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Tumor regressive effect of grape juice (Enoant®) on ehrlich ascites carcinoma of mice

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Summary

Enoant® is a nutritional reinforcement produced from the grape's stem, peel and seeds. In recent years grape products such as wine and grape juice have acquired a great importance because of their polyphenol components, which have a strong antioxidant effect. Those antioxidant effects of polyphenols have an ability to inhibit proliferation and induce apoptosis in cancer depending on the types of cancer and application doses. In our study we have aimed to investigate the tumor regressive effects of Enoant® in solid EAC tumor model in Balb-c mice. Animals were randomly divided into 2 groups in this study. 0.5 ml of Enoant® was administered daily to ENT group and the same volume of NaCl 0.9% was administered to the control group. Animals continued to receive those applications till sacrification day. On the 8th day 2 × 10^6 EAC cells in 0.5 ml NaCl 0.9% were injected subcutaneously into the mice's napes. On day 22 all animals were sacrificed under ether anesthesia. Using PCNA immunohistochemical staining, TUNEL technique, we observed the proliferative and apoptotic cell density changes in tumor tissues as well as the effect of Enoant® on these two phenomena. Dietary Enoant® significantly regressed tumor development in mice. It has been observed that the administration of Enoant® displayed positive effects on EAC tumor's weight and size when compared with control group animals. Mean tumor weights' meaningfulness was $p < 0.01$ and mean short-long diameters' meaningfulness were $p < 0.05$ and $p<0.01$, respectively. It has been determined that while the PCNA index was low ($p < 0.05$) in the Enoant® administered group, the apoptotic index that has been established with TUNEL technique was high ($p < 0.01$). As a result, Enoant® has a regressive function on EAC tumor cells. By inducing apoptosis, ENT inhibited the development of tumors. It is thought that ability of ENT was welded from its strong antioxidant polyphenol component. Because of that the use of Enoant® as a dietary supplement is thought to be a factor for inhibiting cancer development.

Keywords: Enoant®, apoptosis, PCNA, EAC

The black grape is a fruit which has antioxidant, anticancer, antiproliferative, apoptotic, antibacterial, antiviral properties and which organizes cholesterol metabolism and is inductive for detoxification enzymes (2, 4, 10, 32). Recently, products of grapes such as red wine, grape juice and grape seed extract have acquired a great importance due to their polyphenol components, which have a strong antioxidant effect (1, 32). In cancer studies the most attractive and the most studied polyphenols are resveratrol, quercetin, catechin, epicatechin and their dimmers and polymers called proantocyanidines (1, 4, 32).

Resveratrol is a bioflavonoid, which is present in grape, red wine and peanut (14). It has been reported that resveratrol, which has also been shown to have potential antimutagenic activity, inhibits hydrocarbon--induced skin cancer in mice at the onset and promotion steps. It is thought that its anticancer activity arises from its abilities to induce apoptosis, modulate suppressor genes and its effects on the cell cycle (29, 32). A flavonoid, Quercetine has shown its anticancer property by hindering tumor development in human-originated prostate, colon and stomach cancer cells. In addition to that it has inhibited tumor progress and development in melanomas and intestinal tumors of mice (32). It has been proposed that catechins, which is a polyphenol and found in vegetables, fruits and particularly in tea have a potential anticancer property. Catechins have inhibited development in human cell lines originating from breast prostate cancer (32). Furthermore, the low number of prostate cancer incidence in some Asian countries is attributed to the usage of green tea (18). It is reported that epicatechins inhibit proliferation and induce apoptosis in cancer cells (29). Proantocyanidines are polymers having high molecular weights with flavon-3-ol ((+) catechin and (–) epicatechin) units (7).

Grape seed extract includes dimers and trimers of (+) catechin and (–) epicatechin (6, 27). Moreover, as well as having antioxidant, vasodilator, anti-inflammatory, anticarcinogenic and antiallergic properties, it has been reported that they inhibit lipooxygenase and COX (1, 2, 22). It is thought that the therapeutic effects of proanticyanidines result from their strong antioxidant property.

Polyphenols in grape and grape seeds reportedly display positive effects, particularly on cancer, by inducing apoptosis, inhibiting the synthesis of DNA and affecting the expression of some enzymes (cyclooxygenase and protein kinase) which play a role in cancer development (21, 32). Singh et. al. have indicated that grape seed extract evidently inhibits human prostate tumor development (30). It has been shown in cell culture studies that grape seed extract inhibits advanced human prostate cancer development and induces apoptosis (8).

Enoant® (ENT) is a concentrated grape juice derivate from grape stems, peels and seeds. It is obtained from Cabernet Sauvignon genus black grapes, particularly rich in polyphenol content, which are especially grown in the Crimea region of Ukraine. While the polyphenol ratio in red wine is 1-2 g/l the ratio in ENT is 71.1 g/l. Besides its polyphenols content, it contains some trace elements such as iron, potassium, magnesium, organic acids, and vitamin B. Its polyphenol content includes bioflavonoids such as quercetin, cathechin, tannin, proanthocyanidines, epicatechin, anthocyanidis and such non-flavonoids as resveratrol and acid derivatives. Reports claim that even alone these substances have a very strong antioxidant property.

ENT is a nutritive reinforcement used especially because of its antioxidant compound content and diversity. Moreover, it is considered that those compounds it includes might have many different biological activities for cancer avoidance.

The aim of the study was to observe the dynamic effects of ENT's influence on the apoptotic and proliferative cell density of Ehrlich ascites carcinoma (EAC) tissue in mice. Furthermore, we consider whether the anti-tumor effects of ENT are due to induction of the tumor cell apoptosis or not. In summary, experimental data relating to the ability of ENT to regress tumor development will be offered in this study.

Material and methods

Ethical clearance. The protocol used in this study for the use of mice as an animal model for cancer was approved by our university's Ethics Board.

ENT content analysis. 23 µg/ml of resveratrol, 130 µg/ml of quercetin, 1.47 mg/ml of cathechin, 0.88 mg/ml of epicathechin in ENT were determined by Anadolu University Plant Medicine and Scientific Research Center-Eskişehir with HPLC analysis.

Animals. Female Balb-c mice with a mean body weight of 30 g were used in this study. These animals were bred in Istanbul University Veterinary Faculty Morphology Department. They were housed in polypropylene cages in a controlled environment (12 hour dark/light cycle) and fed with standard laboratory chow and were given tap water *ad libitum*.

Induction of Erhlich's ascites carcinoma (EAC). Solid ascites carcinoma in mice was induced by injecting 2×10^6 Ehrlich's ascites carcinoma cells in 0.5 cm^3 NaCl 0.9% (s.i.) from the napes of the animals. Before the administration of EAC cells to mice animals were divided into two groups. The control group: 0.5 ml of 0.9% NaCl was administered daily to the animals in that group (n = 14) with the gavage method. ENT group: 0.5 ml of ENT was administered daily to the animals in that group (n = 14) with the gavage method.

The animals in both groups received those applications for 7 days. On the 8th day EAC cells were administered to the animals in both groups. The respective applications to both groups were continued for 22 days. Tumor developments were determined with the palpation method.

Evaluation of tumor tissues. On Day 22 tumor developments were determined in all animals. At this day, after sacrificing animals under ether anesthesia, tumor tissues were extirpated from the ENT and control groups.

Extirpated tumor tissues were weighted and their short-long diameters were measured with the help of a caliper. Independent samples t-test (SPSS 8.0, SPSS for Windows Advanced Statistics Release 8.0, 1997) was used in the comparison of groups. The value of $p < 0.05$ was accepted as statistically significant.

The tissues were fixed in 10% formaldehyde solution, paraffin embedded and sectioned at 5 µ thickness and collected on Poly-L-Lysine coated glasses.

Tumor cell density of proliferation were recorded by counting cells in a G1/S phase determined by PCNA immunohistochemistry. Likewise in those sections taken from tumor tissues density of apoptosis was determined with TUNEL technique using a Chemicon International, Germany-S7101-Kit.

Immunohistochemical staining of proliferating cell nuclear antigen (PCNA). Immunohistochemical stainings were performed using Indirect Streptavidin Peroxidase technique (Zymed Histostain Plus Broad Spectrum, South San Francisco, CA, US). Paraffin embedded sections were placed on Poly-L-Lysine coated slides and dried at room temperature. Sections were de-waxed, rehydrated through a graded alcohol series and washed with 0.01 mol/L of PBS (phosphate-buffered saline). Endogen peroxidase activity was quenched by a 10 minute application of 0.3% H_2O_2 prepared using PBS and sections were placed in antigen retrieval solution (0.01 M of citrate buffer, pH 6.0). Heat induced epitope retriever was performed in that solution with a 700 watt microwave oven for 10 minutes. The tissue samples were incubated with primer anti-PCNA antibody (Santa Cruz-7907-rabbit polyclonal) in a 1 : 100 dilution, for 50 minutes at room temperature. After rinsing three times with PBS, sections were incubated first in biotinated secondary antibody and then in HRP Streptavidin, each at room temperature for 15 minutes. Later sections were incubated 5 minutes in DAB (diaminobenzidine; Zymed) for the development of product reaction and counterstaining was performed with hematoxylin. For negative control sections, instead of primer antibody PBS was applied.

Tunel. TUNEL was performed by using an Apop Tag Plus in situ apoptosis detection kit (Chemicon International, Germany-S7101). 5 µ sections of paraffin blocks prepared from tumor tissues were incubated overnight at 37°C in a drying oven. In brief, paraffin sections were de-waxed, rehydrated through a graded alcohol series and washed with PBS. Subsequently, tissues were digested with 20 µg/ml proteinase K at room temperature for 15 minutes. For quenching of endogenous peroxidase sections were incubated at room temperature for 5 minutes in 2-3% H_2O_2 prepared using PBS. They were incubated with equilibration buffer at room temperature

for 30 minutes. Next, Tdt enzyme was applied to all sections and sections were kept in 37°C drying oven for an hour. Later sections were treated at room temperature 10 minutes with stop/wash solution, which is manufactured with the kit. Then they were washed 3 changes of PBS for 1 minute and anti-digosigene-peroxidase was applied 30 minutes at room temperature. Specimens were washed in 4 changes of PBS for 2 minutes. For the development of product reaction sections were covered with DAB substrate (diaminobenzidine) and reactions were determined at 3-6 minutes. After the samples were rinsed with distilled water, methyl green was used for counterstaining. In the Tdt enzyme treatment step, instead of this enzyme negative control sections were covered with distilled water.

Proliferation, apoptotic indices and statistical analyzes. Immunohistochemical stainings were investigated and photographed with Olympus light microscope and Bs200Doc digital monitoring system. PCNA-positive cells were used to quantify the proliferation index (percentage of PCNA-positive cells in 1000 cells). Similarly, TUNEL-positive cells were used to quantify the apoptotic index (percentage of TUNEL-positive cells in 1000 cells) (24).

SPSS was used in statistical analyzes performed in this study (SPSS 8.0, SPSS for Windows Advanced Statistics Releae 8.0, 1997). Paired t-test was used in the comparison of groups. The value of $p < 0.05$ was accepted as statistically significant.

Results and discussion

Tumor development. Tumor tissues obtained from animals were weighted and their short-long diameters were measured with a caliper. When the obtained data were evaluated with an independent t-test, the statistical meaningfulness of mean tumor weights and mean long diameter measurements was $p < 0.01$ and mean short diameter measurements was $p < 0.05$. Consequently, the application of ENT had been shown to have a positive effect on weight and size of tumor and when compared with control group, which received nothing, it has inhibited tumor development (tab. 1).

The proliferation and apoptotic indices. PCNA-positive cells in ENT and control groups were observed as a brown reaction in the nuclei. Positive reactions were lesser in ENT group when compared with the control group (fig. 1-2). Moreover, when the ENT and control group are compared, the PCNA index was found to be lower than the control group in ENT applied group. It has been observed that this difference was statistically significant ($p < 0.05$) (tab. 2).

Tab. 1. Mean values of tumor weights and short/long diameters between control and Enoant® groups

	Groups	Mean
Tumor weights (g)	Control n = 8	1.045 ± 0.211
	Enoant® n = 14	0.465 ± 5.262**
Tumor Short Diameter (mm)	Control n = 8	13.8300 ± 1.3544
	Enoant® n = 14	10.4564 ± 0.5612*
Tumor Long Diameter (mm)	Control n = 8	23.431 ± 2.213
	Enoant® n = 14	16.469 ± 1.040**

Explications:* – $p < 0.05$, ** – $p < 0.01$

Tab. 2. PCNA and Apoptic Index between control and Enoant® groups

Groups	PCNA positive cell index (%)	Apoptotic cell index (%)
Control	76.80 ± 2.87	3.68 ± 0.26
Enoant®	58.80 ± 4.48*	5.25 ± 0.50**

Explications: as in tab. 1.

TUNEL-positive cells were observed as brown reactions with the application of DAB chromogene. More apoptotic cells were determined by TUNEL method in the ENT group than in the control group (fig. 3-4). Apoptotic index was found to be significantly higher in the ENT group when compared with the control group ($p < 0.01$) (tab. 2).

The epidemiologic studies performed in many different areas indicate that there is an inverse proportion between the consumption of vegetables and fruits and the risk of cancer (5, 20, 33). Plant originated nourishments involve various phytochemicals with anticancer properties. It is considered that those phytochemicals can reduce the emergence of cancer through several potential bioactivities (35, 36).

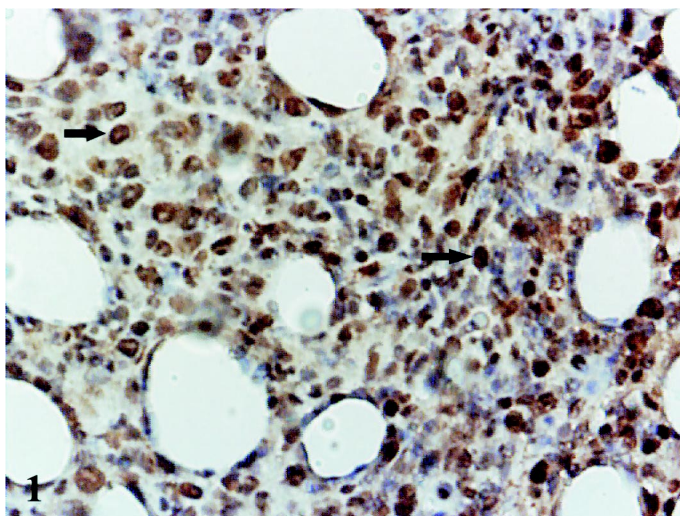

Fig. 1. Control group. PCNA immunoreactions in tumor cells (arrow), × 800

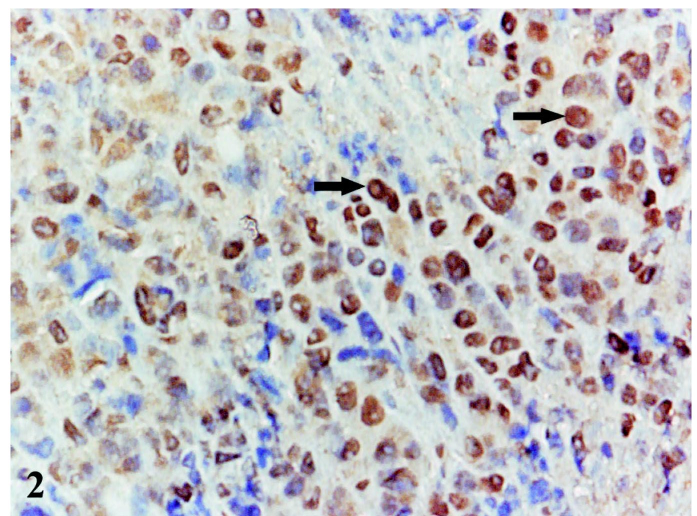

Fig. 2. Enoant® group. PCNA immunoreactions in tumor cells (arrow), × 800

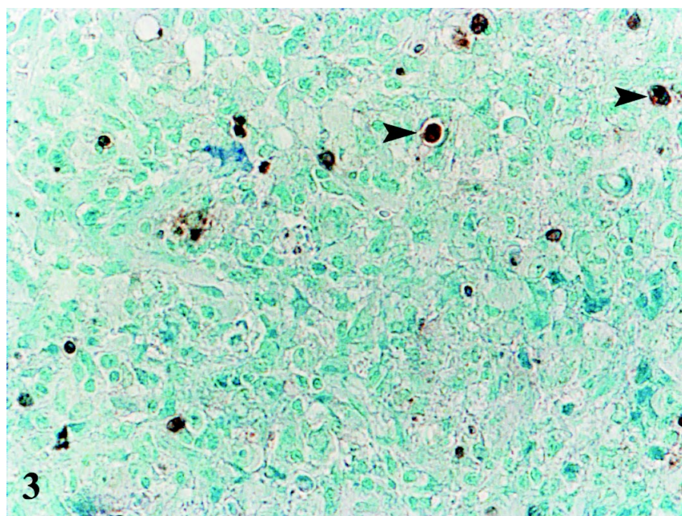

Fig. 3. Control group. Apoptotic cells in tumor cells (arrow head), × 800

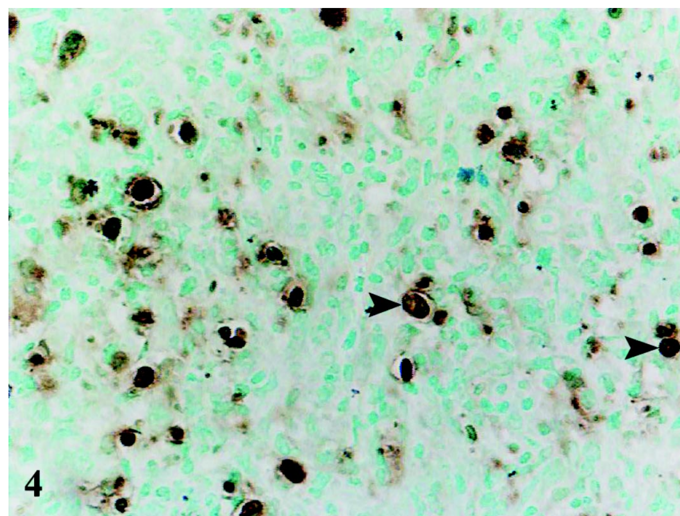

Fig. 4. Enoant® group. Apoptotic cells in tumor cells (arrow head), × 800

It has been observed that those compounds also present in Enoant, which is a concentrated grape juice, have proliferation inhibiting, antioxidant, tumor development inhibiting and apoptosis inductive properties (2, 4, 10, 32). It has been suggested that resveratrol (trans 3, 4, 5-trihydroxystilbene), which is one of those compounds, shows a chemo-preventative effect on chemical carcinogens. Despite knowing that resveratrol has inhibition effects on tumor development, its different *in vivo* and *in vitro* effects specific to tumor type have been exhibited. Resveratrol has inhibited the development of 32Dq210 leukemia cells in vitro and by causing DNA fragmentation it has stimulated apoptosis. However, in vivo administration of 8-40 mg/kg/body of resveratrol has not given the same results upon animals (11).

Guisado et al. (13) have investigated low dose effects of resveratrol upon MCF-7 and MDA-MB-231 human breast cancer lines and have determined that particularly the dose of 300 μM resveratrol suppressed proliferation and induced apoptosis in both cell lines. Although the proliferations were suppressed in both cell lines with the dose of 50 μM resveratrol there was no difference in terms of apoptosis in MCF-7 cells. This result proves that resveratrol shows concentration and cell specific dependent effects on inhibition of tissue proliferation and induction of apoptosis.

In addition, ElAttar et al. (9) have demonstrated that resveratrol inhibits the synthesis of DNA and cell development with the doses of 10 and 100 μM in human oral squamous carcinoma cells. The interesting point of this work was the observation of those effects with the combined usage of resveratrol with quercetin. Although quercetin alone shows a minimal effect with the dose of 100 μM, it indicated a significant effect on cell development and inhibiting of DNA synthesis when applied in combination with resveratrol in lower doses. There is resveratrol and quercetin in ENT that we used in our study. In the EAC solid tumor model that we induced, when compared with the control group, significant regression in tumor development, suppression of tissue proliferation and increase in number of apoptotic cells is observed in the ENT group. Results obtained in this study support the findings of ElAttar et al. who reported that the combined usage of resveratrol and quercetin had indicated a strong effect.

There are several biological properties of flavonoids. It must considered that their cell cycle cell proliferation, oxidative stress inhibition and detoxification enzymes, apoptosis, immune system inductive properties could play a role in cancer chemo-prevention (7, 17, 20). It has been shown that quercetin, which is one of the flavonoids, suppressed proliferation in different cultures of human cancer series. It reportedly inhibited proliferation in HTB43 squamous cell carcinoma (15), meningioma (25) and human tumor cells (31) and delayed breast tumorogenesis.

Proantocyanidines are a mixture of monomers, oligomers and polymers of flavan-3-ols which are known as catechines. They perform as free radical scavenger antioxidants (18). From those antioxidants that can be found abundantly in green tea, the ones with dimeric properties inhibited phorbol myristate acetate induced NF-kB activation in Jurkant T cells (19). Recently Nomoto et. al. has shown that grape-seed procyanidines induced apoptosis in cancer cells and acted as a preventative against colorectal cancer (23). In our study the regression of proliferation and increase in apoptosis in EAC cells with the application of ENT supports these results.

It can be seen that ENT obviously inhibited tumor development in EAC tumors of mice and when it is compared with the control group the difference was statistically meaningful (tab. 1). In the evaluations we have conducted with PCNA marker, which determines cells in G1-S phase (34), we observed that in ENT applied tumor tissues proliferations were suppressed significantly when compared with control group tissues. When we evaluated the apoptotic cell index with the TUNEL technique we determined that this index was higher in the ENT applied group than control group. In evaluations we have conducted according to those results, ENT is found to be quite rich with substances that

possess antioxidant, proliferation inhibiting and apoptosis inducing properties. Because of its non-alcoholic nature and its easy usage and accessibility its beneficial usage in cancer cases should be taken into consideration.

In recent years, the degree of apoptotic activity has been considered to play a role in tumorogenesis (3). The dynamic balance between proliferation and apoptosis sustained a regular number in normal tissue, the dynamic balance between cellular proliferation and apoptosis in normal tissue was destroyed in cancer tissue with an abnormal number and distribution of cellular apoptosis and proliferation (34). The distributive relationship between proliferation and apoptosis disappeared (28). In our study in tumor tissues taken from the control group the density of proliferative cells increased and the density of the apoptotic cells decreased. As it has been reported before, in tumor tissues, while the cell proliferation increases the number of apoptotic cells decreases and the ratio between apoptosis and proliferation is continuously reduced (34).

Conclusion

In studying ENT in the inhibition of EAC in mice we have drawn the conclusion that ENT can suppress the development of EAC tumors. The density of apoptotic cells in the ENT group was higher than in the control group. The extent of apoptosis increased with the degrees of the pathologic change and this change influenced the growth rate of the tumors, which delayed the process of tumorigenesis. In a word, ENT can induce cellular apoptosis causing a delay in the development of tumors. However, cancerous progression is regulated by multigenes and the exact mechanism by which ENT induces the tumor cell apoptosis is unknown (12, 26). It is clear that the relationship between ENT inhibition of EAC needs further study. Additionally, it must be considered that the phytochemicals ENT contains can show different effects on several cancer lines and their effects on those different cancer types must also be investigated.

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