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Hematological Indices for Mice Bearing Ehrlich Tumor When Treated by Liposomes Encapsulated Hemoglobin and Irradiation

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TUMOR vasculature frequently fails to supply sufficient levels of oxygen to tumor tissue resulting in radioresistant tumor. To improve therapeutic outcome radiotherapy (RT) may be combined with liposomes encapsulated bovine hemoglobin (LEBH) as an artificial oxygen carrier. Hematological parameters and lipid peroxidation (LPx) can be indicators for tumor prediction and treatment. The aim of the present work is to considered as investigate and compare the effects of combination between radiotherapy and liposome encapsulated bovine hemoglobin as artificial oxygen carriers through hematological parameters and lipid peroxidation (LPx). This study was done through five groups of Male Swiss albino mice's bearing Ehrlich Ascites Carcinoma (EAC) .

Blood samples were collected from mices of different groups into a tube containing heparin (heparinized tube).

Erythrogram, (LEBH+RT) group shows promising values in most measurements comparable to (RT) group and control group. Meanwhile, (Control) group shows low erythrogram values if compared to (Normal) group, but (LEH) shows the highest erythrogram values when compared to all investigated groups.

The hematological studies suggest that LEBH may have the potential of synergistic action with radiotherapy based on the tumor oxygenation effect of LEBH.

Keywords: Liposomes, Hypoxic tumor, Radiotherapy, Ehrlich ascites carcinoma (EAC), Erythrogram, Lipid peroxidation (LPx).

Hemoglobin (Hb) has been encapsulated using lipid bilayer membranes to form Hb vesicles (HbV), in order to produce a blood like hemoglobin-based Oxygen carriers (HBOC) where the oxygen carrying Hb is not dissolved in plasma[1, 2].

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Artificial oxygen carriers lack blood types, are free of potential infectious pathogens, and can be stored much longer than red blood cells (RBCs)[3].

Hemoglobin (Hb)-based oxygen carriers are classified into acellular chemically modified Hbs and encapsulated Hbs [4, 5]. The cellular structure of HbV has characteristics that resemble those of RBCs. It has lipid bilayer membranes that prevent the direct contact of hemoglobin with blood components and the endothelial lining, thus shielding the side effects of molecular hemoglobin[6, 7]. HbV particles are eventually captured by the phagocytes in the reticuloendothelial system and are metabolized through existing physiological pathways[8–10].

Mounting evidence has disclosed that hypoxia protects tumor cells against cytotoxic actions of radiation therapy or chemotherapeutic agents [11, 12]. 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) (3), oxygen (O_2)-mimetic chemical radiosensitizer[13,14], perfluorochemicals (PFCs)[15,16], modified hemoglobin[17], and hypoxic cytotoxin and mild hyperthermia[18]. 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[19] suggests that HBO has been most effective, showing the highest odds ratio, as a hypoxic modifier.

Hematological profile is measured all over the world to estimate general health, because it is a reliable indicator and is a simple, fast and cost-effective test.[20] In addition, the hematological profile is considered to be one of the factors affecting pregnancy and its outcome[21,22] .

Hematological parameters are a very useful tool in alarming by several tumors. The white blood cell count (total and differentials) and packed cell volume predict disease severity and mortality risk[23-26]. For example, elevated WBCs counts predict a worse prognosis in patients with cancer or coronary artery disease and anemia predicts increased risk of death of cancer patients with heart failure[27-30].

Many conditions will result in increase or decrease in the cell populations. Some of these conditions may require treatment, while others will resolve on their own. Some diseases, such as cancer (and chemotherapy treatment), can affect bone marrow production of cells, increasing the production of one cell at the expense of others or decreasing overall cell production. Some medications can decrease WBCs counts while some vitamin and mineral deficiencies can cause anemia. The CBC test may be ordered on a regular basis to monitor these conditions and drug treatments.

Cancer is considered as a multifactor disease, where oxidative stress may be involved in both initiation and promotion of multi-step carcinogenesis. Reactive

oxygen species (ROS) can accelerate DNA damage, stimulate pro-carcinogenesis, initiate lipid peroxidation (LPx), inactivate antioxidant enzyme systems and thus can modulate the expression of genes related to tumor promotion[31,32].

Excessive production of free radicals cause macromolecular damage and can induce lipid peroxidation *in vivo*[33]. Malondialdehyde (MDA) the end product of lipid peroxidation (LPx), are seen to be higher in cancer tissues than in non-diseased organ[34].

Therefore, this work examines liposomes encapsulating bovine hemoglobin as potential oxygen carriers to increase the efficiency of radiation effect on tumors where oxygen dept in tumor area is considered one of the primary factors of tumor resistance to radiation effect. To assess the feasibility of using LEBH as oxygen transporters, hematological parameters and lipid peroxidation were examined on Mice Bearing Ehrlich Ascites Carcinoma.

Material and Methods

Characteristics of liposome encapsulated hemoglobin

The characteristics of liposomes encapsulated bovine hemoglobin (LEBH) are very important to be determined before *in vivo* study, thus the mean particle diameter, phase transition temperature, hemoglobin concentration, encapsulation efficiency, pH and relative osmolarity are carried out (work is not presented here).

Cell culture and tumor model

Ehrlich asites tumor was chosen as a rapidly growing experimental tumor model[35] where various experimental designs for anticancer agents can be applied. Ehrlich ascites carcinomas cells (1×10^6 cells), obtained from National Cancer Institute "NCI" – Cairo University were intraperitoneally injected into female Swiss albino mice as a donor. Ascites fluid was collected from the donor mice on the 7^{th} day after injection. The Ehrlich cells were washed twice and then re-suspended in 0.05 saline (5×10^6 viable cells). Male Swiss albino mices (obtained from animal house at National Cancer Institute, Cairo University) were then injected subcutaneously in their right flanks where the tumors were developed in a single and solid form. Apalpable solid tumor mass (about $\geq$100 mm^3) was developed within 12 days[36, 37]. Tumor growth was monitored post-inoculation until the desired volume was reached.

Animal care

In vivo studies were done on a total of 50 male Swiss albino mice 8 weeks old, weighing 22–25 g, purchased from animal house at National Cancer Institute, Cairo University. Each group (Ten animals) were housed in plastic cages in a well-ventilated room (26 ± 2 °C) 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 [38] and approved by the Animal Ethics Committee at Cairo University.

Classification of animals

In vivo studies are done on a total of 50 male Swiss albino mice. The experimental animals are divided randomly into 5 equal groups of ten mice each as follows: Group (1): The Normal (mice neither have tumor nor receive treatment) group. Group (2): The control (untreated) group. Group (3): LEBH injected group (mice injected with LEBH only). Group (4): Radiotherapy (RT) group (mice receive radiation dose only). Group (5): Combined treatment of LEBH and RT (mice receive radiation dose after administration with LEBH). Animals were anesthetized with thiopental sodium (40 mg/kg) was administered intraperitoneally (IP) to each mouse, and the dose of LEBH administrated to each mouse in group 3 and 5 was equivalent to 10 mg/kg which were suspended in saline and sonicated for 10 min before injection to get a homogenous suspension then directly injected intravenous (IV) into the tumor interstitial coordinates using insulin syringe. Two groups (4 and 5) of mices were specified to receive radiation dose (20-Gy, single shot). Group (4) 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 (5) received 10 ml/kg of LEBH 30 min before the same radiation dose as group (4) at the same circumstances [39].

Blood sampling

A blood sample is collected from mices of different groups (after 21 days of starting treatment) using a dry, sterile disposable syringe and needle. The blood is dispensed into heparinzed tubes. The specimens were labeled with identification number.

Laboratory analysis

Blood count was performed using a SK9000 Hematology auto Analyzer (Labomed, Inc, USA). Standardization, calibration of the instrument, and processing of the samples were done according to the manufacturer's instructions.

Procedures

Each blood sample is mixed well and then approximately 20 µL is aspirate by allowing the analyzer's sampling probe into the blood sample and depressing the start button. Results of the analysis are displayed after about 30 seconds, after which the analyzer generated a paper copy of the results on thermal printing paper.

Lipid peroxidation (LPx) estimation

Lipid peroxidation (LPx) in blood is observe by the formation of malonaldialdehyde (MDA) as one of the main products of lipid peroxidation using Thiobarbituric acid (TBA) and measured as described by Yoshioka *et al* [40].

Statistical analysis

The results are expressed as mean ± SD. Differences between groups are assessed by t-test analysis of variance. The changes are considered statistically significant if $p < 0.05$.

Results and Discussion

Hematological parameters

In Fig. 1, group (LEBH+RT) shows high value comparable to (RT) and (Control) groups although (LEBH+RT) group receive the same radiation dose as (RT) group, also (LEBH) group achieved the highest value of the different groups.

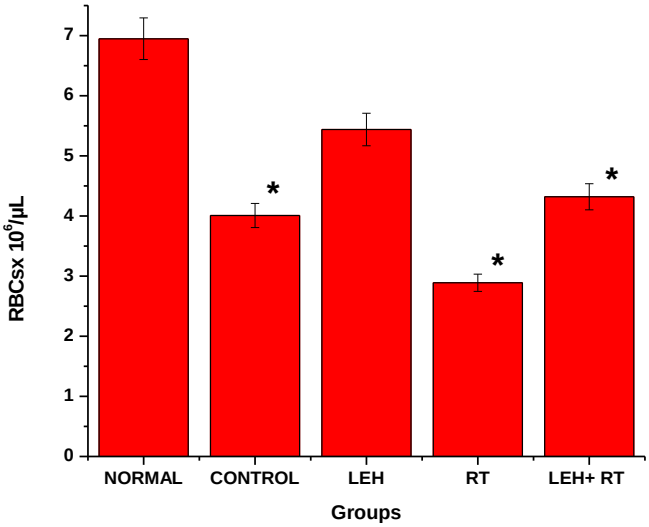

Fig. 1. RBCs concentration for investigated groups, *P<0.05 (Normal vs. other groups).

In the erythrogram (Table 1), (LEBH+RT) group show high values in most measurements comparable to (RT) group. Also, (Control) group show low erythrogram values comparable to (Normal) group, but (LEBH) show the highest erythrogram values comparable to all investigated groups.

TABLE. 1. Erythrogram for investigated groups (mean ± SD).

Group	HGB g/dl	HCT %	MCV fl	MCH pg	MCHC g/dl	RDW-CV %
NORMAL	9.6±2.77	29.8±2.54	42.8±5.23	13.8±2.51	32.3±4.72	8±0.76
CONTROL	9.2±3.04	24±3.98	60±6.34	22.9±3.78	38.3±5.76	19.7±3.41
LEH	12.3±2.3	28.3±3.5	52.1±4.62	22.6±3.04	43.4±5.16	16.6±4.05
RT	5.4±0.9	15.4±2.16	53.45±4.09	16.8±2.50	31.75±3.78	17.9±3.51
LEH+ RT	8.3±1.1	26.3±2.76	60.9±3.12	19.2±2.38	31.5±2.83	20±3.07

HGB: the total amount of hemoglobin in the blood; HCT: the fraction of the blood made up of RBCs; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW-CV: red blood cell distribution width coefficient of variation.

In Fig. 2, there is high significant increase in WBCs value for (Control) and (LEH) groups which reach to about seven folds than the normal group. But (LEBH+RT) group showed the nearest value from the (Normal) group and lower values than (RT) group.

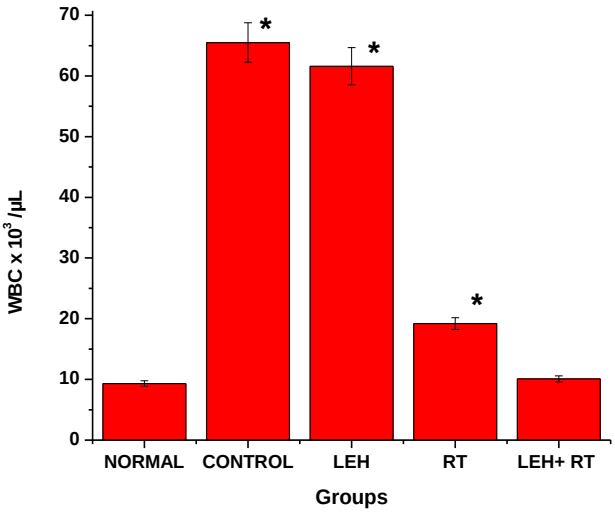

Fig. 2. WBCs concentration for investigated groups, *P<0.05 (Normal vs. other groups).

In the leukogram (Table 2), there was a significant increase in values for WBCs differential cells in control and LEBH Groups. But (LEBH+RT) group showed also the nearest values from the (Normal) group and lower values than (RT) group.

TABLE. 2. Leukogram for investigated groups (mean ± SD).

Group	LYM# x 10^3 /UL	MID# x 10^3/UL	GRAN# x 10^3 UL	LYM%	MID%	GRAN%
NORMAL	6.4±1.77	1±0.12	1.9±0.31	68.7±4.9	10.5±3.15	20.8±3.95
CONTROL	28.1±3.35	20.4±2.01	17±3.15	42.8±5.87	31.1±2.66	26.1±2.0
LEH	23±2.25	18.6±3.72	20±2.7	37.4±3.5	30.2±2.99	32.4±3.51
RT	8.6±1.80	5.9±0.86	4.7±1.75	44.6±5.1	30.5±3.12	24.9±1.78
LEH+ RT	5.8±1.69	1.9±0.2	2.4±0.5	57.2±3.88	18.7±2.70	24.1±3.42

LYM: lymphocyte absolute count; MID: mid-range absolute count (includes monocytes, eosinophils and basophils); GRAN: Granulocyte absolute count.

Malondialdehyde (MDA) concentration

The lipid proxidation resulted in different investigated groups was assessed through measurement of MDA concentrations in blood samples of different group (Fig. 3).

As shown in Fig. 3, the high value of MDA concentration was for (Control) group followed by (RT) group. But (LEBH+RT) showed the lower value for different investigated groups including also (Normal) group.

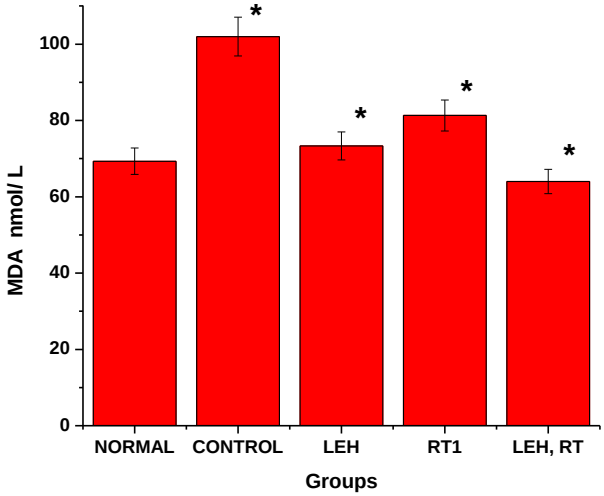

Fig. 3. MDA concentration for investigated groups. *P<0.05 (Normal vs. other groups).

Discussion

Hematopoietic stem cells are highly sensitive to ionizing radiation [41]. Hematopoietic recovery depends on the percentage of residual hematopoietic stem cells. As shown in erythrogram parameters, there was a considerable decrease in the hematological constituents in RT group in agreement with Benkovic *et al* [42]. The decrease in the values of erythrogram parameters following radiation exposure may be due to direct destruction of mature circulating cells, or leakage through capillary walls and reduced cell production[43].

Although (LEBH+RT) group receive the same radiation dose as (RT) group, but (RT) group showed lower erythrogram values than (LEBH+RT) group, this support that LEBH enhances radiation therapy and improves tumor hypoxia [39].

In leukogram (Table 2), the high values of leukocytes for Control and LEBH groups are an indication for the consequence of tumor growth [44]. In otherwise, obtained results of (LEBH+RT) group confirm that LEBH played an important role in healing tumor and improves radiation effect more than radiation effect alone[39].

Lipid peroxidation has been suggested as one of the molecular mechanisms involved in radiation induced damage [45]. In the present study, the increased level of MDA for control and RT groups an index of lipid peroxidation may be due to the free radicals attack on cell membrane phospholipids and circulating lipids [46].

Radiation is the main source of Reactive Oxygen Species (ROS) which lead to lipid proxidation and elevation of MAD level [47] , that appears clearly in (RT) group otherwise (LEBH+RT) group although they receive the same radiation dose as (RT) group.

In control group, the highest MDA value and extreme oxidative damage is as a result of tumor growth [48]**.** Also**,** the activation process of leukocytes is accompanied by the intensive production of reactive oxygen species [49].

It was observed that tumor cells produced more peroxides when they proliferate actively after inoculation of tumor [50]. This rise in peroxides indicates the occurrence of intensification of oxygen free radical production [51].

Conclusion

Liposome-encapsulated bovine hemoglobin is effective for tumor oxygenation and so enhancing radiation therapy not only against tumor growth in mice (data not shown in present work) but also in hematological parameters. The results suggests LEBH have a radical scavenging effect *in vivo* by encountering free radicals after tumor inoculation by inhibiting of membrane lipid peroxidation (LPx). Finally, our Findings clarify that LEBH may have the potential of synergistic action with radiotherapy.

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المؤشرات الدموية للفئران الحامله لورم الأيرلش عندما تم علاجها باستخدام الليبوزمات المغلفه للهيموجلوبين وبالتشعيع

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كثيرا ما تفشل الأوعية الدموية للأورام فى توفير مستويات كافية من الأكسجين إلى الأنسجة السرطانية مما يؤدي إلى أورام بها نقص من الاكسجين ومقاومه للعلاج الاشعاعى. ولتحقيق أفضل النتائج للعلاج الاشعاعى ممكن الجمع بينه و بين الليبوسومات المغلفه للهيموجلوبين من البقر باعتبارها ناقلات صناعيه للأكسجين. ومن المعروف أن المؤشرات الدمويه وتأكسد الدهون هى عوامل هامه فى التنبؤ بالأورام وعلاجها . ولذلك كان الهدف من هذا البحث هو التحقيق من تحسن النتائج العلاجيه عند الجمع بين العلاج الاشعاعى والليبوسومات المغلفه للهيموجلوبين من البقر وذلك من خلال مقارنة نتائج المؤشرات الدمويه وكذلك تأكسد الدهون فى المجموعات العلاجيه المختلفه . وتم تقسيم المجموعات العلاجيه عشوائيا لخمس مجموعات من ذكور الفئران البيضاء السويسرى ثم تم تجميع عينات الدم من المجموعات العلاجيه المختلفه فى انابيب تحتوى على الهيبارين ثم تم قياس المؤشرات الدمويه وتركيز المالوندیالدهايد (MDA) الناتج من تأكسد الدهون فى كل عينه ثم تم تحليل النتائج . وعند فحص نتائج خلايا الدم الحمراء أظهرت مجموعة الليبوسومات المغلفه للهيموجلوبين من البقر مع العلاج الإشعاعى نتائج جيدة فى معظم القياسات مقارنة بمجموعة العلاج الاشعاعى ومجموعة الكنترول و أظهرت ايضا مجموعة الكنترول قيم صغيرة فى مخطط خلايا الدم الحمراء مقارنة بمجموعة الفئران الطبيعيه , بينما مجموعة الليبوسومات المغلفه للهيموجلوبين من البقر مع العلاج الاشعاعى أظهرت أعلى قيم بين كل المجموعات فى مخطط خلايا الدم الحمراء.

وعلى ذلك فان هذه الدراسه قد أوضحت التأثير العلاجى المناسب فى الجمع بين الليبوسومات المغلفه للهيموجلوبين من البقر مع العلاج الاشعاعى و هذا التأثيرناتج من زيادة مستوى الاكسجين فى الخلايا السرطانيه .