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Anticarcinogenic Effect of Grape Seeds Extract Against Ehrlich Ascites Tumour in Mice

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Abstract: Sixty female Swiss mice were used to study the hematological, biochemical and pathological alterations that following the usage of grape seeds extract (GSE) as anticarcinogenic for Ehrlich ascites tumour (EAT). They equally divided into 3 groups: 1st group kept as normal control. The 2nd and the 3rd group were injected intraperitoneally with 2.5×10^6 EAT cells. The third group fed on GSE in the diet until death of all mice (Endpoint). GSE treated group revealed an increase in the mean survival time (MST), increasing life span (ILS) percentage and treated vs positive control (T/C) percentage. Hematological studies revealed macrocytic normochrmoic anaemia with granulocytic leucocytosis in mice bearing tumour cells. A significant decrease in total protein, albumin levels and A/G ratio without a change in globulin level and a significant increase in creatinine level and blood urea nitrogen. While a significant increase in AST, ALT, ALP and MDA levels and decrease in GSH and CAT activities were observed in EAT bearing mice. Using of GSE in the third group ameliorates nearly all of the hematological and biochemical changes toward normal in compared to EAT non treated group. Histopathological examination of liver and kidneys confirm these results.

Key words: Ehrlich Carcinoma · Mice · Grape Seeds Extract · Hematology · Biochemical · Antioxidant

INTRODUCTION

Grape seeds considered waste product of the grape industry. These wastes contain many components as resveratrol (a naturally occurring polyphenol) and anthocyanidins, which play beneficial effects including anti-oxidant, anti-inflammatory, chemopreventive and anticancer activities[1]. Tumour is uncontrolled growth of abnormal cells anywhere in a body. Malignant tumor is a major burden of disease worldwide [2]. Tumor cell invasion of surrounding tissues and organs is the primary cause of morbidity and mortality for most cancer cases [3].

Oncologist interested by rodents tumours which are a case of point where the metabolic and biochemical changes can be studied and they have become the basis of most cancer chemotherapy. The transplant ability of certain tumours in rodents has provided a useful tool for basic cancer research. The Ehrlich ascites tumour (EAT) one of these tumours which provides a reasonably homogenous sample of malignant tissue. EAT is available in large quantities and grows at fairly predictable rate, allows a more reproducible inoculum's which is critical in quantitative chemotherapeutic studies [4]. The basis of cancer chemotherapy lies in understanding of biochemical abnormalities during metabolism of malignant cell. Exploitation of metabolic differences between tumour and host tissue has become one way of treating tumours effectively [5]. The aim of the present work is to estimate the anticarcinogenic effect of grape seeds extract on EAT in Swiss mice through evaluation of survival analysis, hematological, biochemical and histopathological examinations.

MATERIALS AND METHODS

Experimental Animals: A total of 60 adult female Swiss mice (average 22 g body weight) were obtained from the laboratory animal farm of Veterinary Medicine at Zagazig University in Egypt. All mice were reared under strict standard hygienic

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measures and were fed on a balanced ration and water *ad libitum* one week before the experiment for acclimatization.

Ehrlich Ascites Tumours Cells: EAT cells parent lines were kindly obtained from the National Cancer Institute of Cairo University. The tumour line was maintained by serial intraperitoneal transplantation of EAT 2.5×10^6 tumour cells/0.2 ml in female Swiss mice.

Preparation of GSE: Grape was obtained from the local market. Grape (Vitis *vinifera*) seeds (GSE) were separated from the grape manually. GSE was dried at 70°C in a dry oven for several hours, then grinded to powder using a grinder [6]. GSE was mixed uniformly with the standard diet powder at concentration of 10% [6].

Experimental Design: Sixty female Swiss mice were divided randomly into three groups (20 mice per group). Group 1 was kept as the normal control; Groups (2&3) injected intra-peritoneal by 2.5×10^6 EAT cells. Then the third group kept on 10% GSE diet until death of all mice (Endpoint).

Survival Analysis: Five mice from each group were kept under daily observation for survival analysis. The endpoint of experiment was determined by the spontaneous death of all mice. Results are expressed as a percentage of mean survival time (MST) of treated animals over the MST of the control group (treated vs positive control, T/C %). The percentage of increased life span (ILS) was calculated in accordance with the following formula: ILS % = (T-C)/C × 100 where T represents the MST of treated animals; C represents MST of the positive control group. In accordance with the criteria of the National Cancer Institute, a T/C result that exceeded 125% and an ILS result that exceeded 25% indicated that the drug have a significant antitumour activity [7].

Blood Sampling: At the end of the second week after EAT cells inoculation into 2nd and 3rd groups, 10 mice from each group were used for blood collection from the retro-orbital venous plexus into two samples. 1st blood samples were taken in EDTA tubes for hematological analysis. 2nd blood samples were taken without anticoagulant in a sterile test tube for separation of serum which was used to measure the biochemical parameters.

Hematological Examination: Complete blood count was evaluated in an automatic cell counter (Hospitex Hemascreen 18, Italy).

Biochemical Examination: Activities of serum aspartate transaminase (AST) and alanine transaminase (ALT) were assessed according to [8]. Alkaline phosphatase (ALP) was assayed by [9]. Serum reduced GSH was determined according to the method of [10]. While, malondialdehyde was determined according to the method of [11]. Catalase (CAT) was determined by the method of [12]. Total protein was measured according to[13] and albumin was measured according to [14]. While, serum globulin was calculated by subtracting the obtained albumin value from the total protein as described by [15]. Creatinine was measured according to [16]. Serum urea nitrogen was determined according to the method described by [17].

Histopathological Studies: Small specimens were taken from liver and kidney. The specimens fixed in 10% buffered neutral formalin solution, dehydrated in gradual ethanol (70-100%), cleared in xylene and embedded in paraffin. Five-micron thickness paraffin sections were prepared and then routinely stained with hematoxylin and eosin (H&E) dyes. The sections were mounted with Canada balsam and covered with cover slide to be ready for histopathological examination [18].

Statistical Analysis: The data obtained from this investigation were statistically analyzed using one way ANOVA (f) test [19]. Means at the same column followed by different letters were significantly different and the highest value was represented with the letter (a).

RESULTS AND DISCUSSION

The Ehrlich tumor is a rapidly growing carcinoma with potent aggressive behavior and it is able to grow in almost all strains of mice [20]. Cancer cells can spread through the blood and lymph systems and lodge in other organs (tumour metastasis) [21].

This experiment was designed to illustrate the effect of GSE on amelioration of the hematological, biochemical and antioxidant changes which became uncontrollable in EAT bearing mice. (Table 1) revealed that, the MST and ILS percentage were reduced with a marked increase in body weight and distended abdomen in EAT implanted mice (gp.2) (Fig. 1). This result may be come from multiplication and tumor growth which creates ascetic fluid rich in free neoplastic cells [22]. This result was in agreement with Badr *et al*. and Salem *et al*., [23, 24] who mentioned that accumulation of ascetic fluids in the peritoneal cavity of EAT implanted mice could be due obstruction of the lymphatic system by tumor cells or

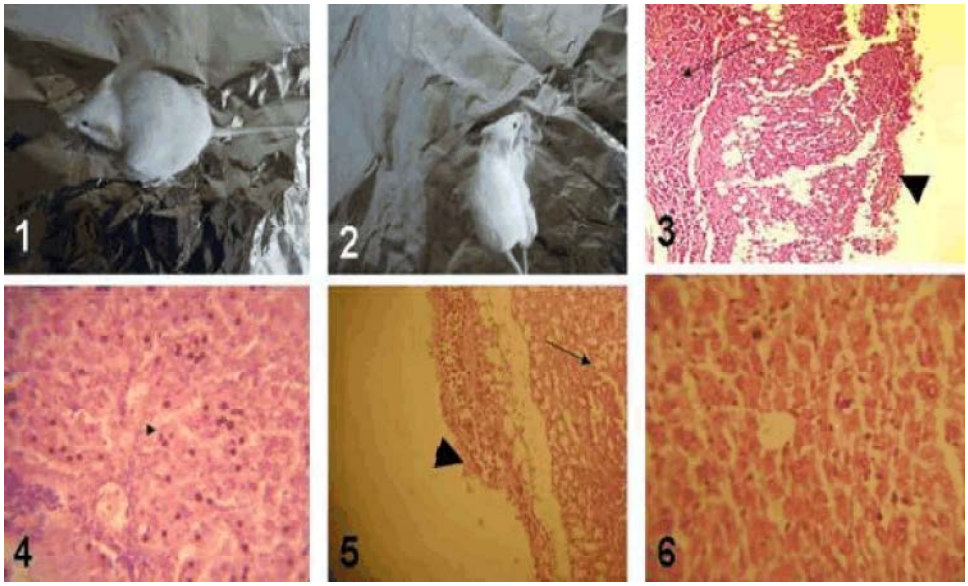

Photo 1: Mice bearing tumor cells (Gp.2) showed marked distention of abdomen due to rapid growth of Ehrlich ascites tumor

Photo 2: Mice treated with GSE (Gp.3) orally in the diet showed a moderate distention of the abdomen, indicating a little amount of ascites

Photo 3: GP.2- peritoneum of mice showing clusters from neoplastic cells (arrow head) with pressure atrophy and necrosis of the surrounding renal parenchyma (arrow) H&E,X 300

Photo 4: GP.2-Liver of mice showing invasion of the hepatic parenchyma with neoplastic cells which appear deeply chromatic with numerous mitotic figures X 300

Photo 5: GP.3- peritoneum of mice showing apoptosis and necrosis of neoplastic cells (arrow head) with mild degenerative changes in some renal tubules (arrow) H&E,X 300

Photo 6: Gp.3 Liver of mice showing individual coagulative necrosis

Table 1: body weight and survival analysis of normal mice (control), EAT and EAT+GSE groups (mean values ±SE).

	Parameters				
Groups	Body weight (g)	Range of survival time (day)	MST	ILS (%)	T/C (%)
Control	21.86^{c}± 0.35	-	-	-	-
EAT	28.80^{a}± 0.53	11-15	13	-	-
EAT+GSE	24.96^{b}± 0.22	19-28	23.5	80.76	180.76

MST mean survival time
ILS percentage of increasing life span (day)
T/C percentage of treated animals vs positive controls
EAT Ehrlich ascites tumour
GSE grape seeds extract

Table 2: Erythrogram of normal mice (control), EAT and EAT+GSE groups (mean values ±SE)

	Parameters					
Groups	RBCs (×10^{6} µl)	Hb (g %)	PCV (%)	MCV (fl)	MCH (pg)	MCHC %
Control	9.75^{a} ± 0.24	13.84^{a} ± 0.17	45.46^{a} ± 0.84	46.72^{b} ±0.36	14.24^{b} ± 0.22	30.48^{b} ± 0.25
EAT	7.57^{c} ± 0.19	11.80^{c} ± 0.20	38.20^{b} ± 0.67	50.58^{a} ±0.39	15.62^{a} ± 0.12	30.89^{ab} ± 0.02
EAT+GSE	8.62^{b} ±0.30	12.85^{b} ±0.19	40.26^{b} ±0.69	47.06^{b} ±1.48	15.02^{ab} ±0.46	32.01^{a} ±0.70

RBC red blood corpuscles
Hb hemoglobin
PCV packed cell volume
EAT Ehrlich ascites tumour
MCV mean corpuscular volume
MCH mean corpuscular hemoglobin
MCHC mean corpuscular hemoglobin concentration
GSE grape seeds extract

Table 3: Leucogram of normal mice (control), EAT and EAT+GSE groups (mean values + SE)

	Parameters			
Groups	TLC ($X10^3$ / µl))	lymphocyte ($X10^3$ / µl))	MID ($X10^3$ / µl))	GRA ($X10^3$ / µl))
Control	7.90 [c] ±0.06	6.91 [c] ±0.11	0.64 [b] ±0.04	0.35 [b] ±0.02
EAT	11.02 [a] ±0.19	8.98 [a] ±0.13	1.39 [a] ±0.01	0.64 [a] ±0.05
EAT+GSE	8.98 [b] ±0.21	7.81 [b] ±0.20	0.74 [b] ±0.05	0.43 [b] ±0.03

TLC total leucocytic count LYM lymphocytes
MID monocytes and some eosinophils GRA neutrophils, eosinophils and basophils
EAT Ehrlich ascites tumour GSE grape seeds extract

Table 4: Proteinogram, creatinine and urea of normal mice (control), EAT and EAT+GSE groups (mean values + SE)

	Parameters					
Groups	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G Ratio	Creatinine (mg/dl)	Urea (mg/dl)
Control	7.11 [a] ±0.04	2.99 [a] ±0.03	4.11 [a] ±0.06	0.73 [a] ±0.02	0.66 [c] ±0.04	38.39 [c] ±0.50
EAT	5.57 [c] ±0.07	1.39 [c] ±0.02	4.17 [a] ±0.08	0.34 [c] ±0.01	1.06 [a] ±0.02	64.62 [a] ±0.83
EAT+GSE	6.78 [b] ±0.14	2.51 [b] ±0.15	4.26 [a] ±0.23	0.62 [b] ±0.06	0.84 [b] ±0.03	50.55 [b] ±2.22

A/G Ratio Albumin / globulin Ratio EAT Ehrlich ascites tumour, GSE grape seeds extract

angiogenesis, which was detected in the EAT bearing peritoneal wall or even due to the hyper permeability of micro vessels in the wall of peritoneal cavity [25]. Increasing percentages of MST, ILS and little increase in body weight and little distended abdomen in the treated GSE group (gp.3) (Fig. 2), that could be due to grape-based products which consider excellent sources of various anticancer agents[26].

Regarding the results of hematological studies (Table 2) showed a significant decrease in erythrocytic count in EAT group which may be due to the suppressive effect of EAT on erythropoiesis in bone marrow [27]. This anemia was classified as macrocytic normochromic anemia. It may be appeared as a result of direct deficiency in the folic acid due to increasing in proliferation of the EAT cells as it is necessary for synthesis of DNA and RNA for new proliferated tumor cells [28]. From the same side, this anemia may be due to the indirect decrease in folic acid due to deficiency of thiamine in the liver and blood of mice bearing EAT which play an important role in folate metabolism [29]. This result also present in the GSE treated group when compared to normal while seemed to be improved if compared with EAT group.

Table (3) illustrates total (TLC) and differential leucocytic count (Lymphocyte, MID and granulocyte) in the experimental groups. Results of leucogram revealed that, there was a granulocytic leucocytosis observed in EAT group which might be owned to the acute stress or inflammatory response that resulted from the multiplication of Ehrlich tumor cells [30].

In order to investigate the effect of EAT on proteinogram as shown in (Table 4). A significant decrease in total proteins, albumin levels and A/G ratio without alterations in globulin level in EAT group and to a lesser extent the GSE treated group as compared with normal mice. This may be attributed to increased mitotic division of cancer cells with increasing withdrawal of body fluid and capillary permeability which permit the run off plasma proteins into the peritoneal cavity and may also be due to liver necrosis [31,32]. In addition to, total proteins may decrease in animals with hepatic disease, hypoproteinemia and hypoalbuminemia may be due to excessive nephritis [33]. These results were confirmed by increased creatinine and blood urea nitrogen levels in these groups that may be attributed to renal tissue damage as a result of cancer cell invasion [32]. Otherwise, GSE treated group showed amelioration to proteinogram, urea and creatinine toward normal in comparing to EAT group where grape seed extract protects the hepatic cells from injuries, improves the liver functions [34] and reduce kidney function disturbances [35].

At the same time, the experimental data (Table 5) showed the effect of EAT cells on the liver functions. A significant increase in ALT, AST and ALP were prominent in the EAT group than in GSE group when compared with the control group. Our results are in accordance with [36] who reported an increase of hepatic transaminases in EAT bearing mice referring to hepatic damage as a result of cancer cell metastasis [37]. Also, the presence of large tumour masses and the associated

Table 5: Some serum Enzymes and antioxidants of normal mice (control), EAT and EAT+GSE groups (mean values + SE)

	Parameters					
Groups	AST (U/l)	ALT (U/l)	ALP (U/l)	MDA (µmol/ml)	GSH (mg/dl)	CAT (µg/ml)
Control	31.41 ^{c} ±0.82	20.89 ^{c} ±0.50	17.40 ^{c} ±0.49	51.96 ^{c} ±0.59	46.39 ^{a} ±0.45	41.84 ^{a} ±0.47
EAT	67.37 ^{a} ±0.69	42.41 ^{a} ±0.62	68.49 ^{a} ±3.81	75.27 ^{a} ±1.11	29.35 ^{c} ±0.54	23.23 ^{c} ±0.67
EAT+GSE	48.99 ^{b} ±1.40	32.61 ^{b} ±1.19	39.83 ^{b} ±1.09	61.79 ^{b} ±1.63	39.63 ^{b} ±1.87	31.64 ^{b} ±1.40

AST	Aspartate aminotransferase	ALT	Alanine aminotransferase
ALP	Alkaline phosphatase	MAD	Malondialdhyde
GSH	Reduced glutathione	CAT	Catalase
EAT	Ehrlich ascites tumour	GSE	Grape seeds extract

long lasting necrosis are considered a metabolic overloading on the liver tissue as indicated by Tofani *et al*. [38]. EAT +GSE group showed significant decrease in plasma levels of ALT, AST and ALP toward the normal values [39]. These results suggest that grape seeds extract protects the liver from injuries and improves the hepatic functions when compared to EAT bearing mice[6].

Antioxidant results (Tables 5) revealed significant decrease in the antioxidant activities of GSH and CAT while a significant increase in MAD level in EAT and GSE groups as compared with normal group. Lipid peroxidation, an autocatalytic free radical chain propagating reaction, is known to be associated with pathological conditions of a cell [40]. Increased lipid peroxidation would cause degeneration of tissues. Lipid peroxide formed anywhere in the primary site, it would be transferred through the circulation and provoke damage by propagating the process of lipid peroxidation [41]. It was also reported that the presence of tumours in the human body or even in experimental laboratory animals were known to affect many functions of the vital organs, especially in the liver, even when the site of the tumour does not interfere directly with organs function [42]. Malondialdehyde (MDA), the end product of lipid peroxidation was estimated to be higher in cancer tissue than in normal [43]. Glutathione reduced (GSH), an important non-protein thiol, plays a significant role in protecting cells from neoplastic process. In addition, GSH plays a role as an endogenous antioxidant molecule that is found particularly in high concentration in liver and known to have a key function in the protective process [41]. The present study confirmed the finding of Sreelatha *et al*. and Tohamy *et al*. [44, 45] who reported that the enhancement of lipid peroxidation in EAT-bearing mice is a consequence of depletion of GSH to certain critical levels. The enzyme present in free radical scavenging system is catalase (CAT). The main function of CAT is to provide a defense against the hydrogen peroxide. The inhibition of CAT activity as a result of tumor growth was also concluded by Choudhury *et al*. [46]. Our results coincide with that obtained by Samudrala *et al*. [47] who found an increase in MDA and decrease in the activities of GSH and CAT in EAT bearing mice. On the other side, GSE treated group showed an improvement in the antioxidant activity when compared to EAT group. As, GSE supplementation may protect cells and tissues from oxidative damage that could be due to their strong antioxidant activities of scavenging reactive oxygen. Similar results obtained by Madi and Chis *et al.* [48, 49] who proved that oral administration of grape seed extract improved CAT level and reduced the levels of lipid peroxides and enhanced the antioxidant defense against reactive oxygen species.

All the previous results confirmed with histopathological examination. In EAT group, the hepatic cells showing invasion of the hepatic parenchyma with neoplastic cells (Fig.4). Renal tissue showed pressure atrophy from neoplasticcells and necrosis of the surrounding renal parenchyma (Fig.3).while treated mice with grape seeds extract showed mild degenerative changes in some renal tubules (Fig.5) with individual coagulative necrosis in hepatic cells (Fig.6). Our results agreed with Kapoor *et al.,* Badr *et al*. and Miranda *et al*. [32, 50-52] who reported that there were alterations in hepatic and renal tissue as a result of invasion of EAT cells causes large areas of necrosis, congestion and mononuclear cell infiltration in liver and kidney sections. While the third group, mild changes appear in the liver and kidney which may be due to protective effect of GSE on the liver. These results are in agreement with El-Beshbishy *et al*. and Kasdallah *et al*. [53, 54] who found that resveratrol reduced hepatic tissue injury.

CONCLUSION

It could be concluded that using of GSE have antitumor activity against EAT in Swiss mice. It improves the hematological and biochemical changes in the treated mice toward the normal when compared to EAT non treated mice.

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