



CONTROL OF EHRlich TUMOR METASTASIS IN MICE LIVER BY ELECTROMAGNETIC WAVES, CERATES CERATES SNAKE VENOM AND METHOTREXATE

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Abstract

The use of chemotherapy for cancer treatment is a successful modality. However, this treatment has its side effects, which limits its applicability. The aim of the present work is a comparative study of side effects of treatment of *Cerastes cerastes* snake venom and methotrexate on tumor progression in the primary site (thigh of the animal) and in secondary sites (metastasis) formation in the liver of mice. This will be achieved through histological, biochemical and tumor volume investigations on two main groups, (healthy animals and tumor bearing animals) to monitor side effects of the used methodologies on normal tissue and cancerous tissue. A total of 122 mice were used, divided into two groups, "A" and "B". Group A: normal control mice, was given saline 0.2 ml. Group B: tumor bearing mice, were subdivided into 3 subgroups: B1: received no treatment; B2: was interperitoneally injected with snake venom (0.75 µg/g); B3: was given 2.8 mg/kg methotrexate as standard chemotherapy. Histological examination of liver using hematoxyline and eosin (H& E), as well as blood analysis were performed. Results showed that exposure to ELF- MWs at resonance frequency 4.5 Hz, 2G was quite effective in limiting tumor growth and proliferation, with minimal effect on non- involved tissues. Its use proves to be much safe for treatment of primary tumor site and to secondary sites.

Keywords: Ehrlich Tumor Metastasis, Cerates Cerates Snake

Introduction

Scientific research has been concentrated mainly on cancer and its treatment,

most treatment protocols start with surgery followed by chemotherapy and/or, radiotherapy. Recent research is now targeting tumor cells with

alternative ways using natural products or changing the radiation protocol by using electromagnetic waves at a certain resonance frequency, aiming to decrease the hazards of chemotherapy and radiotherapy on healthy tissues.

The electromagnetic wave is a transverse wave. It has two components; an electric field (E), which oscillates sinusoidal in time, and space, and a magnetic field (B), which oscillates in a perpendicular plane with the same frequency. The electric field is always perpendicular to the magnetic field (Jackson, 1962).

4.5 Hz QAMW waves are very promising for the treatment of tumors either in primary site state and/or as a preventive agent for metastasis formation in the secondary site (liver, kidney and spleen) (Fadel *et al.*, 2010).

It is verified that Ehrlich tumors implanted in female mice exposed to 4.5 Hz square amplitude modulated waves (QAMW) at a rate of 1h daily for a period of 3 weeks showed inhibited tumor growth and increased animal survival (Fadel *et al.*, 2003).

Materials and Methods Ehrlich ascites tumor

The tumor was prepared in the National Cancer Institute (NCI), Cairo University, Egypt, maintained in Swiss albino mice in the ascitic form. Tumor cells were collected by aspiration with Pasteur pipette. A suspension of 1×10^6 cells /ml was prepared. The animals were injected intramuscularly into the left flank with 0.2 ml of this suspension.

Animals

Female balb/c mice of average weight 18-20g each and of about 5 weeks age were utilized. Animals were housed under normal environmental conditions of temperature (25-30°C) and humidity 60%. Food and water were provided *ad Libitum*. The animals were divided into two main subsets of experiments namely A and B, 6-8 animals were taken from each group as sample for biochemical and histological examinations.

Subset A: were used as control and divided into 2 equal subgroups as follows: A₁ was intraperitoneally injected with 0.2 ml saline, A₂ was whole body exposed to 4.5 Hz square wave magnetic field of intensity 2 Gauss for a period of 7 days (d) at a rate of 2h/d. Subset B: mice were IM injected in the thigh with 0.2 ml Ehrlich tumor suspension and divided into 3 equal subgroups as follows B₁ received no treatment till day 23 (PI), B₂ was whole body exposed to 4.5 Hz square wave magnetic field at day 15 PI for a period of 7 days at a rate of 2h/d and B₃ was interperitoneally injected with methotrexate starting at day 15 PI every other day till day 23 PI in a dose of 2.8 mg/kg.

Magnetic field exposure system

A power square wave generator was manufactured at the Electronics Workshop in Faculty of Science, Cairo University, Egypt. This generator can produce square wave currents from 0-5 Ampere (A) in the frequency range 0-10 Kilo Hertz (KHz) (Fadel, 2003). The

generator is controlled by computer software with interface.

Chemicals

Glucose, cholesterol and albumin Kits were obtained from LINEAR CHEMICALS, S.L, Spain. Methotrexate used as parental form and is manufactured and purchased by EBEWE pharma.

Liver Histology

Liver was dissected and kept in formaldehyde 10% for histological examination. From each animal the liver was trimmed then fixed in formalin 10%, washed in water then in ascending grades of alcohol (for dehydration). Tissues were cleared in xylol, embedded in soft then hard paraffin to form "blocks". Tissue sections were cut from the blocks at 5mm thickness using microtome. Sections were mounted on glass slides, stained using standard "Hematoxyline and Eosin" staining procedure (Culling, 1974). Sections were then examined using Olympus (500) microscope attached to image analysis computerized system, magnification 200, 400 and 1000.

Biochemical measurements

Animals were fasted for 12 h before sampling, anesthetized by ether, and then blood samples were withdrawn from the heart. Blood samples were centrifuged at 3000 rpm for 5 min. and serum was separated in clean sterile Eppendorf tubes. Serum glucose, cholesterol and albumin levels were measured according to Young, (2000).

Biochemical analysis concentration was automatically calculated by slim spectrophotometer (SEAC Company) at the wavelength recommended in the kit.

Cell counter *Danam* manufactured in France and provided by LAB Top Company was used for determination of total and differential WBC count. It was represented by lymphocyte %, monocyte% and granulocyte%. Differential, total WBC count, and glucose level were measured at the same day of sampling.

Determination of tumor size

The tumor size volume (V) of the thigh (Ning *et al.*, 1994) was calculated from the measured values of the tumor dimensions (a, b and c) everyday starting day 10 PI till the end of experiment. The volume of tumor was calculated according to the following relation:

$$V = abc^2/6$$

Where

V= volume of tumor

a=length

b = width

c=height of the tumor.

$p=22/7$

The measurements were done by vernier caliber within ± 0.05 mm.

Statistical analysis:

All data were expressed as the mean \pm standard error of mean. Data were subjected to one way ANOVA followed by Tukey Kramer multiple comparison test using Graphpad InStat; version 2004, Purdue University, Indiana, USA.

$P < 0.05$ was used as criterion for significance.

Results

Histological study of liver of Non-tumor bearing animals

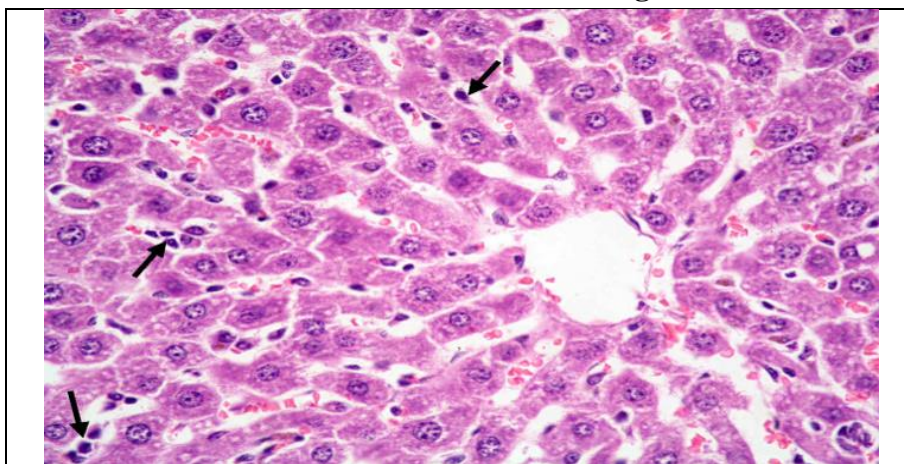


Figure (1a) A photographic cross section for a normal liver shows clear arrangement of the classic hepatic lobules, with cords of hepatocytes radiating around the central veins, enclosing sinusoids in between the hepatic cords are observed. Hepatocytes are seen polygonal, acidophilic with central vesicular nuclei. Kupffer cells are shown among the sinusoidal lining (magnification $\times 400$).

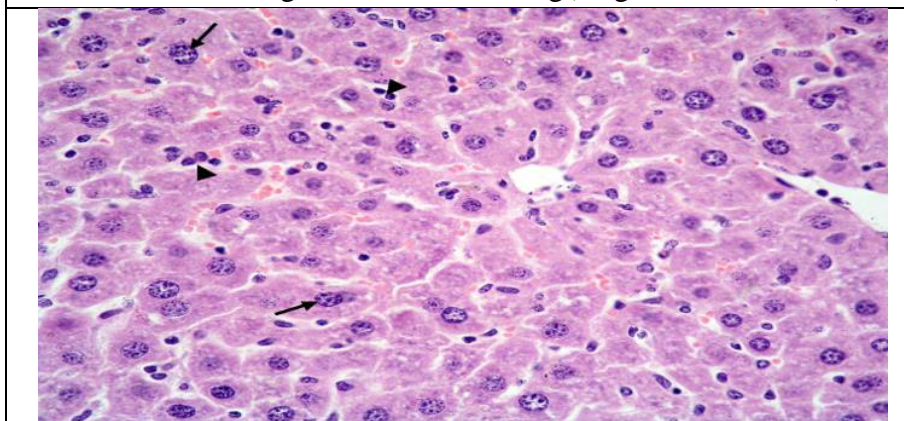


Figure (1b) liver section of group A₁ After exposure of whole body of normal mice for magnetic waves (4.5 Hz) hepatocytes appeared normal with marked increase of Kupffer cells lining the sinusoids. Nuclei of numerous hepatocytes exhibited many nucleoli (magnification $\times 400$).

Histological study of liver of tumor bearing animals

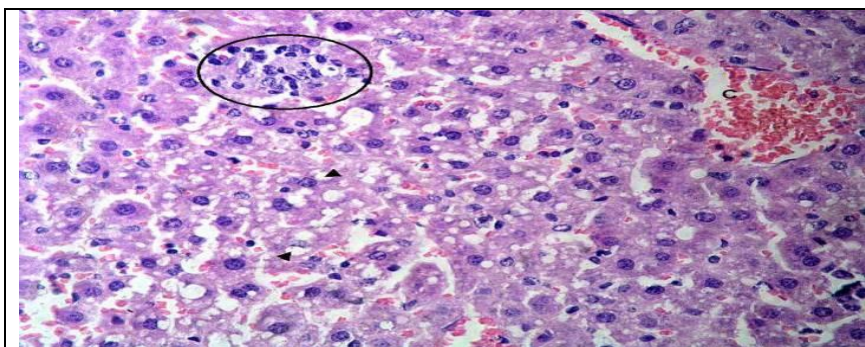


Figure (2a) Liver section of control tumor bearing mice .It is noticed that scattered aggregates of malignant or atypical cells are present in several areas within the liver distributing the normal architecture. Kupffer cells were numerous. Malignant cells showed marked disturbance of nuclear / cytoplasm ratio and nuclear polymorphism. Some degenerated hepatocytes exhibited fragmentation of their nuclei as well as margination of their chromatin .

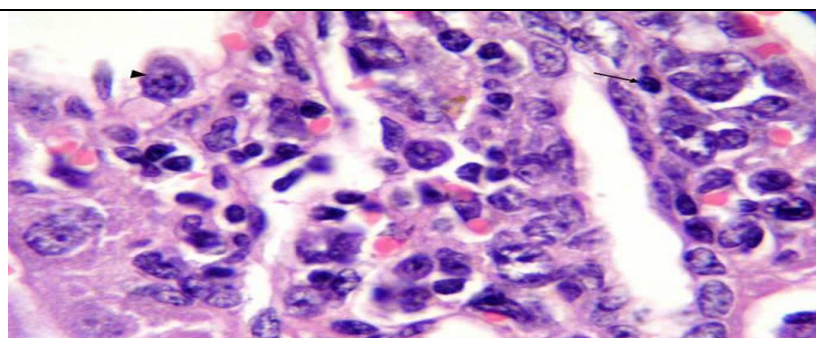


Figure (2b) Tumor-bearing mice exposed to magnetic wave (4.5Hz) showed mild affection of hepatocytes but with marked increase in Kupffer cells in many areas. Malignant cellular aggregates showed marked apoptotic changes with some giant hyper chromatic nuclei. Numerous nucleoli were seen in many hepatocytes with very numerous Kupffer cells

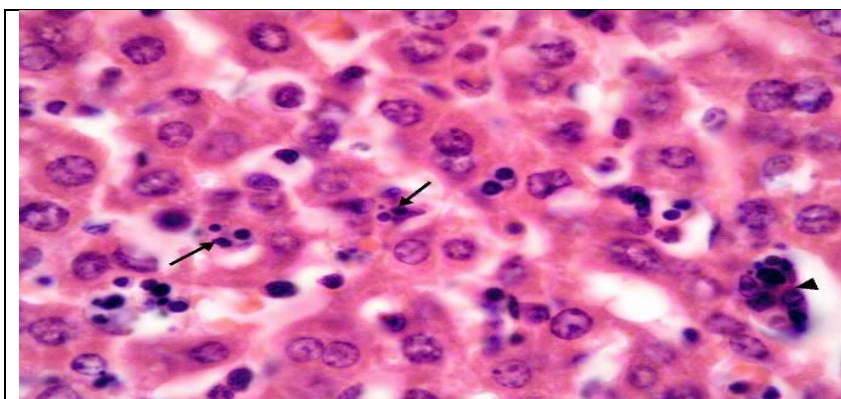


Figure (2c) Liver section of tumor bearing mice *received* methotrexate (2.8 mg/Kg) shows loss of hepatic architecture with numerous apoptotic cells. The central zone of the lobule showed marked vacuolations as well as the peripheral zone with marked lymphatic congestion . Apoptosis of the tumor cells was marked and many giant cells were seen

Biochemical findings

Table (1) Effect of magnetic waves (4.5 Hz) on total leukocytes , lymphocyte % , monocyte% and granulocytes % in normal mice

Group	Total leukocyte count (no/mm ³)	Lymphocytes (%)	Monocyte (%)	Granulocytes (%)
Control (0.2 ml saline)	12433±1708	87.7± 1.7	5.9±0.8	4.9±0.8
Magnetic field (4.5 Hz 2hrs/day for 7 days)	11543 ±1352	78±4.2	* 12.6±2.4	*12.2±2

Data are represented as mean ± standard error.

Each value represents a mean value of 6-7 samples.

**Significantly different from control group*

Table (2) Effect of magnetic wave (4.5 Hz) on serum glucose, Cholesterol and Albumin levels in normal mice

Group	Glucose concentration mg/dl	Cholesterol concentration mg/dl	Albumin concentration g/dl
Control (0.2 ml saline)	120.3±30.3	81.2±4.6	3.5±0.2
Magnetic field (4.5 Hz 2hrs/day for 7 days)	*89.6±21	*62.6±5.2	3.1±0.2

Data are represented as mean ± standard error.

Each value represents a mean value of 6-7 samples.

*Significantly different from control group

Table (3) Effect of magnetic wave (4.5Hz), methotrexate (2.8 mg/Kg), on total leukocytes , lymphocyte %, monocyte% and granulocyte % on normal and tumor bearing mice

Group	Total leukocyte count /mm ³	Lymphocyte %	Monocyte %	Granulocyte %
Control (0.2 ml saline)	12433 ± 1708	87.7 ± 1.7	5.9 ± 0.8	4.9 ± 0.8
Control tumor bearing mice	9280 ± 1333	66.6 ± 5.9	22.3± 1.8	15.8 ± 2.2
Magnetic field (4.5 Hz 2hrs/day for 7 days)	14667 * ± 990	71.3 ± 4.9	17.3*±1.9	10.8 ± 2.2
Methotrexate (2.8 mg/Kg)	13060 *± 2310	81.4 ± 2.7	13.1 * ± 1.6	8.9 ± 1.4

Data are represented as mean ± standard error.

Each value represents a mean value of 6-7 samples.

*Significantly different from control group

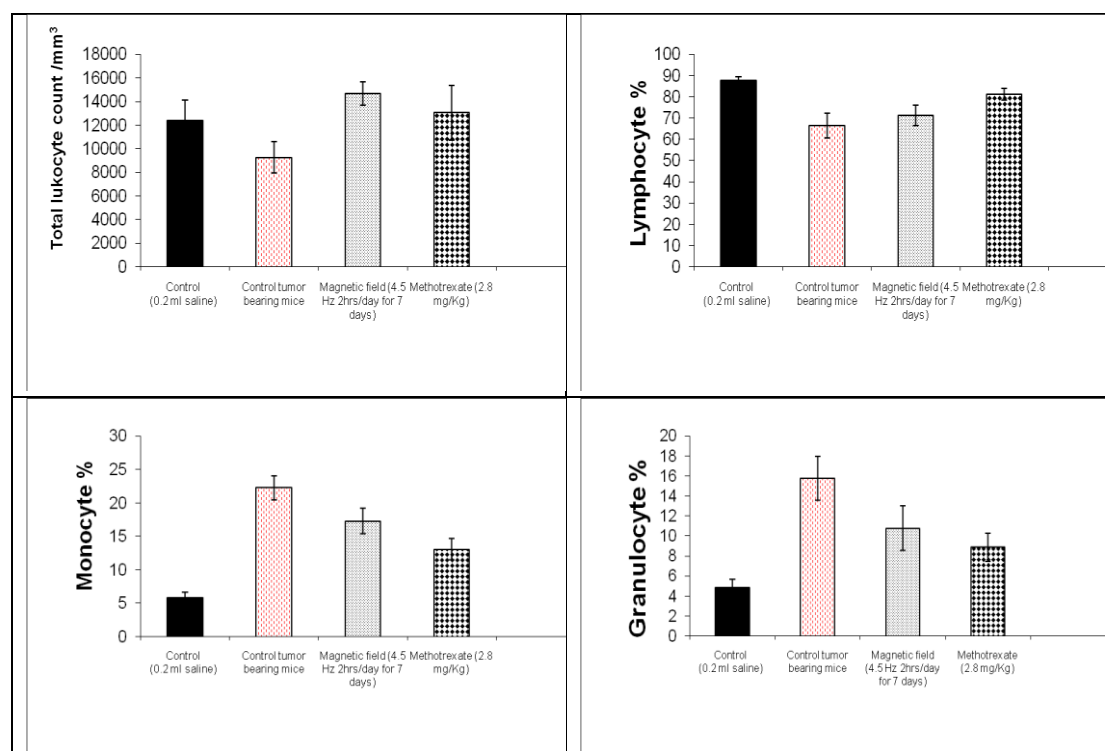


Figure (3) Effect of magnetic wave (4.5Hz), and methotrexate (2.8 mg/Kg), on total leukocytes, lymphocyte %, monocyte% and granulocytes % on tumor bearing mice

Data are represented as mean \pm standard error.
Each value represents a mean value of 6-7 samples.
*Significantly different from control group

Table (4) Effect of magnetic wave (4.5 Hz), and methotrexate (2.8 mg/Kg) on serum glucose, Cholesterol and Albumin levels in normal and tumor bearing mice

Group	Glucose concentration mg/dl	Cholesterol concentration mg/dl	Albumin concentration g/dl
Control (0.2 ml saline)	120.3 \pm 30.3	81.2 \pm 4.6	3.5 \pm 0.2
Control tumor bearing mice	120.3 \pm 30.3	79.4 \pm 8.5	2.5 \pm 0.2
Magnetic field (4.5 Hz 2hrs/day for 7 days)	131 \pm 19.5	86.9 \pm 4.3	3.0 \pm 0.3
Methotrexate (2.8 mg/Kg)	123.3 \pm 16	76.4 \pm 5	3.2 \pm 0.3

Data are represented as mean \pm standard error.
Each value represents a mean value of 6-7 samples.

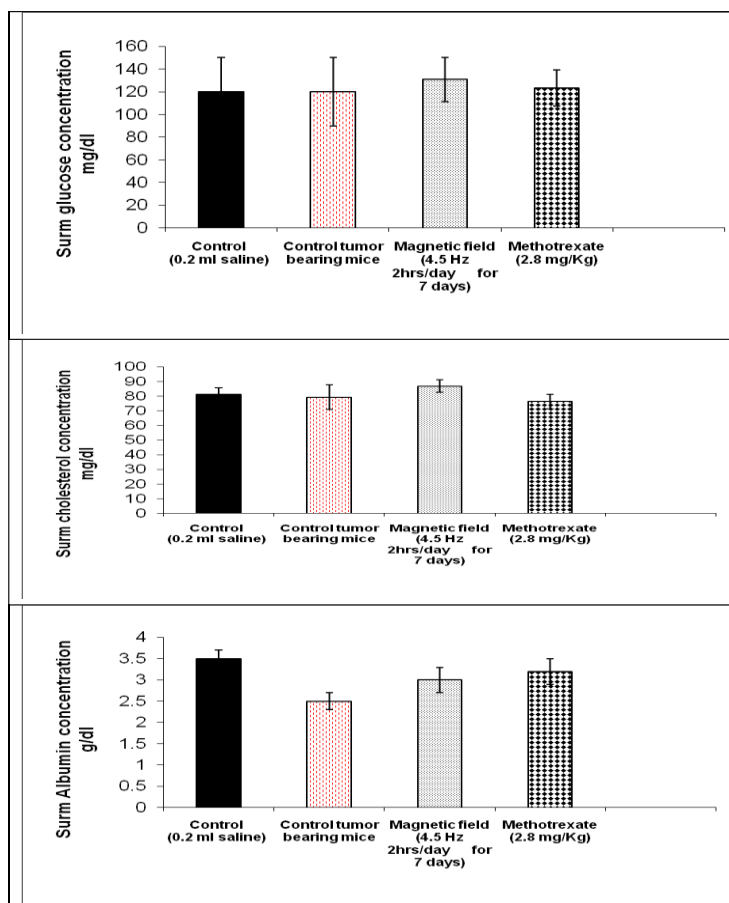


Figure (4) Effect of magnetic wave (4.5 Hz), and methotrexate (2.8 mg/Kg) on serum glucose, Cholesterol and Albumin levels in tumor bearing mice

Data are represented as mean \pm standard error.

Each value represents a mean value of 6-7 samples.

Table (5): Effect of magnetic field (4.5 Hz) and methotrexate (2.8 mg/Kg) on tumor volume size mm³.

Group	Tumor Volume size mm ³
Control tumor bearing mice	0.808\pm0.1
Magnetic field (4.5 Hz 2hrs/day for 7 days)	*0.373\pm0.04
Methotrexate (2.8 mg/Kg)	*0.357\pm0.04

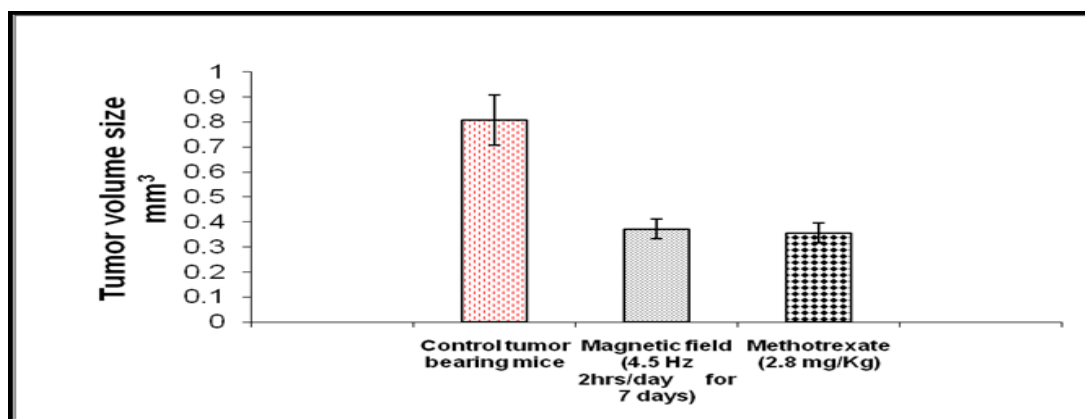


Figure (5) Effect of magnetic wave (4.5 Hz) and methotrexate (2.8 mg/Kg) on tumor volume size mm³.

Data are represented as mean \pm standard error.

Each value represents a mean value obtained from 14 animals

*Significantly different from control group.

Discussion

The data recorded in the control group indicated that no changes in the histological patterns of sections of the liver of mice injected with saline.

Whole body exposure of normal animals to 4.5Hz, 2G magnetic waves resulted into formation of hepatocytes with multiple nucleoli. One may not be able to criticize whether these changes can be considered as a primary step for tumor formation (Domenico and Sergio, 2009) /or just an increase in response of internal activity of hepatocytes to the influence of magnetic field. The first reasoning needs more investigation, including determination of liver tumor markers. This is a probable interpretation of incidence of cancer as a result of exposure to ELF magnetic waves. This is in accord with the findings of occupational study of Gerald *et al.*, (2005) that reported an increasing risk

of brain tumor and leukemia for children born close to or at a distance less than 600 meters from power lines. They found that epidemiological exposures to magnetic fields higher than 0.3 μ Tesla (3 mG) cause toxic and carcinogenic effects (Kheifets *et al.*, 2009). All these epidemiological studies were concerned with sine wave magnetic fields of frequencies 50 Hz or 60 Hz generated from power lines (Feytching *et al.*, 2005). Moreover, the volume of these developed hepatocytes is similar to normal, as compared with malignant hepatocytes, which are usually smaller in size, and more easily stainable (Turler *et al.*, 2000). The first reasoning is a very important point for future research since it will help scientists to criticize whether exposure to magnetic field can be a detrimental of malignancy or enhanced regenerative process (Behari *et al.*, 2010). One may state that the effect of ELF (extremely

low frequency) magnetic waves on biological cells is the generation of induced electric currents from ionic movements (Feytching *et al.*; 2005). These biocurrents will generate bioelectric potential in the cells, which will interfere with its running metabolic process (Hashish *et al.*; 2008). Therefore, one may find that ELF magnetic wave can have two effects: either an increase of metabolic activities within the cells, and/or changing or stopping these activities. The first reasoning is a rationale medical treatment based on magnetotherapy (Newell *et al.*, 2000). While the later reasoning supports the new findings of toxicity of ELF magnetic fields (Behari *et al.*, 2010). The frequency and form of the magnetic wave used in the present work is 4.5 Hz and its shape was square. These waves induce electric currents and waves inside the treated tissue, which inhibit Ehrlich tumor growth (Fadel, 2005 and 2010). At this frequency the applied induced electromagnetic waves inside the tissues cause destructive interference with the metabolic bioelectromagnetic waves generated during malignant cellular division, which affects the ionic motions evolved in the process (Fadel, 1998). Moreover, epidemiological studies include cases of intrauterine children and newly born children who were in continuous exposure to the magnetic field (Feytching *et al.*, 2005), however in the present investigation, treatment of tumors by magnetic waves in adult animals continued for a limited period of one week at a rate of 2h/day.

Significant decrease of serum glucose level was observed in normal animals exposed to 4.5 Hz, 2 G magnetic field. The significant decrease of glucose level in blood serum exposed to the magnetic field, indicates a high consumption rate of glucose in order to provide energy to those injured cells and to enhance the repair mechanism (Tu and Giltner, 1974). Significant decrease of serum glucose level was also observed in normal animals exposed to 4.5 Hz, 2 G magnetic field in contrary with these results are the findings of Elferchichi *et al.*, (2010) who used static magnetic field on rats 1hr/day for 15 consecutive days which displayed increase in both plasma glucose level and cholesterol level this may be due to different animal species and different type of magnetic field. In this study significant increase in granulocyte % was observed and non-significant difference in total leukocyte and lymphocyte in normal mice exposed to magnetic field. Further support to the present results was the finding of Hashish *et al.*, (2008) using static magnetic field and extremely low frequency electromagnetic field for whole body exposure in mice, in addition of the results of Cakir *et al.*; (2009) using ELF-EMF 50 Hz on rats that were exposed to EMF for 50 .The increase in granulocyte % indicates that there is relation between exposure of extremely low frequency electromagnetic field and the oxidative stress through distressing redox balance leading to physiological disturbances Hashish *et al.*, (2008). The tumor bearing mice were concerned with the

effects of chemotherapy (Methotrexate) and 4.5 Hz, 2 G magnetic waves on Ehrlich tumor implanted in the thigh (primary site) and in the liver (secondary site)metastasis. These treatments were consequently followed by investigation of tumor growth at the primary site (thigh) and histological examination of liver sections as a secondary site (metastasis), in addition to estimation of some biochemical parameters of the blood. This is clear from the rate of tumor growth in the primary site in the thigh, where aggressive growth of the tumor occurred in a similar manner to that of untreated animals. The results also indicate that the growth of tumor volume in the thigh of animals treated with either 4.5 Hz, 2G magnetic waves or with Methotrexate was minimal as compared to that of untreated group. Similar growth rate of tumor in the primary site for both Methotrexate and 4.5 Hz magnetic waves occurred. However, as a result of the toxic effects of chemotherapy (Mycek *et al.*, 2000), a massive destruction of hepatocytes at the hepatic lobules occurred and supported by the findings of Zachariae, (1990), an observation, which didn't occur in animals, treated with magnetic waves. The effect of Methotrexate differs from the results obtained from using Methotrexate in combination with hydroxychloroquine for its antireumatic effect as the tested drugs didn't show hepatic toxicity and this is due to using hydroxychloroquine that reduced the dose of methotrexate, thereby reducing its hepato-toxic effects (Shashikumar *et al.*; 2010). In

this study significant increase in total leukocyte count was observed in all tumor bearing groups. However this result is compatible with the significant increase in total leukocyte count that was observed by Nakamura *et al.*; (2002) who used methotrexate on pulpal inflammation in rats.

The increase in total leukocyte count of tumor bearing mice treated with Methotrexate but no change was observed in plasma albumin level, these results were in compatible with the result of Rofe *et al.*; (1994) using Methotrexate therapy in tumor bearing rats where there are decrease in total white cell count and decrease in plasma albumin level. This incompatibility may be due to difference in dose administrated, species of animals and environmental conditions. Also, non significant change in serum cholesterol level was noticed for tumor bearing mice in all treatments.

The formation of aggregates of tumor cells in the liver of group exposed to magnetic field in-addition to the formation of cells exhibiting multinucleoli are markers for the start of repair mechanism (Fadel *et al.*, 2003). Further support to the present results (repair mechanism)was the finding of Truler *et al.*; (1998,2000) who have studied the effectiveness of low level direct current in liver metastasis tumor growth, and Traitcheva *et al.*; (2003) who have studied the lethal effect of extremely low frequency pulsed electromagnetic field on human cancer cells. More investigations are needed to confirm the afore-mentioned observations. The

results of the change in tumor size after exposure to magnetic waves are in agreement with other published data (Fadel *et al.*, 2009).

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