EFFECT OF LOW INTENSITY ULTRASONIC ON EHRLICH SOLID TUMOR IN VIVO

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Abstract

Purposes: To determine the effect of low intensity ultrasonic on Ehrlich solid tumor in vivo. **Study Design:** It is a randomized controlled study. **Methodology:** Thirty one mice were divided into 2 experimental groups. Experimental group I (which started after 4 weeks from induction of Ehrlich solid tumor) contained 17 mice, which subdivided into treated group contained 10 mice, and the control group which received shame ultrasonic and contained 7 mice. Experimental group II (which started after 2 weeks from induction of Ehrlich solid tumor) , contained 14 mice, which subdivided into treated group contained 7 mice, and the control group which received shame ultrasonic and contained 7 mice. Comparison in between both groups in each experiment was done for measuring tumor cell size and tumor necrotic area through histopathology examination, and for measuring Vascular Endothelial Growth Factor (VEGF) expression through immune histo chemistry examination. Also comparisons between treated groups of 2 experiments were done. **Results:** The study revealed that there were significant differences in the measurements of Tumor cell size between treated and control groups in 1st and 2nd experiment. For tumor necrotic area measurements and for VEGF there were highly significant differences between treated and control group in 2nd experiment, and no significance difference in 1st experiment. And for all variables there were significant difference in between 1st and 2nd experiment **Conclusion:** Low intensity ultrasonic particularly targeted the vascular structures of tumor, and may prevent further tumor growth.

Keywords: Ultrasonic, Antitumor, VEGF, Angiogenesis

Introduction

The tumor growth depend on the development of angiogenesis factors resulted in that sprouting of new blood vessels from pre-existing vessels to supply the tumor , the survival and growth of solid tumors depend on the development of blood vessels formed as a result of angiogenesis . Tumor blood vessels were known to be structurally and functionally different from those in the normal tissues (Folkman., 2001).

Many studies had shown that the maturity of blood vessels within a tumor varied and that the more immature vessels were more sensitive to anti vascular therapies (Gee et al., 2003). The fragile, poorly functioning tumor vessels, whose formation was activated by (angiogenesis switch), were likely to be more sensitive to ultrasonic application (Wood et al., 2005).

For many years, ultrasound (US) had been used for clinical imaging, as well as for therapeutic action in physical therapy (Barnett et al., 1997). The parameters of (HIFUS) were different from low intensity (US) which was used for clinical therapy fields (Baker et al., 2001) The low intensity US power levels generally range from 0.2 to 2.6 w/cm-2 (Demmink et al., 2003). In vitro, the frequency of low intensity US raised the tissue temperature from 1to8^0 (Robertson and Baker. , 2001).

Jin et al., (2000) were reported that photodynamic therapy , with low intensity US (0.51 W/cm $^{-2}$,1 MHz ,and with duration 10 min) was used to inhibit the growth of cutaneous squamous cell carcinoma in mice , but the author concluded that the mechanism of the response remained unclear. Hazle et al., (2002) and Melodelima et al., (2003) had used higher intensities of US for treatment of experimental canine prostatic neaplasia, and human esophageal cancer respectively.

Each minute of insonation with physiotherapy US beam had a potent antivascular effect and significantly decreased the vascular perfusion of the murine melanoma (Wood et al., 2005). Because the US induced antivascular effect were unlikely to be dependent upon specific biochemical pathway used by various drug therapies, it was conceivable that US could be used to disrupt vessels of different maturities. (Bunte et al., 2006). So the aim of this study was to investigate the anti tumor effect of low intensity ultrasonic on tumor vasculature of solid tumor (Ehrlich tumor).

Justification of the study

As a tumor grows , the up regulation of angiogenic factors result in the sprouting of new blood vessels from pre-existing vessels to supply the tumor , but these new vessels fail to mature into a normally functioning vasculature (Carmeliet and Conway ., 2001) .The recent tumor blood vessels were fragile and leaky, their endothelial cells remained loosely associated,

there was continued degradation of the extra cellular matrix. Also the basement membrane was not functional, had a non uniform distribution and demonstrated irregular branching and arteriovenous shunts. (Haroon et al., 1999)

In considering the development of cancer therapies, a tumor should be considered to have two cellular compartments, one containing the tumor cell and the other is the endothelial cells of the vascular structures within the tumor, for anti cancer therapy to be effective, each compartment may be selectively targeted. (Jain., 2002). While high intensity ultrasound treatments had targeted tumor cells, little attention had been given to the use of low intensity physiotherapy ultrasound to disrupt different tumor vasculature. Using the low intensity ultrasonic in tumor treatment may provide non invasive and more economic method of tumor treatment

Hypothesis

1) There was no significant difference in tumor cell size and tumor necrotic area length in between experimental group 1(treated with physiotherapy US after 4 weeks of induction of tumor) and control group.
2) There was no significant difference in tumor cell size and tumor necrotic area length in between experimental group 2(treated with physiotherapy US after 2 weeks of induction of tumor) and control group.
3) There was no significant difference in (VEGF) expression of mice bearing Ehrlich tumor through immuno histo chemistry in between experimental group 1(treated with physiotherapy US after 4 weeks of induction of tumor)) and control group .
4) There was no significant difference in (VEGF) expression of mice bearing Ehrlich tumor through immuno histo chemistry in between experimental group 2(treated with physiotherapy US after 2 weeks of induction of tumor)) and control group.
5) There was no significant effect of the onset of application of low intensity US (after 2 and 4 weeks of induction of Ehrlich solid tumor) on tumor cell size, tumor necrotic area length and (VEGF) expression.

Methodology

This experiment was conducted in experimental animal lab of the biology department of national institute of cancer, Cairo, Egypt. Experimental design**:** The design of this study is controlled randomized single blind study. Throughout the experiment 4 mice were excluded, two of which died during grasping throughout the insonation from asphyxia, the other two died from gastrointestinal microbial disease.

Table (1): The experimental design

Groups	Treated	Control	Total
Experiment (I)	10	7	17
Experiment(II)	7	7	14
Total	17	14	31

Experiment group I: Contained 17 mice. In this group the experiment started after 4 weeks from induction of Ehrlich solid tumor. The mice were divided into two groups, treated group contained 10 mice, which were treated with physiotherapy ultrasonic, and the control group contained 7 mice which received shame ultrasonic.

Experiment group II: Contained 14 mice. In this group the experiment started after 2 weeks from induction of Ehrlich solid tumor. This group was divided into two groups, treated group contained 7 mice, which were treated with physiotherapy ultrasonic, and the control group contained 7mice which received shame ultrasonic.

Experimental animals: Thirty five male mice 6-8weak old with average weight 18-25 g, were used for this study, 4 mice were died throughout the study. The animals were housed in standard mice cages and were fed a balanced diet of proteins and fibers. The all mice were kept at the same conditions of temperature, humidity and light, and subjected to comprehensive veterinary care. The thirty five mice were inoculated subcutaneously with 10^6 single cell suspension isolated from Ehrlich solid carcinoma. Cells were injected using a volume of 0.5 ml into the right flank of mice.

Instrumentation: Therapeutic ultrasounds unite (ProSound ULS-1000-Medserve Limited. Prior Hall Business Center. United Kingdom).Light microscope**:** Meiji metallurgical microscope, model ML8000. This model is capable of magnifications of 40, 50, 100, 200, 400X. Light microscope was used for histo pathological analysis of the specimens, and VEGF Study. Micrometer: was used as a diameter to measure tumor cell size.

Treatment procedures: Animals preparation; a depilatory cream was used to remove hair coat from the tumor site, after 3 min of application of the depilatory cream , the right flank of each mouse was cleaned by water. So the right flank of each mouse was free from hair.

Ultrasound unite operational sequence: The 2 cm^2 transducer head was inserted in the PROBE socket 1 so that 8 prongs aligned correctly with the 8 receptor holes in the PROBE socket 1.The investigator pressed the ON /OFF button to turn on the power, so the display panel would illuminate .At this time would select the treatment parameter. Then ultrasonic coupling gel was applied to the skin and to the head of ultrasonic. When all setting was set, the investigator pressed the Start /Stop button. Animal fixation; each mouse was fixed in dorsal recumbence position by a veterinary assistant, so

the sagittal anatomical plane was used for insonation. Tumor insonation; the tumor site was insonated by a physiotherapy ultrasonic machine with the following parameters, 3 MHz, at 2.5 W/cm^2, continuous output, 2-cm^2 transducer head, 5 minutes, four times/week, for one week. With moving the transducer in circular pattern. The head of ultrasonic was placed in water for 3 min after each 1min of insonation to overcome the overheating. Each mouse in control group was received a shame ultrasonic in which the ultrasonic machine is turned off. Histopathological procedure: At the completion of the experiment, a thoracatomy was performed in each mouse. Then the tumor was excised and emersion-in neutral buffer formalin 10% and processed by conventional method. Paraffin wax blocks were sectioned at 3-5 u and stained by hematoxyline and eosine stain. The following criteria were searched and recorded; Tumor cell size. Tumor necrotic area, Tumor cell viability, Tumor giant cell number, The amount of vasculature either in the stroma or in-between the muscle bundles, Presence or absence of necrotic cell, Presence or absence of hemorrhage and edema.

Immuno histochemistry procedures: Tumor sections were cut onto saline coated slides and dewaxed by paraffin wax. The paraffin sections were dewaxed by xylene the put in descending concentration of ethyl alcohol then washed by water. The slides treated with 0.3% hydrogen peroxide for 30 min at room temperature (RT) to remove intrinsic peroxidase activity that may give false positive reactions when detecting the secondary antibody conjugated peroxidase. Slides were then washed with PBS pH 7.4. To reduce background during staining, the slides were soaked in 1% normal fetal bovine serum (FBS) for 30 min at RT. During the incubation, the primary antibody (VEGF) was diluted at 1:1000 .After washing of the diluted FBS with PBS, the primary antibody dilution was added to designated sections. The primary antibodies were incubated for 1 hour at 37° C in a humid chamber, and then at RT (18-20° C) overnight. The primary antibody was washed extensively the next day, using PBS (3-5 washes 10-30 min each). The second antibody, anti-mouse peroxidase conjugate, was diluted in PBS at 1:4500 and added to the sections for one hour. At this point all sections were encircled with a wax pen. After extensive washing, the DAB reagent [Ultravision, Fermont, CA, USA] was used to stain according to the manufacturer's recommendations. Briefly, diluted DAB reagent was used at 50 µl/section for 30 min at RT, and then washed with DW. Haematoxylin stain was then used to counterstain for 15 min at room temperature. The slides were washed with DW, dried, mounted with gelatin glycerol, covered and examined to detect the degree of VEGF expression (mild, moderate, and sever).

Data collected through data collection sheet was analyzed statistically using Descriptive statistics, Independent t test, Non parametric study (Mann-whiteny).

Results: Thirty five mice were incubated with solid Ehrlich tumor four of them were died before starting the treatment. The remaining thirty one mice were used and classified into two experiments groups. Experiment group I which include ten mice were treated by low intensity ultrasonic after 4 weeks from the incubation and seven mice act as control group for this experiment. Experiment group II which include seven mice were treated by low intensity ultrasonic after 2 weeks from the incubation and seven mice act as control group for this experiment. After completion of the treatment course selected histopathological and immune histochemistry (VEGF) evaluations were done. The results and analysis of the data were presented and discussed in this chapter according to the following sections. Experimental animals' characteristics, Histopathological results which included (tumor cell size and tumor necrotic area size), Immunohistochemistry (VEGF) study results. Ultrastactural observation.

1. Experimental animals characteristics: Thirty five male mice were investigated in the study. Their ages ranged from 6-8 weeks with mean age 6.77 ±0.8 weeks, their weights ranged from 20-25g with mean weight 22.33 ±1.84 g.

2. Histopathological results: a. Tumor cell size: Tumor cell size for 1st experimental groups: The data presented in figure (1) showed that the mean tumor cell size of Ehrlich solid tumor for treated and control groups for the 1st experimental study were 4.34mm and 4.9mm respectively with standard deviation for treated and control groups were ±0.60773and ±0.29439 respectively. The independent t-test revealed that there were significant difference in tumor cell size between the treated and control groups , where **t** was -2.522 and **p** value was 0.025 and **df** equal 16 at level of significance was 0.05.

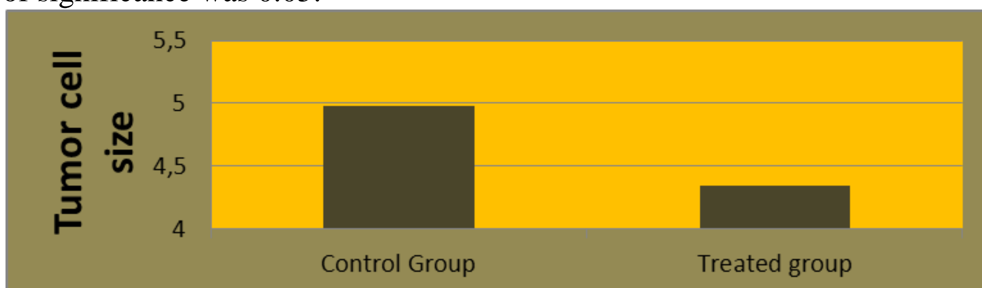


Figure (1): Mean of tumor cell size for treated and control groups in 1st experimental.

Tumor cell size for 2nd experimental groups: The data presented figure (2) showed that the mean tumor cell size of Ehrlich solid tumor for

treated and control groups for the 2nd experimental study were 1.1857 mm and 1.8857 mm respectively with standard deviation for treated and control groups were ± 0.38048 and ±0.5928 respectively. The independent t-test revealed that there were significant difference in tumor cell size between the treated and control groups, where **t** was -2.629 and **p** value was 0.025 and **df** equal 13 at level of significance 0.05

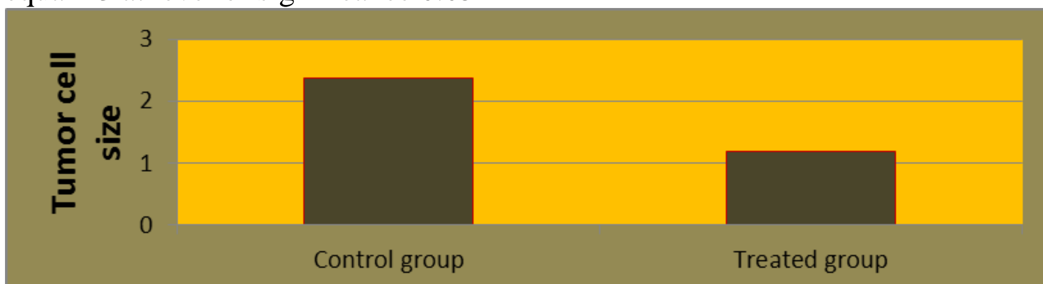


Figure (2): Mean of tumor cell size for treated and control groups in 2nd experimental

Tumor cell size for 1st and 2nd experimental groups: The independent t-test revealed that there were significant difference in the tumor cell size between treated groups in 1st and 2nd experimental studies, where t was 13.141 and p value was 0.000 and df equal 14.895 at level of significance 0.05 .

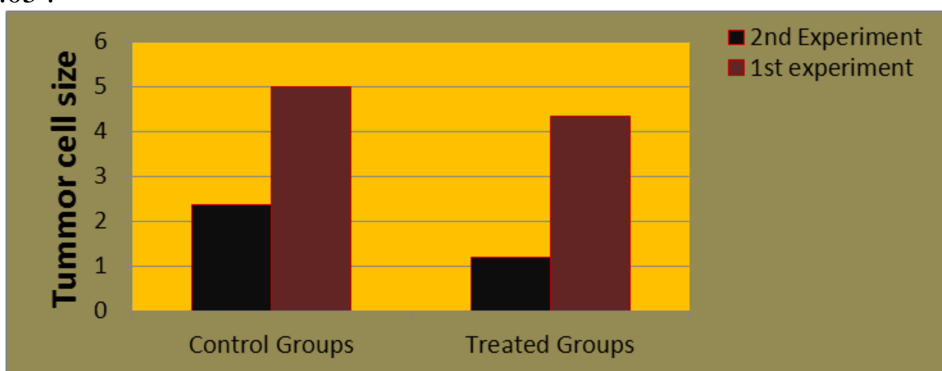


Figure (3): Mean of control and treated groups of tumor cell size for 1st and 2nd experiment

Tumor necrotic area: Tumor necrotic area for 1st experimental groups: The data presented figure (4) showed that the mean tumor necrotic area of Ehrlich solid tumor for treated and control groups for the 1st experimental study were 1.7mm and 1.8857 mm respectively with standard deviation for treated and control groups were ±0.3055and ±0.3579 respectively. The independent t-test revealed that there were no significant difference in tumor necrotic area between the treated and control groups, where t was -1.117 and p value was 0.286 and df 16 at level of significance 0.05.

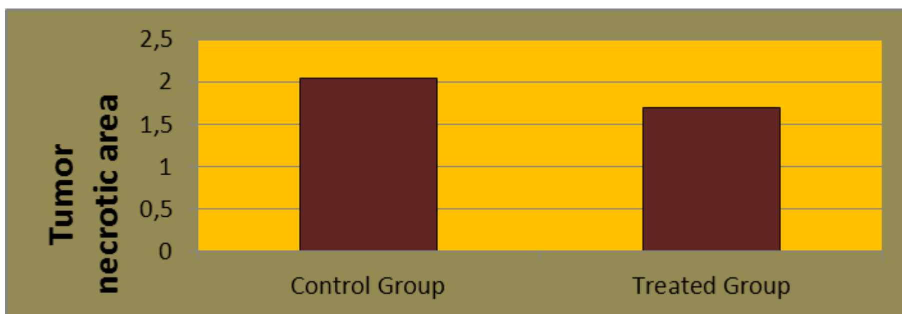


Figure (4): Mean of tumor necrotic area for treated and control groups in 1st experimental

Tumor necrotic area for 2nd experimental groups: The data presented figure (5) showed that the mean tumor necrotic area of Ehrlich solid tumor for treated and control groups for the 2nd experimental study were 0.2481 mm and 0.7861 mm respectively with standard deviation for treated and control groups were ± 0.07071 and ± 0.16184 respectively. The independent t-test revealed that there were significant difference in tumor necrotic area between the treated and control groups, where t was -8.053 and p value was 0.000 and df was 13 at level of significance 0.05.

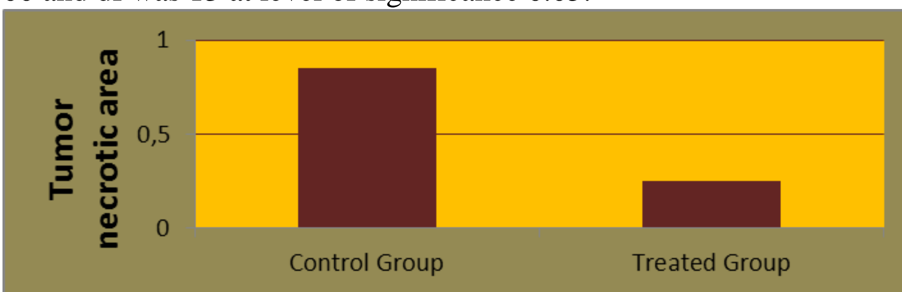


Figure (5): Mean of tumor necrotic area for treated and control groups in 2nd experimental.

Tumor necrotic area for 1st and 2nd experimental groups**:** The independent t-test revealed that there were significant difference in the tumor necrotic area between treated groups in 1st and 2nd experimental studies, where t was 14.479 and p value was 0.000 at level of significance 0.05 .

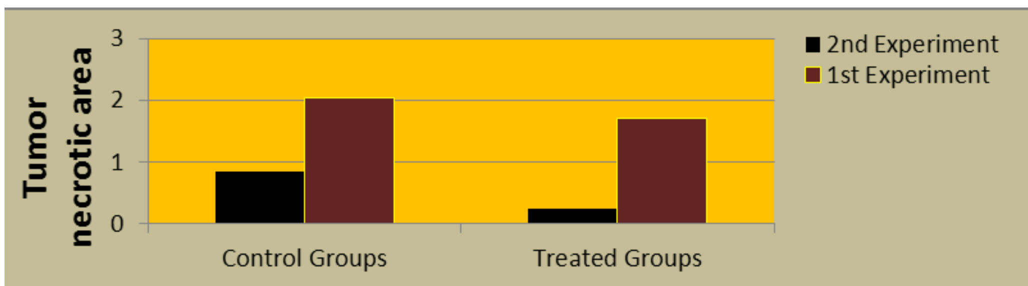


Figure (6): Mean of control and treated groups of tumor cell size for 1st and 2nd experiment.

Immuno histo chemistry (VEGF) study results. VEGF for 1st experimental groups: The percentage of strong VEGF expression of Ehrlich solid tumor for treated and control groups for the 1st experimental study were 100% and 100% respectively . The Non parametric Mann Whitney test revealed that there were no significant difference in VEGF expression between the treated and control groups for 1st experimental groups , where z was 0.000 and p value was 1.00 at level of significance 0.05.

VEGF for 2nd experimental groups: The percentage of mild VEGF expression of Ehrlich solid tumor for treated group for the 2nd experimental study was100% . While figure (19) and table (8) showed that the percentage of mild , moderate, and strong VEGF expression of Ehrlich solid tumor for control group of the 2nd experimental study were 0% , 28.6% and 71.4% respectively . The Non parametric Mann Whitney test revealed that there were significant difference in VEGF expression between the treated and control groups , where z was -2.793 and p value was 0.005 . at level of significance 0.05 .

VEGF for 1st and 2nd experimental groups: The Non parametric man whitenty test revealed that there were significant difference in the VEGF expression between treated groups in 1st and 2nd experimental studies, where z was -4.00 and p value was 0.000 at level of significance 0.05.

Ultrastractural observation; For 1st experimental study; Ultrastractural observation of the Ehrlich solid tumor cells appeared infiltrating the skeletal muscles of mice in both control and treated groups. It appeared as microscopic islands of malignant cells deep in-between the muscles bundles and adipose tissues as shown in (Fig 7) surrounded by strands of connective tissues and vascularzed stroma.

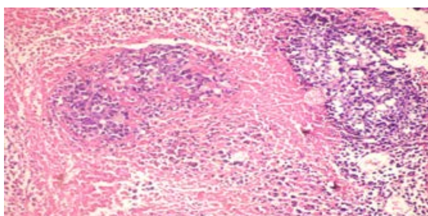
Other portions of skeletal muscles revealed only scattered tumor cells. There was considerable inflammatory reaction represented by few numbers of polymorph leukocytes cells infiltrations as shown in (Fig 8). The tumor cells showed polymorphisms (ovoid, spindles, round shape) with large prominent basophilic nucleus and basophilic cytoplasm as shown in (Fig 9).

There were considerable numbers of mitotic divisions and large numbers of tumor giant cells as shown in (Fig 10). There were multiple fat vacuoles in the centers of tumors islands in all examined groups. The tumors cells are viable either in the center or peripheral the solid masses and the volume were same in both treated and control animals. Some bundles of muscles showed Zenker's necrosis and even in some cases showed liquefied necrosis (Fig 11).

Although some blood vessels in the stroma of the tumor or in-between the tumor cells showed necrosis in their wall , there is no any signs of hemorrhages or edema in-between the tumors islands or in the muscle

bundles. There were metastatic tumor cells in some blood vessels as shown in (Fig 12).Only few cases showed dilated blood vessels in the tumor mass.

Vascular Endothelial growth factor (VEGF) expression were evaluated both control and treated mice via immune histo chemistry. VEGF was strongly expressed in both treated and control groups with no differences in the expression as shown in (Fig 13).



Figure(7): Islands of tumors cells invading the muscle bundles in 1st experiment control and treated. H&E stain X 100.

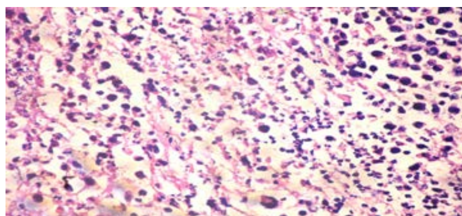


Figure (8): Inflammatory cells aggregations around and in-between the tumor cells in 1st experiment for control and treated groups. H&E stain X 200.

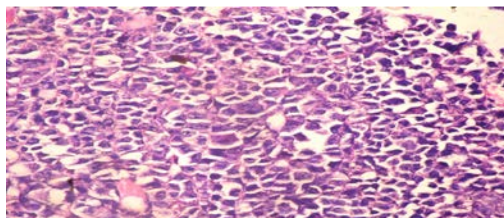


Figure (9): Tumor cells appearance in 1st experiment for control and treated groups. H&E stainX400.

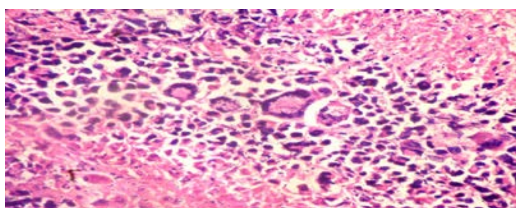


Figure (10): Large numbers of tumor giant cells in-between the tumor cell in 1st experiment for control and treated groups. H&E stain X 400.

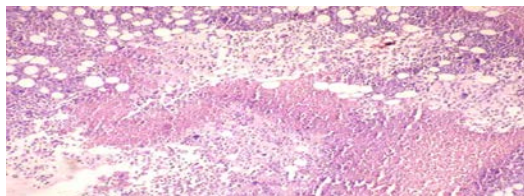


Figure (11): Zenker's and liqufactive necrosis of muscle bundles. Notice the fat vacuoles in between the tumor cells in 1st experiment for control and treated groups. H&E stain X 200

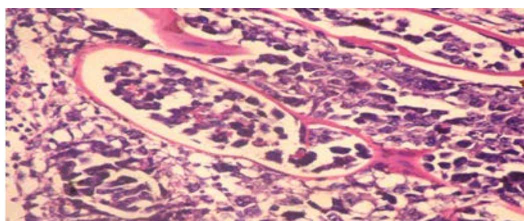
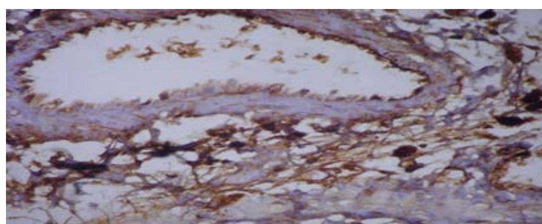


Figure (12): Metastatic tumor cells in some blood vessels in 1st experiment for control and treated groups. H&E stain X 200.



Figure(13): Strong expression of VEGF in the endothelial lining of blood vessels. in 1st experiment for control and treated groups in 1st experiment. Immunohistochemistry staining X 200.

For the 2nd experimental study; In the 2nd experiment where as the therapy began at 2 weeks from the induction of the tumor the treatment groups showed decrease in the size of the solid mass of the tumors. The tumors cells appear smaller in size with pyknotic nuclei (Fig 14) with decrease in the mitotic division and scanty numbers of tumor giant cells was detected. The viability of the tumor cells were decreased if compared with the 1st experiment especially in the center of the tumor mass (Fig 15).

The vasculature was decreased either in the stroma or in-between the muscle bundles. There were necrosis and vasculitis in the blood vessels of some cases especially in the group treated with 3 MH as shown in (Fig 16) accompanied with edema around the vessels and in-between the muscle bundles as shown in (Fig 17). Muscle bundles in both control and treated groups showed Zenker's necrosis in some areas. The tumor in the control group was more or less the same as in the first experiment but the size was smaller and numbers of tumors cells were decreased. The tumor cells

showed polymorphisms with large prominent basophilic nucleus and basophilic cytoplasm.

There were considerable numbers of mitotic divisions and large numbers of tumor giant cells. The tumors cells are viable either in the center or peripheral the solid masses. The expression of VEGF was decreased in treated group if compared with the control group or to the 1st experiment (Fig 18&19).

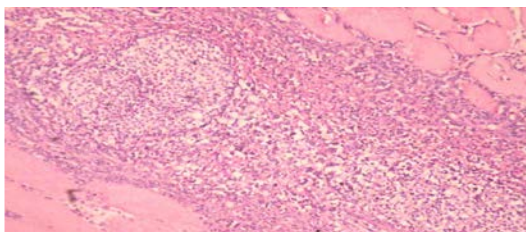


Figure (14): Muscle of mice of treated group showing decrease in numbers and size of the tumor cells with pyknosis of the nucleus. H&E stain X 200.

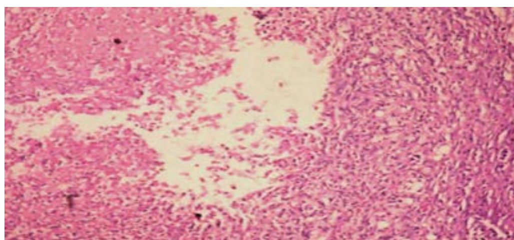


Figure (15): Muscle of mice of treated group in 2nd experiment showing necrosis and disappearance of tumor cells in the center of the tumor. H&E stain X100.

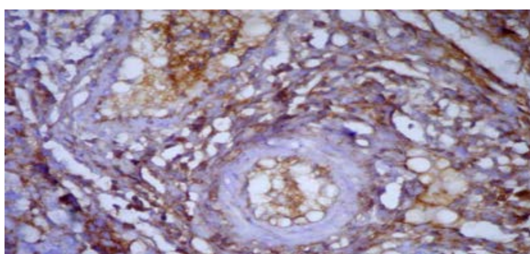


Figure (16): Blood vessel in muscle of mice of treated group in 2nd experiment showing necrosis and vasculitis. Notice the edema around the blood vessel. H&E stain X200.

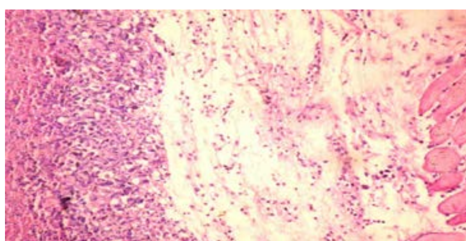


Figure (17): Muscle of mice of treated group in 2nd experiment showing edema in-between the muscle bundles. H&E stain X200

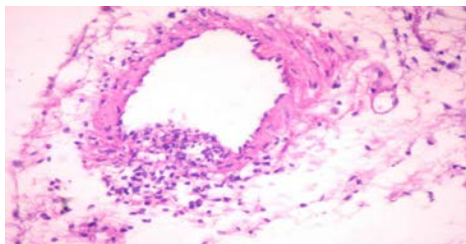


Figure (18): Muscle of mice of treated group in 2nd experiment showing mild expression of VEGF in the wall of the blood vessels. Immunohistochemistry staining X 200.

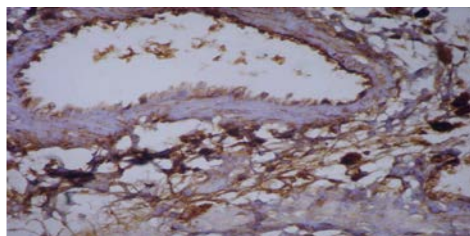


Figure (19): Muscle of mice of control group in 2nd experiment showing strong expression of VEGF in the wall of the blood vessels. Immunohistochemistry staining X 200

Discussion

The investigations were done in two occasions , the whole procedure were done after 4 weeks of inculation of Ehrlich solid tumor in 1st experiment for treated and control groups , the measurement were taken for 2nd experimental group which receive treatment after 2 weeks of inculation of Ehrlich solid tumor for control and treated groups. The repetition of study for 2nd experiment was done to detect the effect of early intervention with ultrasonic for treatment of tumor.

The current study was done on the Ehrlich solid tumor because of its simple ability for induction in mice, also this type of Ehrlich tumor which appeared originally as a spontaneous breast canrcinoma in a mouse, can resemble the breast cancer. (Ferreia et al., 2007)

Also the physiology of solid tumors differs from that of normal tissues in a number of important aspects, the majority of which stem from differences between the two vasculatures. Compared with the regular, ordered vasculature of normal tissues, blood vessels in tumors are often highly abnormal, distended capillaries with leaky walls and sluggish flow. Tumor growth also requires continuous new vessel growth, or angiogenesis. These physiological differences can be problems for cancer treatment; for example, hypoxia in solid tumors leads to resistance to radiotherapy and to some anticancer drugs. However, these differences can also be exploited for selective cancer treatment. **(Martin and Giaccia**., 1998)

In this study several variables which could have influenced the results were controlled. For example, the animals were housed in standard mice

cages and were fed a balanced diet of proteins and fibers. The thirty five mice were kept at the same conditions of temperature, humidity and light, and subjected to comprehensive veterinary care.

This study was limited by the use of the Ehrlich solid tumor which classified as a malignant tumor type (Ferreia et al., 2007). Because of biological variation of mice and to reach the desired number of mice in this study, the investigators start the study with 45 mice.

Within the limitation of this study, in the first experiment the independent t-test revealed that there were significant difference in tumor cell size between the treated and control groups , where t was -2.522 and p value was 0.025 df equal 13.731 at level of significance was 0.05, while in the second experiment the independent t-test revealed that there were significant difference in tumor cell size between the treated and control groups , where t was -2.629 and p value was 0.025 and df equal 10.226 at level of significance 0.05. These result shown that the physiotherapy ultrasonic can decrease the tumor cell size in Ehrlich solid tumor significantly after inculation of tumor by four and two weeks.

This was in line with the earlier finding of (Kremkau., 1988) which Studied the effects of ultrasound on several solid tumors in experimental animals and concluded that the tumor growth rates can be reduced. This study was generally consistent with a thermal mechanism of action of ultrasonic but this study examined the effect of ultrasonic in combination X-irradiation. Also examined the combined effects of ultrasound and cancer chemotherapy drugs leukemia.

The current result findings were in agreement partially with (Frederick., 2005), Who studied the effect of treating cancer by ultrasound through three approaches (a) ultrasound alone, (b) ultrasound in combination with radiotherapy, and (c) ultrasound in combination with chemotherapy. With the first approach the results had varied. In some cases, decreased growth rates or regressions of tumors have been reported; in other cases, either no effect had been observed or growth had been increased. With the second approach, for some tumors, combined treatment had produced greater effects on tumors than had x-ray alone, whereas in other tumors the addition of ultrasound had produced no change. With the third approach, enhancement of the effects of drugs had been observed in melanoma and mouse tumor cells treated with ultrasound and several anticancer drugs. The mechanism of action in most (but not all) cases had appeared to be absorption of heating.

Most of this study were done in vitro and didn’t measure the tumor cell size accurately, but in present study the tumor cell size was measured under light microscope with micrometer that revealed the tumor cell

accurately not the apparent whole tumor growth and the survival rate as in previous studies.

In addition, the present study measure the necrotic area caused by Ehrlich solid tumor, the independent t-test revealed that there were significant difference in tumor necrotic area between the treated and control groups in second experiment , where t was -8.053 and p value was 0.000 and df was 8.231 at level of significance 0.05. This result shown that the physiotherapy ultrasonic can decrease the necrotic area caused by Ehrlich solid tumor significantly after incubation of tumor by two weeks.

Shang et al, (2006) was in agreement with the present study results, they investigated the bi frequency ultrasonic on killing tumor cells. The results showed that the bi-frequency ultrasound exhibited an improved effect of killing tumor cells than single frequency ultrasound. This study was carried in Vitro so can detect tumor cell necrosis by different color changes between living and necrotic cell and didn't measure the tumor necrosis area size.

While there were several studies investigated the tumor cell necrosis by high intensity ultrasonic (High focused ultrasonic) in animal and in human, there were limited study that investigated the tumor cell necrosis by the low intensity ultrasonic. This was well elaborated in study of (Vaezy et al., 2000) which investigated the potential efficacy of high-intensity focused ultrasound for the treatment of uterine fibroid tumors in a nude mouse model. They concluded that a single high-intensity focused ultrasonic treatment resulted in an average reduction in tumor volume of 91% within 1 month of the treatment. Histological analysis of tumors treated with high-intensity focused ultrasound showed coagulation necrosis and nuclear fragmentation of tumor cells.

While there were little study examined the antitumor effect of low intensity ultrasonic, there were many studies examined the antitumor effect of ultrasonic combined with antitumor medication, (Feril et al., 2009) examined the effects of echo-contrast agents (ECAs) on ultrasound (US)-induced apoptosis and cell lysis. Therefore, this study showed that Optison™ and YM454 were effective in augmenting the US-induced cell killing, but not Levovist™. Another result indicated that cavitations played a role in the augmented effects and that inertial cavitation appears necessary for Optison TM and YM454 to effect their actions

This in line with study in which the result showed that a 30 s ultrasonic irradiation by 1 or 3 MHz ultrasound applied locally to the tumor significantly enhanced accumulation of Pluronic in the tumor cells. The data indicated targeting of Pluronic micelles to the tumors; the degree of targeting was enhanced by a local tumor sonication. (Gao, et al., 2004)

According to literature review, there were many studies investigate the relationship between tumor growth and VEGF but there were no study investigate the effect of physiotherapy ultrasonic on VEGF. In the present study the Non parametric Mann whiteny test revealed that there were significant difference in VEGF expression between the treated and control groups in the second experiment, where z was -2.793 and p value was 0.005 at level of significance 0.05.

This result would be in line with (Bernard et al., 2009) who examined the effects of low-power ultrasound, the anti-cancer drug cisplatin, and their combined application in two lines of human ovarian carcinoma cells, A2780 and A2780cis. It was shown that a combined effect of ultrasound and cisplatin was more effective than that of ultrasound or cisplatin alone. This study also was performed in vitro and didn't study the effect of ultrasound on tumor blood vessels.

The antivascular effect of ultrasound was examined by (Wood et al 2007) this study was aimed to determine if physiotherapy ultrasound (US) affected the fragile and leaky angiogenic blood vessels in a tumor. In 22 C3HV/HeN mice, a subcutaneous melanoma (K1735[22]) was insonated (1, 2 or 3 min) with continuous 1-MHz low-intensity (spatial-average temporal-average = 2.28 W cm^{-2}), physiotherapy US. Contrast-enhanced power Doppler US observations were made and histogram analyses of the images were performed. A linear regression analysis showed that each min of insonation led to a 25% reduction in tumor vascularity; the antivascular activity persisted for 24 h. Histology demonstrated disruption of vascular walls and tumor cell death in areas of vascular congestion and thrombosis. Physiotherapy US particularly targeted the vascular structures, and the effects on tumor cells appeared to be secondary to the resultant ischemia. This study found that there was an effect on tumor blood vessels, but didn't examine the VEGF expression changes in response to the ultrasonic.

This result was supported by (Wood et al., 2009) who concluded that Insonation of the tumor at a higher frequency amplified the heating of the neoplasm and led to greater disruption of the tumor vasculature; 3-MHz ultrasound was more efficacious than 1 MHz for antivascular cancer therapy.

Studying the effect of ultrasonic on VEGF was very limited , and there was little amount of study examined the effect of hyperthermia alone on the VEGF. Kong et al., (2001) concluded that the application and scheduling of hyperthermia combined with other therapeutics (*e.g.,* liposomes, antibodies, and viral vectors) was effective for the treatment of cancer. The result of this study can illustrate the mechanism under which the ultrasonic can produce reduction of VEGF expression as the ultrasonic thermal effect can produce the hyperthermia which alone can produce significant reduction of VEGF expression.

Since all previous research had focused on the effect of ultrasonic on tumor growth and didn't concentrate on the time of intervention with ultrasonic, the current study concentrates on the investigation of the time of intervention with ultrasonic. In this study the results shown that the independent t-test revealed that there were significant difference in the tumor cell size between treated groups in 1st and 2nd experimental studies, where t was 13.141 and p value was 0.000 and df equal 14.895 at level of significance 0.05. Also the independent t-test revealed that there were no significant difference in tumor necrotic area between the treated and control groups in the 1st experiment in which the treatment started after 4 weeks of induction of tumor , where t was -1.117 and p value was 0.286 and df 11.659 at level of significance 0.05 . And there were significance difference in the tumor necrotic area between treated groups in 1st and 2nd experimental studies, where t was 14.479 and p value was 0.000 at level of significance 0.05. Also there were significance difference VEGF between treated groups in 1st experiment and 2nd experimental studies, where z were -3.148 and p value were 0.002 respectively at level of significance 0.05. This result shown that low intensity physiotherapy ultrasonic antitumor effect were highly enhanced and confirmed when applied early as much as possible. This may be due to recent tumor vasculature characteristics.

This results were supported by many studies that investigate the role of time of intervention in cancer treatment. Fadel et al., (2005) investigate value for the inhibiting resonance frequency (4.5 Hz) of electromagnetic radiation for solid tumor implanted in mice. He concluded that early treatment of the tumor by extremely low frequency electromagnetic field (ELF-EMF) gave better results than delayed treatments.

This were well illustrated in study of (Hrazdira., 1998) which aimed to determine which phase of the cell cycle is most sensitive to ultrasonic action; and whether and in which way ultrasound can influence components of the cytoskeleton. HeLa cell monolayers grown on glass cover-slips in DEM medium were used in all experiments. For proliferation studies, the cell monolayers were trypsinized and the cells were resuspended in fresh medium. The structure of the cytoskeleton was studied by means of the indirect immunofluorescence method. The cells were sonicated by a continuous wave ultrasound of 0.8 MHz at low SA intensities (0.05, 0.1 and 0.5 W/cm^2) for 5 and 10 min. The analysis of proliferation demonstrated that cells were most sensitive when undergoing M- and S-phases of the cell cycle. The ultrasonically induced disassembly of cytoskeleton components was most marked in microtubules and microfilaments due to depolymerization of basic proteins (tubulin and actin). The reaction of intermediate filaments was distinctly weaker. And concluded that in-vitro treatment of tumor cells with low intensity ultrasound resulted in partial

inhibition of proliferation as well as in partial disassembly of all components of the cytoskeleton. Ultrasonically induced changes of the cytoskeleton seem to be non-specific and temporary.

In the current study the ultrastractural observations illustrated the following change in the tumor cell, 1) decrease in the mitotic division and scanty numbers of tumor giant cells. 2) The viability of the tumor cells were decreased because of, decreasing in the vasculature either in the stroma or in-between the muscle bundles .3) There were necrosis and vasculitis in the blood vessels accompanied with edema around the vessels and in-between the muscle bundles.4) Muscle bundles showed Zenker's necrosis in some areas, this ultrastracural changes in the tumor cell was attributed to the heating or mechanical effect of ultrasonic or both of them.

This was in line with study of (Bunte et al., 2006) who concluded that the ultrasonic application could produce, vascular channels dilation, with interstitial edema, and necrosis of neoplastic cells in examined mice which were inculated with melanoma.

Ultrastructural observation in the form of vascular congestion, and inflammation were reported in murine thigh tumors heated to 44°C in a water bath for 30 min (Nishimura et al., 1988), and also in rat gliomas treated with combretastatin A-4 (Eikesdal et al., 2001). Another study had shown that the maturity of blood vessels within a tumor varies and that the more immature vessels were more sensitive to anti vascular therapies (Gee et al., 2003).

Because the morphologic changes observed in this study were similar to those caused by hyperthermia and combretastatin (not involving heat), it is not yet feasible to determine if the observed anti tumor activity was of thermal origin only. Other bioeffects, including cavitation, radiation pressure and other nonlinear effects, may also have a role in disrupting tumor vascularity and reducing its cell numbers (Barnett et al., 1997, 2000)

Ultrasonic was also likely to have been reflected from the rear skin surface and produce heat that may have contributed to the heating of the tumor. Whether the increase in temperature of the tumor was caused by reflected or transmitted US was not known and requires further investigation. Absorption of the US beam at a bone-soft tissue interface would also have a heating effect and resulted in the observed localized muscle necrosis adjacent to the tumor. So the finding of this study could not be assumed to be due to the thermal or mechanical effect alone of ultrasonic, but may be due to both of them.

In summary for anticancer therapy to be effective, the tumor cell management and the endothelial cells of the vascular structures within the tumor management should be combined together (Folkman. , 2001). In present study the results showed that low-intensity US particularly targeted

the tumor growth by acting on the two mechanisms the tumor cell size reduction and acting on the VEGF by producing reduction of their expression which may be the cause for tumor cell size reduction. So the ultrasonic can act on both compartment (the tumor cell size and the endothelial cells of the vascular structures within the tumor). Such a combination of therapies may result in a more comprehensive and effective cancer therapy than if either treatment was used alone.

Conclusion

Within the limitations of this study, The physiotherapy ultrasonic can retard tumor growth and can decrease the tumor cell size, tumor necrotic area and VEGF expression when applied early as much as possible according to the current study protocol. Also for further studies should be undertaken to examine the effect of physiotherapy ultrasonic on different types of tumor. Further research should be undertaken to examine the effect of physiotherapy ultrasonic on similar types of tumor in human being treatment, and retardation of tumor growth in human.

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