).

In the present study the effects exerted by low intensity radiofrequency static electromagnetic fields, on a sarcoma cell line , were investigated.

Materials & Methods

Production of malignant (sarcoma) [MC] and smooth muscle cells (SMC). In this study, the malignant cells were isolated from selected sarcoma described tumors of Wistar rats. Fifteen (7 males and 8 females) Wistar rats, belonging to the fifth generation of a certain couple, 60 days old, were subcutaneously injected by 1 ml of 3,4-Benzopyrene solution (B[a]P) in Tricapryline at a final dose of 10,08 mgr/ml in their right scapula. . After 110 days (maximum 135 days), all the animals developed malignant tumors at the site of injection. All the tumors were histologicaly identified as leiomyosarcomas. The tumors were surgically removed and cut under aseptic conditions into pieces of 0.5cm size. Each pieces was placed immediately in cold Ringer's solution, then sliced down again to smaller pieces of 1 mm size and placed into 5 ml DMEM solution which contained small quantities of trypsin. The pieces in the solutions were kept at 37° C for 4 hours, with gentle mixing every15 minutes. Then they were centrifuged at 900 rpm for 10 minutes and the supernatant was rejected. The remained cells were resuspended in DMEM+10%FBS solution and seeded in plastic coated dishes of 52 mm size and subcultures of these cells were made, and were submitted to histological examination.

In order to verify if these cells are able to induce the same type of malignancy in rats, 4 million of these cells suspended in Hanks Salt solution were inoculated into every Wistar rat. The animals were anaesthetized with Midazolame and Ketamine, and surgical opening was made on the backside to their outer skin layer. The tissue underneath was traumatized by lancing with a sharp blade in order to bring fresh blood to the surface. Malignant cells were then aseptically infused into the operated area, closure of the open site was immediately performed. The animals developed

medium-sized malignant tumors (approximately 12 cm^3 size) within the first 10 days after inoculation.

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

Equipments used: Radio - frequency measurements and static electromagnetic field exposure of cells were performed by a device called MULTI CHANNEL DYNAMIC EXITER 100 V1 (MCDE) invented by K. Havelas and collaborators. The MCDE has been certified by the International Committee of Atomic Energy (E.K.E.F.E DEMOKRITOS, Athens Greece) for its safe use in humans and animals. This device consists of two basic parts: a) a diagnostic part with an EPR spectrometer's characteristics and b) an electromagnetic field generator of various 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) and radio – frequencies (from 1kHz to 1MHz) conducted by a sophisticated software. To use this software, first it is necessary to record the biological target system's frequencies and then, by using a specific algorithm , to calculate the appropriate electromagnetic frequencies that are needed , for the exposure of living target systems or cells (submitted for patent) .

Estimation of Malignant(MC) and Smooth Muscle Cells (SMC) electromagnetic radio - frequencies. A measurement of the MC and SMC biofrequencies was taken by the device described above, before their exposure to EMF. Electromagnetic radio frequency measurements were also taken from the survived MCs after their exposure to EMF for two consecutive days ..

Method of sarcoma cell exposure to EMF. Twelve Petri dishes with 10 ml growth medium each were seeded with the same number($1x10^5$) of sarcoma cells (time zero). The cells cultures were incubated in 37° C at 95% O_2 + 5% CO_2 for 48 hours and then the medium was changed. At 72 hours from time zero, six cell cultures (EMF cells) were placed into a Faraday apparatus at room temperature (RT) and exposed to electromagnetic radiofrequencies from 10 KHz 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 minutes. The other six cell cultures (control cells) remained at RT for the same time as EMF cells without being exposed to EMF. The control and EMF cells were incubated once again at the same conditions as before for about seven hours. At 79h from zero time, the cells of each culture were counted, subcultured suspended at about $1x10^5$ cells per plate and incubated at the same as above conditions. The same procedure was repeated again as it is described above at 96hours from zero time. At 120hours the EMF cells were re-exposed again to the electromagnetic field as before and 24 hours after this exposure, both EMF and control cells in each plate were counted and examined microscopically. Then, to examine their proliferation rate in relation to time, both groups (EMF and control cells) were subcultured and incubated in order to estimate the time until confluence. The cells were then preserved in liquid nitrogen .

Sarcoma cell cycle determination. The preserved in liquid nitrogen EMF and control cells were defrost and subcultured until confluence. Twelve plates were then seeded with the same number of these sarcoma cells and incubated for 24 hours. The EMF cells were exposed to EMFs as before, after 24 h and 48 hours respectively. Six hours after the last session, samples from each plate were taken for testing in a

Bexton Dickinson flow cytometer. Cell samples were also tested for their ability to aggregate human platelets by an Aggregometer (CRONOLOG ,CA-500).

Estimation of platelet aggregation ability of sarcoma cells. The metastatic potential of sarcoma cells was determined from their ability to aggregate platelets before and after their exposure to electromagnetic fields. A total of 120 tests were performed on blood samples taken from six healthy volunteer donors , free from drugs or alcohol for about ten days before tests. The tests of platelets reactions were performed in platelet rich plasma (PRP) of the donors, prepared according to the manual of the apparatus. The CRONOLOG kit was used to verify the normal functional responses of platelets via the three aggregation pathways. Platelet activation and aggregation tests were performed after suspension in human PRPs of 500,000 of EMF-exposed or control cells .

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

Results

Cell proliferation rate. 24 hours after the first and second session of exposure to the electromagnetic fields, the proliferation rate of the sarcoma cells was slightly decreased, in comparison to those of the control (unexposed) cells ($p<0.05$). The microscopic examination also showed that the highest percentage of sarcoma cells was under stress (round-shaped cells with abortive pseudopodia and formation of nuclear membrane blebs). The multiplication rate of EMF-exposed cells was dramatically decreased at a percentage higher than 95%, ($p< 0,00001$ compared to the control) after 48 hours of incubation 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.1).

Also, the survived after EMF exposure, sarcoma cells showed a great difficulty in proliferating according to time till confluence (6 days incubation) in comparison the control cells (3 days incubation until confluence) (fig.2 and 3).

Malignant cells exposed for four repeated tumor cells sessions to the described above electromagnetic fields showed only a 20 % decrease of number of cells compared to the control sarcoma cells.

Sarcoma cell distribution in the cell cycle phases. Flow cytometry revealed that after the 4th exposure to EMF sessions 33% of cancer cells found to be in G0/G1, 9%in S phase, 2% in mitosis and 45% were undergoing apoptosis while the control cells found to be 36% in G0/G1, 38% in S phase, 19% in mitosis and 2% undergoing apoptosis (table 1).

Estimation of tumor cell "metastatic potential" (platelet aggregation ability). The aggregational ability of the control as well as of the exposed to EMF cells was 78%, and was almost equal to the aggregational ability of ADP (82%). According to the above EMF-exposure did not seem to affect significantly the "metastatic potential" of sarcoma cells(fig.4).

Estimation of cell electromagnetic radio - frequencies. Radio - frequencies of unexposed sarcoma cells were ranging between 10.5 to 120.5 KHz, of EMF-exposed sarcoma cells between 10 to 120 KHz and of the smooth muscle cells between 10 to 120 KHz. Spectrum analysis of the above estimations revealed that the radio-frequencies of sarcoma cells exposed to EMF showed significant differences compared to those of the unexposed sarcoma cells (control cells), presenting an almost 70% similarity to the radio-frequencies recorded from smooth muscle cells (data not shown).

Discussion

In the present study the actions of static electromagnetic fields , of low intensity (μT) at radiofrequencies, on (leiomyo)sarcoma cell lines obtained of B(a)P-treated Wistar rats, were investigated. These rat sarcoma cells have the following characteristics:: Sensitivity to antioxidant substances and free radical scavengers indicating that their proliferation is related to the release of reactive oxygen species (11,12,13),their proliferation can be inhibited via administration of COX-2 and 5-LOX inhibitors(14) and they possess the ability to induce (leiomyo)sarcomas when inoculated into Wistar rats.

The results of our experiments indicate that the application of radiofrequency EMFs according to electromagnetic resonance principles, can cause potent growth inhibition of (leiomyo)sarcoma cells (more than 95%).

Antiproliferative effects and significant morphological alterations on human melanoma cell lines have been achieved when cells exposed to low power millimeter waves in the 50-80 GHz frequency rate of the electromagnetic spectrum.(15).Apoptotic effects on human epidermoid cancer cells have also been induced by static electromagnetic fields of 1.95 GHz frequency(16).

In the present study antiproliferative and apoptotic effects have been achieved by exposing sarcoma cells to a static electromagnetic field of low energy waves and frequencies between 10 KHz to 120 KHz. This field is far lower in frequency and power than the upper limits of permitted exposure, being thus, safe for use in animals and humans (17).

From the literature, it is evident that the effect of EMFs are dependent on immediate interactions that affects the electronic spin of the atoms or molecules with uncoupled electrons in their external orbital, enhancing electron spin coupling and thus may help neutralization of free radicals, especially those produced by the activation of arachidonic acid cascades (14,16,18,19,20,21). It is also known that EMFs induce free radicals production that may act as activators of signal transduction pathways (10,19,20).

According to the above it is possible that the effects of these electromagnetic fields could be similar to the effects of antioxidants and free radical scavengers on sarcoma cell lines.

The high percentage of the exposed tumor cells found in apoptosis (45%) in comparison to that of the unexposed , control cells (2%), could be explained as the result of the EMFs effects on cellular membranes activating signal transduction

pathways leading to apoptic gene activation (5,7,16,22) or inactivation of anti-apoptotic genes(15). The low percentage of the cells found in synthesis and in mitosis (9 and 2% respectively) compared to that of the control cells (38 and 19% respectively) indicates that RF EMFs can act as cell cycle inhibitors , possibly to the effects of magnetic fields on DNA-synthesis (23,24,7). There is also evidence that exposure to electromagnetic fields may reduce immuno reactive p53 expression in tumor bearing mice (19), which has been found increased in BaP-induced sarcomas in Wistar rats (25). The lower sensitivity after the fourth exposure of the sarcoma cells to EMFs compared to their sensitivity after the first exposure indicates that the sarcoma cells may develop some type of resistance .

Our finding that the electromagnetic frequency pattern of the sarcoma cells changed after their exposure to EMF and resembled to that of smooth muscle cells, may possibly indicate that some type of sarcoma cell differentiation could take place.

The above is supported by our, yet unpublished data, indicating that these, EMF – treated, sarcoma cells fail to induce tumor development, when inoculated to Wistar rats in comparison to the unexposed sarcoma cells that induce tumors in 100% of the rats.

Nevertheless, the sarcoma cells exposed to EMF seems to retain their "metastatic potential" as they can still efficiently aggregate the platelets. There is also data that the EMFs, as well as the generalized electromagnetic radiation fields, are able to induce differentiation in cancer cells and other types of undifferentiated cells (26, 27).

It must be emphasized that for the first time a low intensity RFs EMF is used and it is essential this electromagnetic field to be carefully designed on the basis of the emitted electromagnetic frequencies from the target cells, in order to be effective. Also, the intensity of the electric field we used was 75 times lower and the intensity of the magnetic field was more than 1800 times less than the average of the international safety standards according to the International Committee of Atomic Energy (E.K.E.F.E DEMOKRITOS, Athens Greece) (17). Because of that, the use of this device as an electronic instrument in cancer treatment seems safe. Unpublished data of ours, from the follow up of tumor-bearing animals and cancer patients exposed to similar with the present investigation EMFs , are very encouraging.

Future targets

Our data concerning the alterations of radio-frequency pattern of sarcoma cells after repeated exposures to EMFs ,indicate that in order to affect the biological system of these cells, it is crucial to make readjustments of the RF, in order achieve a better electromagnetic resonance of cells , as close as possible as can be to that of the normal cells. If this hypothesis is right, it is expected that the final radio-frequencies of the sarcoma cells would be close to those of the smooth muscle cells. In this case the sarcoma cells should loose their malignant phenotype, so that their inoculation to Wistar rats will not cause malignant disease. Experiments going on , in our lab, seems to confirm this hypothesis.

Acknowledgements: This research is supported by funds of the Center for Energy Frequencies Studies in Physical and Mental Balance (Greece), through Research Committee of University of Ioannina. We thank Dr. Georgios Vartholomatos for his excellent assistance in flow cytometry and Nikos Papadopoulos for his contribution of management and administration.

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FIGURES

Fig.1: Sarcoma cells viability after exposure to EMF : 6 hours after exposure (p<0,01), 24 hours after exposure (p<0,009), 48 hours after exposure (p<0,00001). [SCw/oexEMF: Control (non exposed SC). SCwexEMF: Exposed SC].

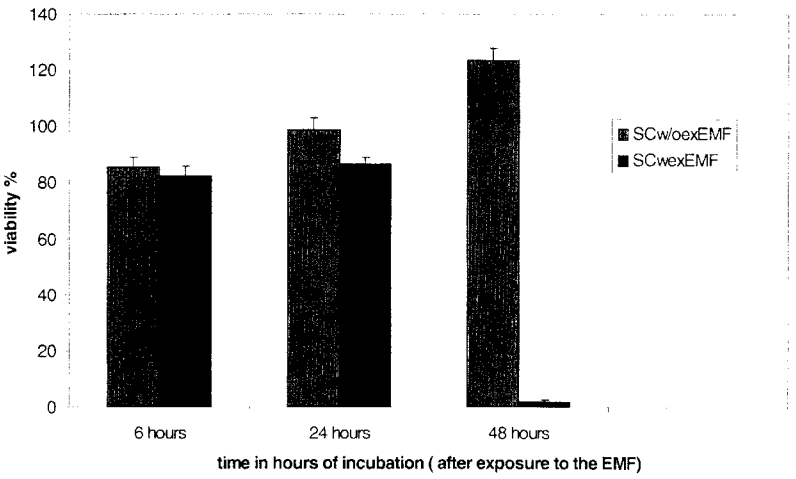


Fig 2. Proliferation rate of sarcoma cells survived after exposure to the EMF(note that the proliferation rate of these cells correspond to 20%, of the proliferation rate, after 72 hours of incubation, of unexposed sarcoma cells[control] , p < 0,001)

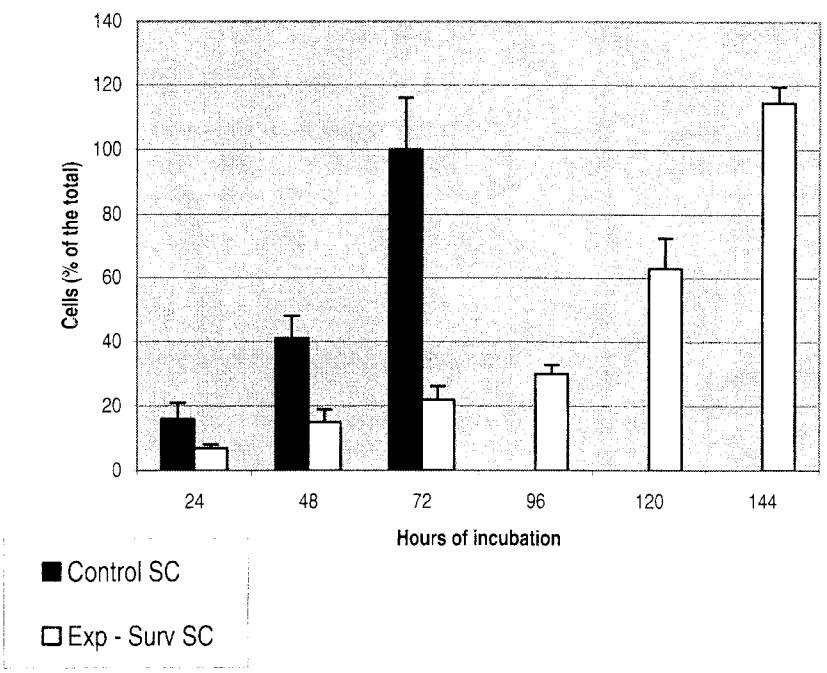


Fig.3 Viability of Smooth Muscle Cells (SMC) non exposed and exposed to the EMF. The non exposed SMC have a rate of growth 10% greater than the exposed ones (p<0,4)

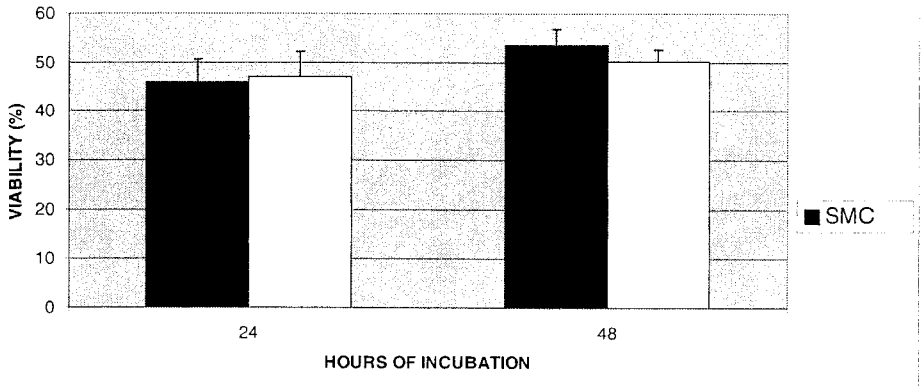
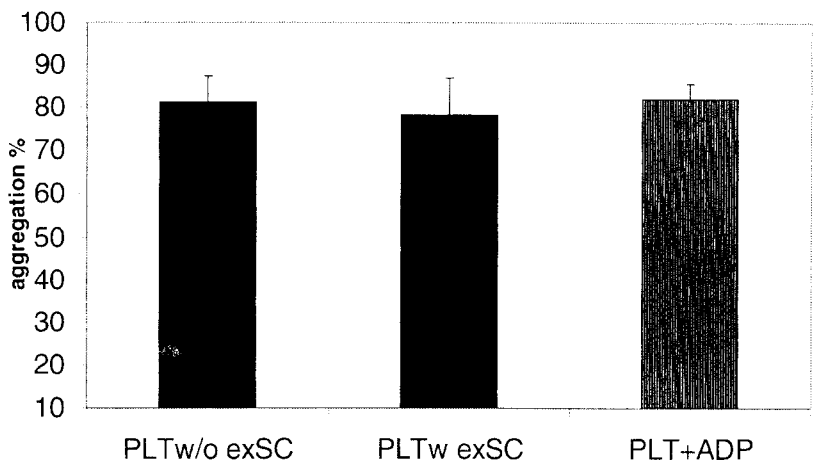


Fig.4: Platelet aggregation induced by exposed SM,non-exposed SM and ADP
(PLTw/oexSC:non-exposed SM,PLTw exSC:exposed SM)



TABLES

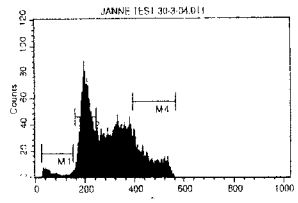
Table 1 : Flow cytometry for non exposed and exposed to EMF sarcoma cells .

The percentage of apoptotic nucleus in the group of control cells is very low (1,79 %) when the percentage of apoptotic nucleus in the group of exposed cells to EMF is much greater (45,34 %) .

Control (non exposed sarcoma cells)

Marker	Left	Right	Events	% Gated	Total
All	0	1023	9461	100,00	49,07
M1	26	149	169	1,79	0,88
M2	15	242	3366	35,58	17,46
M3	262	386	3549	37,51	18,41
M4	391	565	1774	18,75	9,20

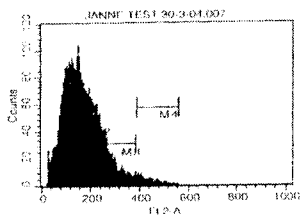
G0/G1 : 35,58%, S : 37,51% , G2/M : 18,75% ,
Apoptosis : 1,79%



Exposed sarcoma cells to EMF

Marker	Left	Right	Events	% Gated	Total
All	0	1023	13232	100,00	17,89
M1	26	149	5999	45,34	8,11
M2	159	242	4396	33,22	5,94
M3	262	386	1232	9,31	1,67
M4	391	565	217	1,64	0,29

G0/G1 : 33,22%, S : 9,31%, G2/M : 1,61% ,
Apoptosis : 45,34%



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M3	262	386	1232	9,31	1,67
M4	391	565	217	1,64	0,29

G0/G1 : 33,22%, S : 9,31%, G2/M : 1,61% ,
Apoptosis : 45,34%