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Subcutaneous Ehrlich Tumor Growth Retardation Using Extremely Low Frequency Magnetic Pulses (ELF MPs)

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Abstract: The objective of this study to investigate the antitumor effect of extremely low frequency magnetic pulses (ELF MPs, 0.7Hz, 0.88±0.02 mT) against subcutaneous implanted Ehrlich tumor in mice. Thirty Swiss Albino BALB/C female mice were equally divided into three groups: normal mice group (GP I), untreated tumor positive control mice (GP II) and ELF MPs-tumor local exposure mice group (GP III) (1h/day, for 3 alternative days). Body weight changes and blood hematological findings of three experimental groups have been assessed. Tumor volume following up and structure changes of tumor tissues form the two tumor bearing mice groups (control and exposed) were scanned under light microscope (LM). ELF MPs caused a reduction in increase percentage of mice body weight, as compared with the untreated control mice. The results also indicated that tumor local exposure to ELF MPs retarded its growth activity. Moreover, ELF MPs can modulate some blood hematological parameters of exposed tumor bearing mice (lymphocytes percentage, hemoglobin concentration and platelet count), with respect to the control mice. Additionally, the exposed tumor tissues LM examinations resulted in less dense tumor cells. It can be concluded that ELF MPS are a promising modality for treatment of tumors and can offer a good improvement in hematological profile in exposed tumor bearing mice to be nearly retuned as normal mice.

Key words: Extremely low frequency magnetic pulses · Ehrlich tumor · Tumor retardation · Blood hematology · Immune response · Histopathological studies

INTRODUCTION

The major burden of disease worldwide is cancer; with 8.2 million approximate deaths in 2012 in accordance with WHO [1]. Cancer is distinguished by un-useful, uncontrolled and invasive multiple cell division. The cancerous cells regenerate again and again inconsistently and may spread to other parts of the body which is known as metastasis [2].The potential use of physical means as an aid and/or alternative fighting modality against cancer has been investigated in many researchers. Recently, intensive efforts have focused on the characteristic features of extremely low frequency electromagnetic fields (ELF EMFs), not only to estimate the basic mechanisms of its interaction to living systems; but also to investigate its potential of practical applications. Furthermore, many scientific studies indicated that external electric and/or magnetic fields at extremely low frequency (<300 Hz) can influence the biological systems, change the cellular proliferation [3], stimulate the ATP production [4] and change the flow of ions throughout the membranes [5]. In this context, Pasquinelli *et al*. [6] stated that pulsing electromagnetic field (PEMF) effects lead to a modification of the multidrug resistance (MDR) of cells in *vitro* and in *vivo*. They also showed an increase in the life span of the mice bearing-murine leukemic doxorubicin-resistant 75 Hz and 20 Gauss PEMF. Zhang *et al*. [7] found that the growth of sarcomas implanted in mice, might be controlled by the application of extremely low frequency (ELF) pulsed-gradient magnetic field with a maximum intensity of 0.6–2.0 T, a gradient of 10–100 T per meter, a pulse width of 20–200 ms and a frequency of 0.16–1.34 Hz. They proposed that such magnetic fields induce apoptosis of cancer cells and arrest neoangiogenesis, preventing a supply developing to the tumor. Another study by Nie *et al*. [8] observed that 2 h/day exposure to low frequency magnetic field (LW-MF) with magnetic flux density of 0.4 T and frequency of 7.5 Hz for 43 days inhibited growth and metastasis of melanoma cancer cells in mice model. Authors suggested that this inhibitory

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effect of the demonstrated magnetic field was dependent up on modulation of LF-MF on immune function, as well as induction of cell cycle arrest and on decomposition of chromatins. In more recent work, Destefanis *et al*. [9] findings indicated that exposure to extremely low frequency electromagnetic fields (ELF-EMF) of frequency 60Hz might affect mitochondrial metabolism and the consequent impact on cancer cell growth in some cell lines, possibly in association with a cell cycle delay. The authors found that a reduction in the cell proliferation, cell numbers and cell viability of several human cancer cell lines, especially MCF10 A and MCF7 cells after long-term exposure to ELF-EMF with magnetic flux density of 1 mT and this effect was related to an increase in mitochondrial activity. In this study, the anticancer efficacy of extremely low frequency magnetic pulses (frequency 0.7Hz, 0.88±0.02 mT) was studied using subcutaneous Ehrlich ascites carcinoma mice model. It has been informed that Ehrlich ascites tumor (EAT) is a rapidly growing experimental tumor model which show greater initial growth and total cell count in female than male mice [10]. Our selection to magnetic pulses is supported by its effective ability in producing micro-currents in the exposed-body's tissues. These micro-currents trigger specific biological responses depending on field frequency.

MATERIALS AND METHODS

Experimental Animals: This experiment was carried out on 30 Swiss Albino BALB/C female mice, their ages ranged between (5-6) weeks and average body weight (27-30g). The animals were housed under standard laboratory conditions with (23-26°C) temperature for one week for adaptation. They were fed with free access of complete mouse diet and tap water *ad libitum*. All experimental procedures were performed in accordance with all international ethics for treatment of animals and with the guide for care and use of laboratory animals published by the US National Institute of Health [11].

Implantation of the Mice with Ehrlich Tumor Cells: For tumor implantation, ascites carcinoma cell suspension of Ehrlich tumor was pipetted into a sterile insulin syringe fitted with a 29½ -gauge needle from the peritoneal cavity of a donor mouse, prepared in the National Cancer Institute (NCI), Cairo University, Egypt. The transplanted Ehrlich tumor cells of concentration ~10^6 cells/ml. suspended in 0.2 ml of sterile phosphate buffered saline (PBS, pH=7.4) were subcutaneously injected into the right lateral thigh of each mouse using the needle. The number of viable tumor cells was 99% as judged by trypan blue exclusion assay.

Experimental Design "Classification of Experimental Animals Groups": The experimental mice (30) were divided equally into 3 groups: GP I Non-tumor-bearing mice (n= 10). Tumor-bearing mice (n= 20): The day of tumor injection was specified as day zero; in approximately 11 days following tumor-implantation, the solid tumor volume in the thigh of the mice was palpable about 0.13 cm^3. On the same day, the Ehrlich tumor injected mice of similar size were then equally divided into 2 groups (n=10 mice/group):

Group II: (the positive control tumor-bearing group): mice of this group were left without any treatment during the course of the experiment
Group III: At day 13 after tumor implantation, mice of this group were treated by local exposure of the implanted Ehrlich tumor to extremely low frequency magnetic pulses (ELF MPs); with (0.88 ± 0.02 mT and frequency of 0.7 Hz). Each mouse exposed 3 times with alternative 3 days (1h/day).

Each group was housed in a separate plastic cage. Cages were with perforated sides and the floor covered with dry wooden chips, the beddings were changed regularly to avoid infection. Animal groups were identified by home cage labeling and marking lines around the tail of each mouse with indelible ink pens.

The Extremely Low Frequency Magnetic Pulses (ELF MPs) Exposure Facility Set-Up: The exposure device was fabricated and manufactured locally in the Biophysics Department research lab, Faculty of Science, Cairo University, Egypt. In this experiment, a power supply of d.c. voltage (15 V) through an electronic switching device is constructed to generate a square pulsed current with different extremely low frequencies in the range from 0.1 to 20 Hz with intervals of 0.1 Hz. The square pulses then allowed to flow in the main set of a magnetic gun (i.e. the magnetic gun was connected with the circuit providing the d.c. current). The magnetic gun was used for tumor local exposure constructed by wounding 1 mm thickness copper wire around the outer surface of a ferromagnetic bar of 1 cm in diameter. During exposure period, each animal was set on a wood board using sticky rubber tapes, which were soft to avoid any pressure/stress. After being fixed, the table with the animal was adjusted 2 cm air

gap between the implanted tumor mass and the tip of the magnetic gun. At this distance, the magnetic flux density was 0.88 ± 0.02 mT which was measured by means of a Gauss/Tesla meter (model 4048, manufactured by F.W. Bell, with probe T-4048.001-USA) of accuracy ± 2 %. The magnetic pulses shape was also displayed using the linear Hall-effect IC sensor on the oscilloscope. The animals were not anesthetized or sedated during exposure so that there was no additional parameter that may interfere with the magnetic field pulses effects. After exposure each mouse was returned to its group in cages subjected to a normal day, receiving the same feeding diet and the environmental conditions as control (lighting, ventilation, temperature and periodical cleaning) throughout the experimental period.

Tumor Volume and Mice Body Weight Estimation: Tumor growth for the two tumor bearing mice groups II and III was monitored over a 30-day experimental period from day 11 to day 30 following tumor implantation. Recording the three mutually orthogonal dimensions of the tumor regularly with a digital caliper (Tricle Brand, Shanghai, China) (accuracy ± 0.01 mm). The tumor volume V was then calculated using Ning *et al*. [12] equation:

$$V(cm^3) = \frac{\pi}{6} * length * width * hieght \quad (1)$$

The body weight of each mouse was checked regularly throughout the period of the experiment using an electronic balance, precision of 0.005 g. At day 18 after tumor implantation, prior to sacrifice mice, the examinations of blood samples from four randomly selected mice from all experimental groups were performed, especially in order to check whether treatment of tumors with ELF MPs may cause any side effects. After that, the four mice from each group were sacrificed by cervical dislocation on the same day and then tumor masses from each mouse were processed for histopathological examinations.

Blood Collection and Hematologic Analysis: Four mice from each experimental group were used for hematological analysis. Blood samples were taken from the venous sinus of the left eye of each mouse and each mouse blood sample of about 200µl was collected separately on anti-coagulated Microtainer Brand vials with ethylene diaminetetra acetic acid (EDTA). Hematological parameters including lymphocyte percentages, haemoglobin (Hb) concentrations and platelet counts were immediately determined using a Sysmex XT-2000i automated blood cell analyzer in a hematology laboratory.

Autopsy and Tumor Tissue Sample Collection for Histopathological Examinations: Four mice from each tumor bearing mice groups were sacrificed by cervical dislocation and histological studies for tumor tissues were performed. The tumor masses were carefully dissected out after sacrificing the mice by cervical dislocation and cut into small pieces to allow good fixation in 10% neutral formalin. They were then embedded in paraffin blocks and sectioned at 4-5 mm thick according to Bancroft and Gamble [13]. All tissue sections were collected on glass slides and Hematoxylin-Eosin stained (H&E). The histological slides were viewed and scanned using light microscope (C x 31 Olympus optic microscope) connected with a digital camera (Canon).

Statistical Analysis: All the data are mean values ± standard errors of the means (SEM). The statistical analysis were done by one-way analysis of variance (ANOVA) with $P<0.05$ as a significant difference between groups, using SPSS software

RESULTS

Tumor Volume Results: The results of the mean tumor volume in cm^3 with the corresponding standrad error of the mean (SEM) as afunction of time (days) follwing tumor implantation over a 32-day period for the 2 tumor bearing mice groups demonstrated was represented in Fig. 1. GpII showed a marked progressive increase in tumor volume continously over time. On the other hand, GP III showed a delay in the mean tumor growth rate compared with the control tumors, although the tumors of this group continued to grow in size. In fact, we have showed that tumors of this group actually enlarge at a slower rate. At day 18 after tumor implantation, the mean tumor volume of GP III was (0.556±0.05 cm^3), which was not statistically significant with respect to control tumors of mean volume (0.618±0.03 cm^3). On day 32-following tumor implantation (i.e. final experiment day), a significant difference (P= 0.038) in mean tumor volume was noticed in GP III, 0.92±0.04 cm^3, compared with the untreated tumor positive control group (GP II, 1.145±0.06 cm^3).

Body Weight Changes: Fig. 2 demonstrates the percentage change (%) in average body weight on day 32 (final body weight) from day 11 (initial body weight) after tumor implantation, for the three experimental mice groups and the results were compared with that of the GP I normal mice. The corresponding initial body weights of all mice groups before any treatment had no significant difference. The mice of GPII exhibited the marked change

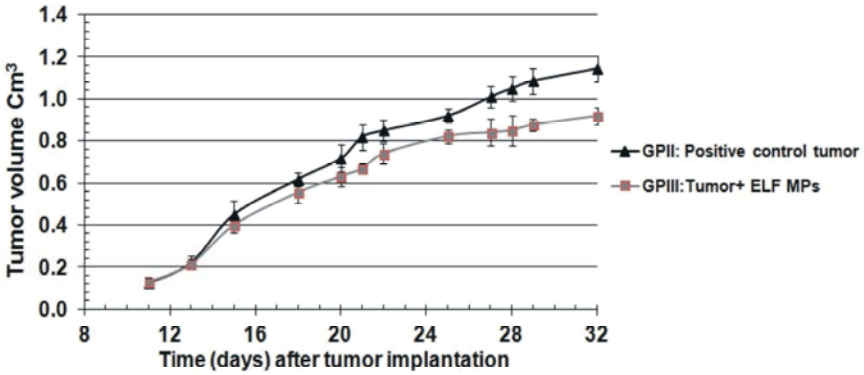

Fig. 1: Time course of tumor volume (cm^3) curves for untreated and treated tumors of GPII and GPIII, respectively. Data are mean ± (SEM) for the surviving animals in each group

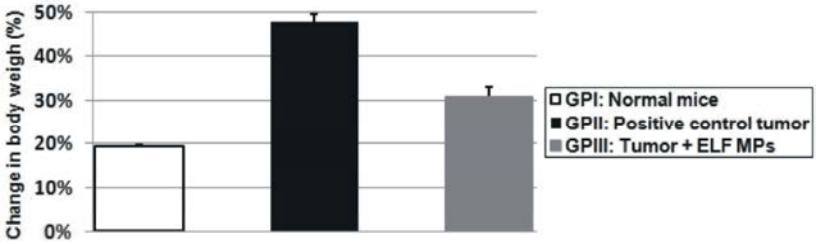

Fig. 2: Bar graphs comparing the percentage change in average body weight on day 32 relative to body weight at (day 11), for the demonstrated mice groups

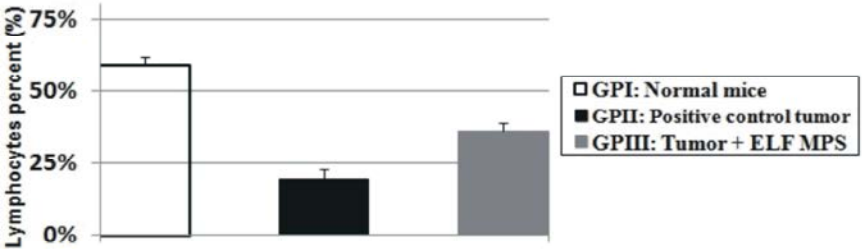

Fig. 3A: Lymphocytes percentage (%) corresponding to each experimental mouse group. Data are mean (4 mice/group) ± SEM

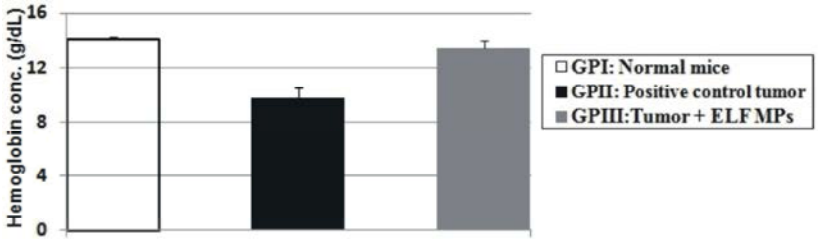

Fig. 3B: Hemoglobin count (g/dl) corresponding to each experimental mouse group. Data are mean (4 mice/group) ± SEM.

in the average body weight, where this group gained (47.8%) increase in the average body weight as shown in Fig. 2. The normal mice of GP I have had the minimum change in body weight with 19.45% body weight gain. On the other hand, mice of GP III exhibited a moderate body weight change with an increase of 30.28% in the average body weight

Hematological Findings

Lymphocytes Percentages: Fig. 3A shows the results of lymphocyte percentage (%) of the three experimental groups, at day 18 after tumor implantation. The average lymphocyte percentage of mice from GP I was observed to be 59.25% ± 0.025. On the other hand, mice from GPII exhibited a very high significant decrease ($P<0.0001$) in

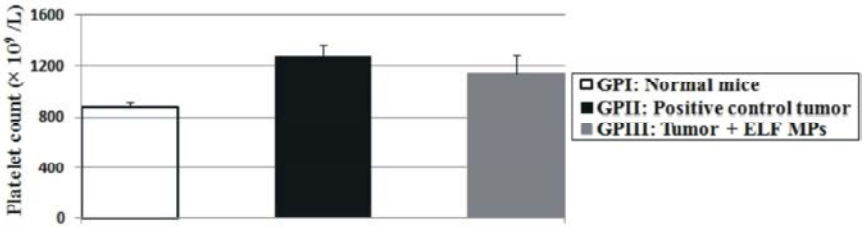

Fig. 3C: Platelet count (×10^9/L) corresponding to each experimental mouse group. Data are mean (4 mice/group) ± SEM

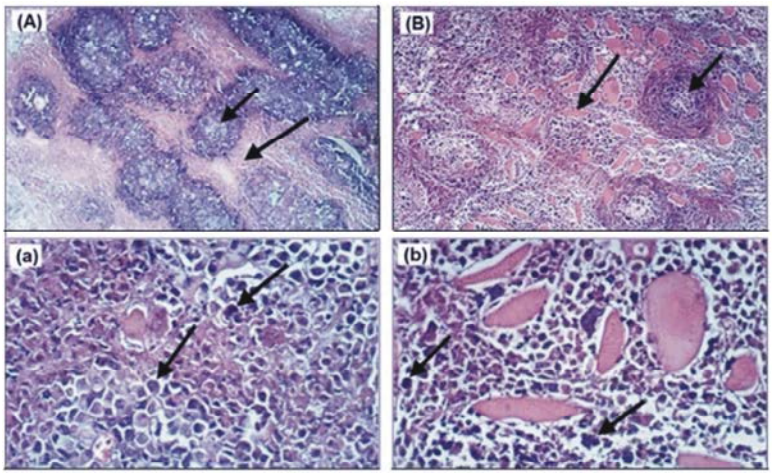

Fig. 4: Representative histological photomicrographs of H&E-stained sections from Ehrlich solid tumor bearing mice obtained of A (100X): untreated GPII showing massive infiltration of neoplastic cells destructing the surrounding myocytes that showing necrosis note the EAC cells showing pleomorphism, anisocytosis and large vesicular nuclei, a (400X): Showing diffuse infiltration of densely compact sheets of neoplastic cells (short arrow) with destructive invasion to the surrounding muscle bundles that showing massive necrosis and myocytolysis, B (100X): ELF Mps-exposed GPIII showing diffuse but not compact non cohesive infiltration of the tumor cells (short arrow) that showing necrosis with less destruction and necrosis of the surrounding myocytes (long arrow), b (400X): showing karyopyknosis and karyolysis of the Ehrlich tumor cells showing less with apparently histological normal of the surrounding myocytes.

the average lymphocyte percentage which was 19% ± 0.038, as compared with that of GP I mice. Interestingly, the average lymphocyte percentage of the mice from GPIII was 36% ± 0.03, which significantly increased (P= 0.008) than that of GP II. It was also detected that the lymphocytes percentage of this group was still smaller than that of mice from GP I, with significant difference (P<0.001).

Hemoglobin (Hb) Concentration: Fig. 3B displayed the concentration of hemoglobin (Hb, g/dl) for mice from all experimental groups. It was noticeable that the mean hemoglobin concentration of mice of GP II (13.46 ± 0.56) significantly (P= 0.001) higher than that of GPII mice (9.8 ± 0.75) and non-significantly (P= 0.439) lower than that of GP I normal mice (14.1 ± 0.24). Moreover, there was a significant difference in the mean Hb concentration value between GP I normal mice and GP II.

Platelet Count: Fig. 3C showing the platelet count (× 10^9/L) for all experimental mice groups. The GP II mice was observed to have an average platelet count of (1271 ± 88) which was significantly (P=0.045) higher than that of GP I normal mice (878.6 ± 36.8). By contrast, GP III exposed tumor bearing mice revealed a mean platelet count of (1142 ± 139.5) which was reduced than that of GP II mice but with no significant difference but also still higher than that of GP I normal mice without significant difference (P= 0.141).

Histophathological Findings: The histopathological examination of untreated Ehrlich tumor tissues from positive control mice of GP II (Fig. 4A & a) revealed diffuse infiltration of densely compact Ehrlich tumor neoplastic cells in between muscle bundles showing extensive destruction and necrosis by the invasive and infiltrative tumor cells. The neoaplastic cells were large

cells with abundant eosinophilic cytoplasm and large hyperchromatic and vesicular active nuclei and generally showing pleomorphism and anisocytosis with karyomegally and frequent mitosis with scarce karyolysis of tumor cells and leucocytes infiltration. On the other hand, The ELF PMF-exposed tumor tissues from mice of GPIII (Fig. 4B &b) showed diffuse, but none compact nor cohesive infiltration of tumor cells with less destruction of the surrounding myocytes.

DISCUSSION

In this study, it was reported the *in vivo* antitumor effect of local exposure of tumor to ELF MPs (0.88 ± 0.02 mT, frequency of 0.7 Hz, 1 h for 3 alternative days). The results registered above displayed that the implanted Ehrlich tumor of GP III exposed mice showed retardation of tumor growth rate, as compared with untreated tumors of GP I positive control mice (Fig. 3). Regarding the results of the percentage change in mice body weight (Fig. 1), it was evidenced that ELF MPs-exposed tumor bearing mice GP III exhibited moderate increase in body weight while normal mice GP I revealed the smallest increase in their body weight. It was notable in this regard that the treatment with ELF MPs caused a decrease in change percentage of mice body weight, as compared with mice of GP II. The reason for this few change percent in the body weight of mice GP III after exposure to ELF MPs, may be not due to the impairment of exposed mice health, rather it may be an indication for the tumor growth retardation in the ELF MPs-exposed tumor bearing mice GP III. In the context of the hematological parameters, Babu *et al*. [14] noticed that tumor bearing mice exhibited alterations in hematological parameters such as a decrease in lymphocytes percentages with respect to normal mice, which is in consistent with the present results. The authors explained these changes as hemolytic of myelopathy conditions induced by malignancy. Nevertheless, the exposure of GP III mice to ELF MPs was found to significantly ($P= 0.0008$) increase the lymphocyte percentage, as compared with GP II (Fig. 3A). Despite this parentage was still smaller than that of normal mice GP I with a significant difference ($P<0.001$), suggesting that ELF MPs exposure tried to increase the immune response of the body.

Concerning the blood Hb concentration (g/dl), it was previously reported that tumor bearing mice exhibited a reduction in red blood cells (RBCs) or hemoglobin production which is indication for anemia [15]. The author interpreted this Hb reduction either due to the iron deficiency or hemolytic or myelopathic conditions. Here, the exposure to ELF MPs could be also beneficial for restoring the blood Hb concentration of exposed tumor bearing mice GPIII to the normal Hb levels of normal mice GP I (Fig. 3B). These results are incompatible with that reported by Alghamdi and El-Ghazaly [16] where they observed that the average number of Hb decreased significantly after exposure of rats to mobile phones compared with the unexposed group. This disagreement with the present results may be due to the variation in the exposure conditions where mobile phones are in the radiofrequency range and to genotype of exposed organisms. Another observation could be in favor of the helpful effects of ELF MPs exposure as antitumor facility. The present results about the blood platelet count indicated that mice of GPII exhibited a significant increase in the platelet count as compared with that of GPI. These results are in line with those reported by Wang *et al*. [17], who found that platelet parameters were all significantly increased following the implantation of 4T1 breast cancer cells into BALB/c mice compared with the normal group. The elevation of platelets counts support cancer progression and its spreading by protecting tumor cells from the act of the immune elimination [18, 19]. Indeed, the effect of ELF MPs on the platelet count was almost indistinct where exposed tumor bearing mice of GP III was found to still have their blood platelet count higher than that in GP I normal mice without significant difference ($P= 0.141$). This platelet count result in the GP III mice was seemed to be enhanced to be reduced than those in GP II mice but with no significant difference (Fig. 3C). The reduction of the platelet count in ELF MPs exposed tumor bearing mice GPIII may be associated with suppression growth of primary implanted Ehrlich tumor and also metastasis suppression. Collectively, all these mentioned hematological results and their changes following exposure to ELF MPs indicate that these ELF MPs can be involved in possible induction of useful bio-effects by improving almost all of the mentioned hematological characteristics of exposed tumor bearing mice GP III toward normal in comparison with the untreated bearing mice GP II.

All the pervious results and the trend of the tumor volumes under the employed treatment as compared with untreated control tumor of GP II can be also confirmed by the histological studies of tumor tissues, 18 days following tumor implantation (Fig. 4). It was noticed that in comparison with untreated tumor cells of GP II, ELF MPs exposed tumors of GPIII exhibited necrosis of tumor cells and relatively disappearance of densely compact neoplastic cells with less destruction of the surrounding myocytes (Fig. 4B). Furthermore, the high

power section (Fig. 4b) revealed pagination in the nucleus (i.e. karyolysis) of the Ehrlich tumor cells with apparently histological normal of the surrounding myocytes. These observations somewhat similar to that reported by Zhang *et al*. [7], who found exposure to ELF pulsed-gradient magnetic field with (0.6–2.0 T, 10–100 T/m, a pulse width: 20–200 ms, 0.16–1.34 Hz) lowered the malignancy degree of S- 180 sarcoma cells implanted in mice and suppressed their rapid and heteromorphic growth as detected under electron microscope. They found in sarcoma exposed cells besides apoptotic morphological features; the host cellular immune response was also enhanced. The more probable explanation for these changes in tumor volume and its histology and in hematological parameters; all of exposed tumor bearing mice is that as previously stated in the metabolic bio-magnetic resonance model (MBMR) suggested by Fadel [20], where the interference of an external electrical or magnetic impulse will affect the ion motion in the biological system, which may inhibit or enhance physiological processes depending on the mode of resonance interaction.

CONCLUSION

It can be concluded that extremely low frequency magnetic pulses (ELF MPs, 0.7Hz, 0.88 ± 0.02 mT) have antitumor effect as detected from tumor volume and histological findings. Also, exposure to ELF MPs of mentioned parameters cause good improvement in hematological profile in exposed tumor bearing mice to be nearly retuned as normal mice.

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