LIPOSOME ENCAPSULATED HEMOGLOBIN ENHANCES THE RADIATION THERAPY EFFECT: HISTOPATHOLOGICAL STUDY

M.I. EL GOHARY, S.A. ALI**#, M.M. ISMAIL*, M.H. GABER***

*Physics Department, Faculty of Science, Al-Azhar University, 11787 Nasr City, Cairo, Egypt
**Biophysics Department, Faculty of Science, Cairo University, Giza, Egypt
#e-mail: saidtaha@aucegypt.edu

Abstract. Liposomes encapsulated hemoglobins are considered as artificial oxygen carriers or blood substitutes that play an important role in many diseases which need an excess of oxygen supply such as tumors. In the present study, the preparation and characterization of liposomes encapsulating bovine hemoglobin (LEBH) as potential oxygen carriers for enhancing radiotherapy treatment are carried out. First, LEBH was synthesized from 1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine by thin film hydration method. The sample of LEBH was characterized using dynamic light scattering (DLS), differential scanning calorimetry (DSC), and transmission electron microscope (TEM). Diameter of liposomes containing hemoglobin was found to be approximately 289.5±5.02 nm as measured by (DLS) and (TEM). DSC scan of LEBH sample showed mean phase transition temperature at 71.94 °C indicating the high stability for LEBH at high temperatures through *in vivo* studies. Histopathological studies through the morphological changes in tumor sections stained with hematoxylin and eosin under light microscope indicated the effectiveness of the combined effect of LEBH with radiation therapy on tumor cells through the extent of induced cytotoxicity, including inflammation, cell apoptosis, cell necrosis, steatosis. The results indicated that LEBH greatly enhanced radiation effect on tumors.

Key words: liposomes encapsulated bovine hemoglobin (LEBH), blood substitute, artificial oxygen carrier, radiotherapy.

INTRODUCTION

A new trend has recently emerged in the HBOCs field, which focuses on the development of nanobiotechnology for encapsulation hemoglobin into nanosized carriers and has shown promise in research and clinical trials [6]. They are types of encapsulated hemoglobin-based oxygen carriers (HBOCs) in nanosize, that Hb molecules are encapsulated with different materials and designed to mimic the features of red blood cells. Hb liposomes are of the most important HBOC

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formulations. Hb liposomes are artificially prepared Hb encapsulation vesicles made of a lipid bilayer. The bilayer is composed of phospholipids, cholesterol, fatty acids, and so on [9, 42]. Hb liposomes with the sizes of 250 nm have received good evaluations in terms of safety and oxygen-carrying capacity in animals, and can even eliminate vasoconstriction [33, 34]. Liposome-encapsulated hemoglobin (LEH) has been developed as a promising artificial oxygen (O_2) carrier [6, 31, 38]. Because of not only its bloodlike O_2-carrying capacity and O_2 affinity but also its long shelf life with stability at room temperature, compatibility with all blood types, and safety in infection or immune reactions.

Hypoxia in the tumor is considered to be one of the main mechanisms of the failure of cancer treatment, as radiosensitivity and/or chemosensitivity of hypoxic tumor cells is lower than in normoxic cells [3]. Mounting evidence has disclosed that hypoxia protects tumor cells against cytotoxic actions of radiation therapy or chemotherapeutic agents [43, 44]. As this is the mechanism of therapeutic resistance of the tumor, there have been various attempts to modify tumor hypoxia to enhance the sensitivity of these therapies, including hyperbaric oxygen therapy (HBO) [28], oxygen (O_2)-mimetic chemical radiosensitizer [24, 28], perfluorochemicals (PFCs) [37, 39], modified hemoglobin [40], and hypoxic cytotoxin and mild hyperthermia [22]. These various methods and/or medicines as hypoxic cell modifiers have been tested in preclinical experiments and clinical trials. A recent meta-analysis of these clinical trials [29] suggests that HBO has been most effective, showing the highest odds ratio, as a hypoxic modifier.

Therefore, this work examines the preparation and characterization of liposomes encapsulating bovine hemoglobin as potential oxygen carriers to increase the efficiency of radiation effect on tumors. The combined effect of LEBH and radiation therapy (RT) can be a new approach for an efficient tumor treatment planning. To assess the feasibility of using LEBH as oxygen transporters, size distribution, stability, encapsulation efficiency, morphology and shape of these LEBH dispersions were examined in addition to some histological studies on tumor cells in mice.

MATERIALS AND METHODS

MATERIALS

1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) lipid was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and bovine hemoglobin (Powder, 64.5 kDa) was purchased from Alfa Easer GmbH & Co KG (Karlsruhe, Germany). All other reagents and solvents were of analytical grade (Sigma, Egypt) and were used without further purification.

PREPARATION OF LARGE UNILAMELLAR LIPOSOMES

Liposomes were prepared by thin film hydration method [2]. 50 mg of dry phospholipids were dissolved in chloroform in a rounded bottom flask. The organic solvent was evaporated under vacuum leaving a uniform thin film on the inner walls of the flask. The film was hydrated with a solution of bovine hemoglobin (6 g/dL) which is dissolved in 10 mM phosphate buffer saline (PBS), pH = 7.2. Multilamellar liposomes were formed by constant vortexing for 4 min. The suspension was afterwards sonicated in a water bath sonicator at 50±1 °C for 30 min [36] resulting a large unilamellar vesicles (LUV) suspension. The suspension was then ultra-centrifuged (Optima Max-E, Ultra Centrifuge, Beckman Coulter, Pasadena, CA) at 50,000 rpm at 4 °C for 30 min [12] to recover and eliminate the top layer containing non-encapsulated Hb. The purified sediment was then diluted using PBS (pH = 7.2) obtaining Hb encapsulating LUV which were used directly for characterization study.

LIPOSOME CHARACTERIZATION

Particle size distribution

The mean diameter of the LEBH and the polydispersity (% Pd) of the distribution were determined by dynamic light scattering (DLS) using a Zetasizer Nanoseries (Malvern Instruments, UK). The refractive index and viscosity of pure water were used as calculation parameters and each sample was measured 3 times using the unimodal model for size distribution. The results values of mean diameter of the liposomes and polydispersity are expressed as a mean ± SD. Size distribution was recorded as a function of volume at 23 °C.

Differential scanning calorimeter

DSC measurements of bovine hemoglobin and LEBH samples using Shimadzu DSC-50 (Shimadzu, Japan) were recorded to detect any change in phase transition temperature (enthalpy and entropy were calculated from the graph). The sample is placed in an aluminum pan as a cell for the sample and for a reference, using nitrogen as an inert atmosphere. Each scan was recorded from 20 °C to 100 °C at a scan rate of 10 °C/min.

Transmission electron microscopy

LEBH size was analyzed by negative stain electron microscopy using a JEM 1230 electron microscope (Jeol, Tokyo, Japan). A drop of LEBH suspension was applied to copper coated with carbon grid and the excess sample was drawn off

with filter paper. An aqueous solution of ammonium molybdate (1% w/v) was used as a negative staining agent. Staining was followed by a 2 min wait at room temperature, removal of the excess solution with a filter paper, and then examination under the electron microscope.

Determination of encapsulation efficiency

The encapsulation efficiency (*EE*) was defined as the fraction of hemoglobin incorporated into LUV relative to the total amount of the hemoglobin and is calculated with equation (1) [11]:

$$EE = \frac{t_{\mathrm{Hb}} - f_{\mathrm{Hb}}}{t_{\mathrm{Hb}}} \tag{1}$$

where t_{Hb} is the total content of Hb in the solution used to prepare the LUV (6 g/dL) and f_{Hb} is the free (non-encapsulated) hemoglobin content in the supernatant after ultracentrifugation.

The concentration of the non-encapsulated hemoglobin was determined by spectrophotometry using Drabkin's reagent (eBioscience, Europe) according to the producer's protocol. The procedure for measuring f_{Hb} was adopted from the method of Zijlstra and Buursma [47]. The amount of hemoglobin was determined by monitoring absorbance at 540 nm [41]. A spectral scan was employed using UV-visible spectrophotometer (Jenway 6405, Japan) with matched 1 cm quartz cells. The concentration of non-encapsulated hemoglobin was calculated according to the equation (2):

$$f_{\mathrm{Hb}} = A \times 33.77 \tag{2}$$

where A is the specimen absorbance and f_{Hb} results in g/dL.

CELL CULTURE AND TUMOR MODEL

Ehrlich ascites tumor was chosen as a rapidly growing experimental tumor model [17] where various experimental designs for anticancer agents can be applied. Ehrlich ascites carcinoma cells (1×10^6 cells), obtained from National Cancer Institute "NCI" – Cairo University, were intraperitoneally (IP) injected into female Swiss albino mice as a donor. Ascites fluid was collected from the donor mice on the 7th day after injection. The Ehrlich cells were washed twice and then re-suspended in 5 mL saline (5×10^6 viable cells/mL). Male Swiss albino mice (obtained from animal house at National Cancer Institute, Cairo University) were then injected subcutaneously with 0.2 mL of suspension in their right flanks where the tumors were developed in a single and solid form. A palpable solid tumor mass (about $\geq$ 100 mm^3) was developed within 12 days [10, 15]. Tumor growth was monitored post-inoculation until the desired volume was reached.

In vivo studies were done on a total of 40 male Swiss albino mice 8 weeks old, weighing 22–25 g. Each group (10 animals) were housed in plastic cages in a well-ventilated room (26±2 ºC) with a relative humidity of (40±2%), 12 h light / 12 h dark cycle and free access to feed and water. All animal procedures and care were performed using guidelines for the Care and Use of Laboratory Animals [27] and approved by the Animal Ethics Committee at Cairo University.

The animals were divided randomly into 4 equal groups of 10 mice each as follows: (1) the control untreated group, (2) the LEBH injected group (mice injected with LEBH only), (3) the Radiotherapy (RT) group (mice received radiation dose only) and (4) the combined LEBH and RT treatment group (mice received radiation dose after administration with LEBH). Animals were anesthetized with thiopental sodium. A dose of 40 mg/kg was administered (IP) to each mouse. The dose of LEBH administered to each mouse in group 2 and 4 was equivalent to 10 mg/kg. LEBH saline suspension was sonicated for 10 min before injection to get a homogeneous suspension then directly intravenously injected into the tumor interstitial coordinates using insulin syringe. Two groups (3 and 4) of mice were specified to receive a radiation dose (20 Gy, single shot). Group (3) were restrained in acrylic holders and received local irradiation to the tumors at a dose rate of 1.2 Gy/min using 4MV X-rays by linear accelerator (Clinac 600C, Varian Medical Systems, Palo Alto, CA, USA) under room air. Then, group (4) received 10 mL/kg of LEBH 30 min before the same radiation dose as group (3) in the same circumstances [25].

HISTOPATHOLOGICAL EXAMINATION AND MORPHOMETRIC ANALYSIS

Tumor slices from animals of each group were fixed instantaneously in 10% formal saline for 24 h. The specimens were washed in tap water, dehydrated in ascending grades of ethanol, cleared in xylene, embedded in paraffin wax (melting point 55–60 °C). Sections of 6 μm thicknesses were prepared and stained with Haematoxylin and Eosin for histopathological and histomorphometric studies. These sections were examined under a light photo-microscope Leica Q 500 IW (Leica DM LB, Cambridge, UK) with position captors and a CCD video camera module N 50 (JVC TK-C 1380, Victor Company of Japan LT, Japan). Images were captured at magnifications of x100 and x400 s. The cytoplasm stained shades of pink and red and the nuclei gave blue color.

The morphometric measurements were done using Leica Quin 500 Image Analyzer (LEICA Imaging systems Ltd, UK) in Pathology Department, National Research Centre, Cairo. The morphometric measurements were carried out with optical magnification of x200 on hematoxylin and eosin stained sections for necrotic areas and viable areas. Areas (in μm^2) of necrotic and viable cells were measured in five random fields per group and the data obtained as mean area ± standard error (SE).

RESULTS AND DISCUSSION

LEBH CHARACTERIZATION

LEBH size distribution

The DSL size distribution of LEBH, as a function of volume at 23 °C, was unimodal and relatively narrow (Fig. 1). The calculated mean diameter is 289.5±5.02 nm. Size is of great importance when one wishes to use these particle-systems in a body to supply increased (or optimized) oxygen concentration. Rapid extravasion from the body by the reticulo-endothelial system (RES) and immunogenic effects are only a few of the problems foreign particles encounter in the body [1, 4, 8, 14, 16, 21, 32, 45, 46]. The mean value of polydispersity (% Pd) of 3 samples of LEBH dispersions was 34.16±0.57%. Therefore, LEBH dispersions can be considered as polydisperse. As a rule of thumb, samples with % Pd ≤ 20% are considered monodisperse [5].

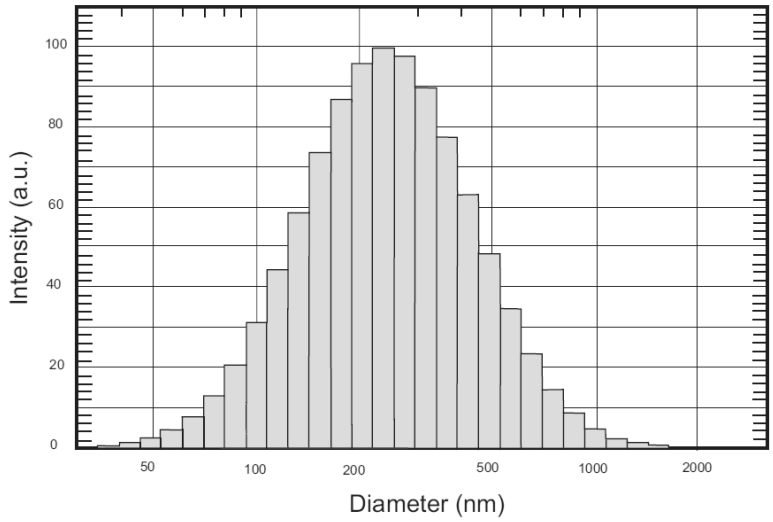


Fig. 1. Size distribution for LEBH.

Transmission electron microscopy analyses

TEM images of LEH are presented in Fig. 2. In TEM images, the characteristic aspects of large unilamellar vesicles (LUV) can be observed [18]. Large unilamellar vesicles are liposomal structures exhibiting a rather empty core that can be used to encapsulate different molecules, such as drugs or proteins [19].

TEM images revealed that LEBH have a diameter in the range of 150–300 nm (50 liposomes scored on TEM images as in Fig. 2A). These results are in good agreement with the ones obtained from dynamic light scattering analyses, while

most nanoparticles-loaded liposomes were spherical shapes and they have less aggregation as shown in Fig. 2B. There is difference between the liposome that encapsulated bovine hemoglobin and other non encapsulated hemoglobin as can be observed in Fig. 2C (the darker colour).

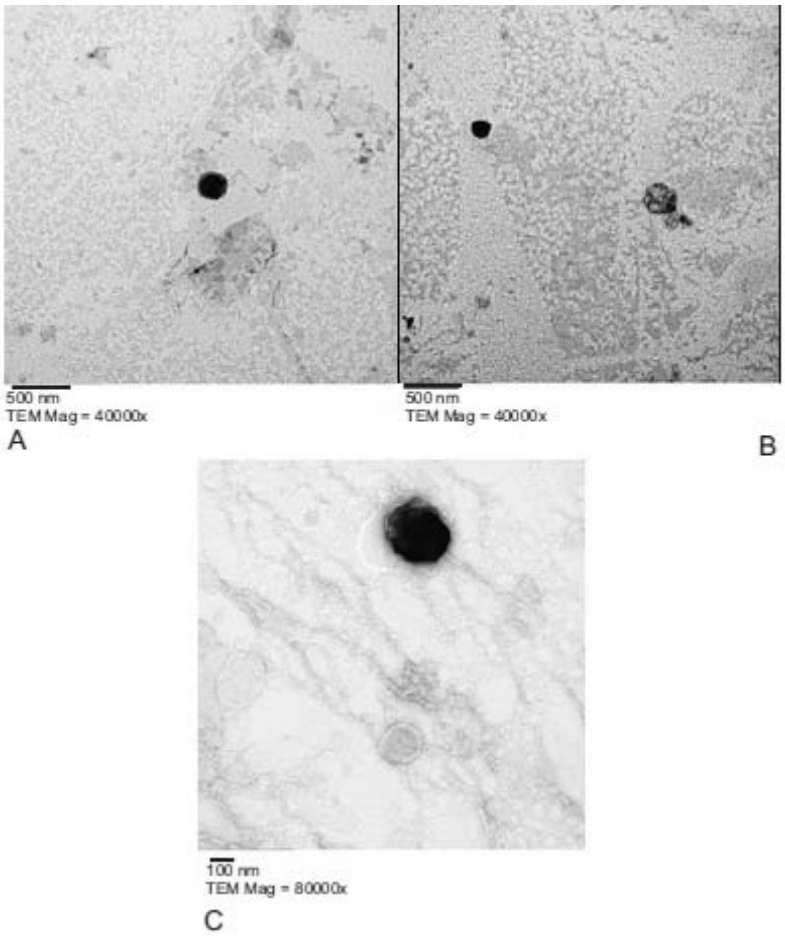


Fig. 2. TEM images revealing shape, structure, and sizes of liposomes encapsulating hemoglobin molecules**.**

Differential scanning calorimetry

DSC measurements were recorded to bovine hemoglobin and LEBH (Fig. 3). DSC scan of LEBH sample shows mean phase transition temperature at 71.94 ^{o}C, an enthalpy change per gram (ΔH) of –1.75 kJ/g and an entropy change (ΔS) of 0.00507 kJ/g·K while pre-transition temperature has disappeared. The higher value of phase transition temperature 71.94 ^{o}C than of DPPC (41 °C) is due to the

presence of bovine hemoglobin which has phase transition temperature (83 ^{o}C) [35]. The higher value of phase transition temperature gives stability for LEBH in high temperatures through *in vivo* studies.

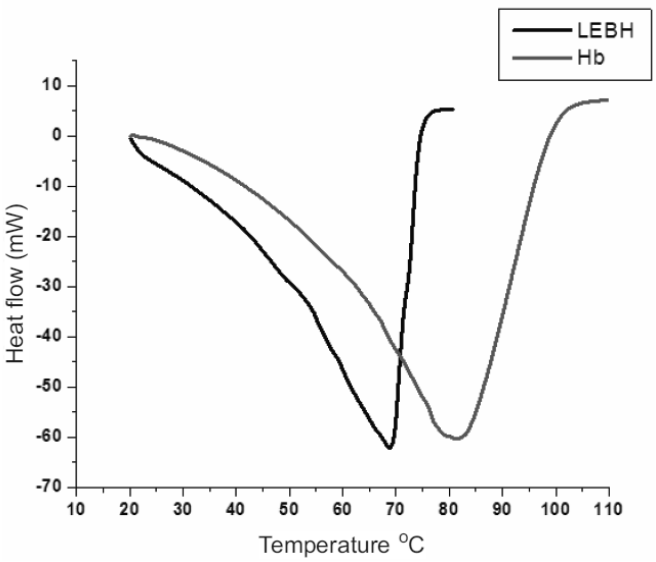


Fig. 3. DSC scan for bovine hemoglobin (Hb) and LEBH.

Determination of encapsulation efficiency

EE of LEBH was derived by eq. (1). Calculated *EE* from three repetitions of experiments reaches 55±0.32% of the total initial concentration (6 g/dL) which is a good efficiency for LEBH.

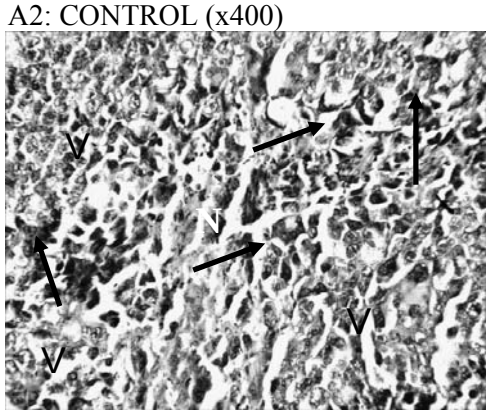
HISTOPATHOLOGICAL EXAMINATION AND MORPHOMETRIC ANALYSIS

Histopathological examination and morphometric analysis were done for the four groups to determine the cell viability and to quantify and compare its amount in different groups. Examination of the entire tumor sections for the various groups revealed marked differences in cellular features with various degrees in tumor cell necrosis.

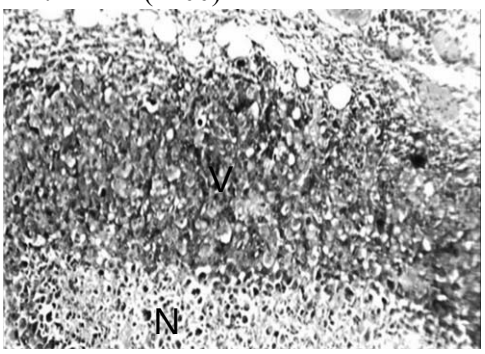
In case of control group (Fig. 4A), the section is characterized by neoplasia, nuclear and cytoplasmic anaplasia, anisocytosis, cellular gigantism and increased nuclear/cytoplasmic ratio. Fig. 4A1 shows higher chromatophilic nuclei, undifferentiated tumor cells with bizarre size and shape, cell proliferation and few spontaneous necrosis. Fig. 4A2 shows entirely viable tumors formed of sheets of polymorphic hyperchromatic tumor cells with irregular giant (cellular gigantism) and few spontaneous necrosis.

A1: CONTROL(x100)

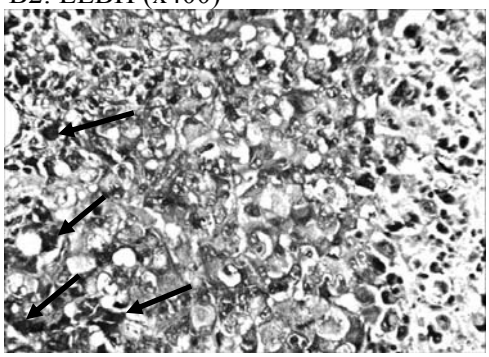
A2: CONTROL (x400)



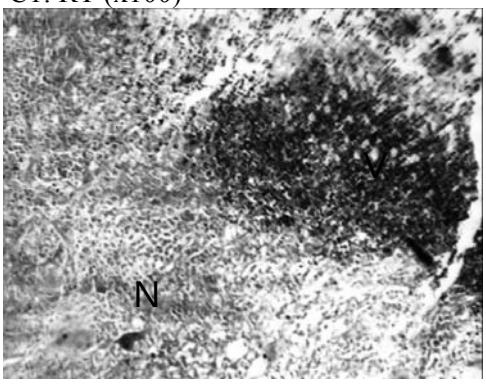
B1: LEBH (x100)



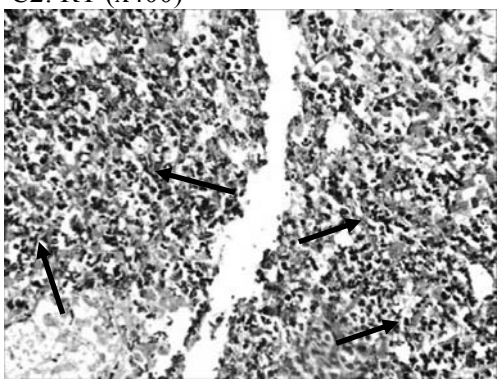
B2: LEBH (x400)



C1: RT (x100)



C2: RT (x400)



D1: LEBH+RT (x100) D2: LEBH+RT (x400)

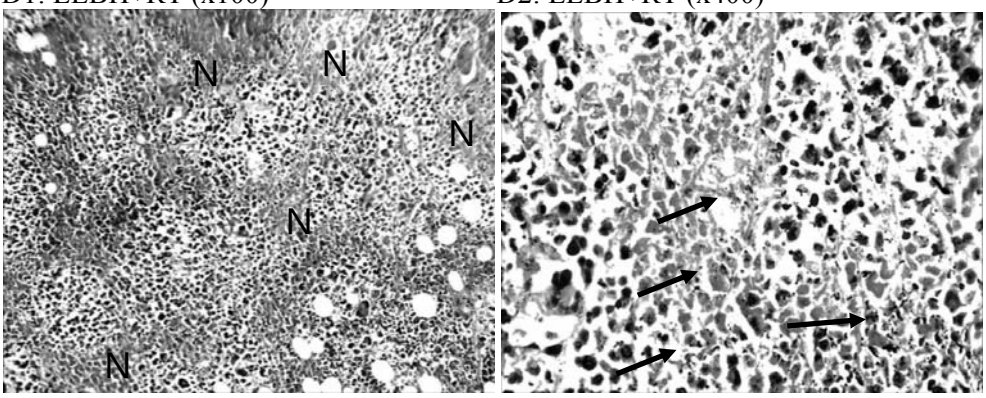


Fig. 4. Histopathological analysis of mice with solid Ehrlich ascites tumor with two different magnifications (×100 and ×400). A1 and A2 for control group (V & N denote viable and necrosis cells respectively, but arrows in A2 denote giant cells). B1 and B2 for LEBH group – mice treated with LEH only – arrows in B2 denote cellular gigantism. C1 and C2 for RT group, mice exposed only to radiation, arrows in C2 denote pyknosis. D1 and D2 for LEBH+RT group, arrows in D2 denote karyorrhexis.

In case of LEBH treated group, Fig. 4B1 presents a large area of neoplastic cells with small necrosis areas. In Fig. 4B2 appears cellular gigantism, nuclear and cytoplasmic anaplasia, anisocytosis, increased nuclear/cytoplasmic ratio, but lower than in the control group (Fig. 4A2), in addition to some necrosis features with eosinophilic cytoplasm.

RT group sections have large eosinophilic necrotic area with differentiated cells and an island of higher basophilic tumor cells (Fig. 4C1). Also, Fig. 4C2 shows cell necrosis containing numerous basophilic shrinking nuclei (pyknosis), few fragmentations of the nuclei (karyorrhexis) distributed irregularly throughout the cytoplasm.

In the case of RT+LEBH group the section is abundant with necrosis containing fragmentation of the nuclei (karyorrhexis) (Fig. 4D1). In higher magnification the section shows many eosinophilic tumor cells, eosinophilic cytoplasm, several destructive fragmentations of the nuclei (karyorrhexis) whereby its chromatin distributed irregularly throughout the cytoplasm (Fig. 4D2).

The quantitative analysis which was done by morphometric analysis as shown in Fig. 5 sustains the qualitative analysis done by histopathological examination.

Based on the above results, the massive area of cell necrosis demonstrated in histological sections and morphometric analysis of the same group (Fig. 4D) and (Fig. 5A and B) verified the high efficiency of the combined effect of radiation dose and LEBH in destruction of tumor cells under the current treatment protocol.

Underdevelopment of the vascular system makes cancer tissue hypoxic and resistant to radiotherapy. Therefore, delivering O_2 to malignant tissue may intensify

radiation therapy. Hypoxia influences outcome not only through radioresistance, but also through promoting more aggressive tumor phenotypes. This is supported by the increased risk of nodal and distant metastases in hypoxic tumors [13, 30].

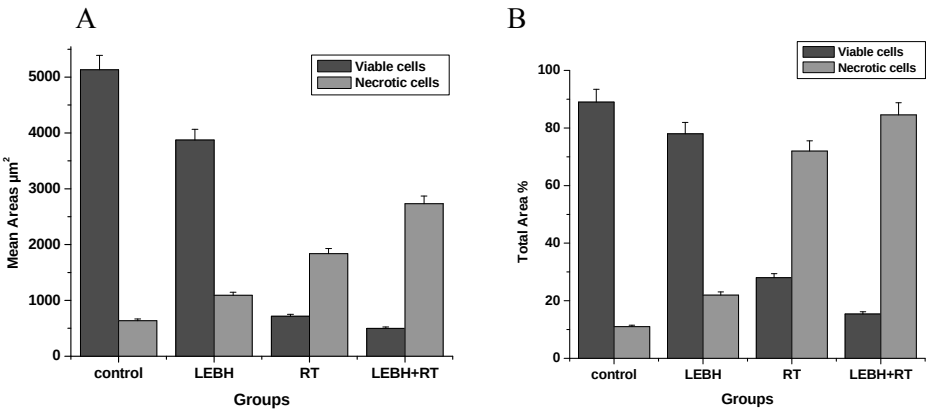


Fig. 5. Morphometric analysis for the investigated groups (five fields per group), a: shows the mean areas±SE of viable and necrotic cells in μm^2 for different groups; b: shows the percentage of the total area covered by viable and necrotic cells for different groups. (Bars denote to standard error).

Recent studies in animal as well as in human tumors have confirmed the presence of significant hypoxic areas [7, 20, 23, 43, 44]. This is important, as the presence of such hypoxic but clonogenic cells in solid tumors consequently takes them to the G1/S phase of the cell cycle, which is resistant to radiation therapy as well as some chemotherapy regimens [25].

As the increased delivery of O_2 to tumor hypoxic cells can be a way of enhancing radiation therapy, the use of PFC emulsions [37, 39] and cell-free hemoglobin (68) with oxygen or carbogen breathing showed positive results [37, 39, 40]. Thus, tumor oxygenation may improve the effects of various antitumor agents on tumor growth, local invasion, and distant metastasis [26].

CONCLUSION

In the current study, we tested the hypothesis that LBEH may enable O_2 delivery to hypoxic cells and thereby enhance the antitumor effect of radiation as shown from the histopathological analysis.

Liposome-encapsulated hemoglobin with high O_2 affinity was effective for tumor oxygenation and enhancing radiation therapy against tumor growth in mice (data not shown in present work). The results suggested that LEBH may have the potential of synergistic action with radiotherapy based on the tumor oxygenation effect of LEBH. Further studies are necessary to understand whether mechanisms other than the target O_2 delivery hypothesis may be involved.

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