Effects of an oral treatment with concentrated grape juice (ENT) on cell NOS (eNOS and iNOS) expression and proliferation in experimentally induced carcinoma in mice

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SUMMARY

The exact role of nitric oxide (NO) in tumour development has not been yet fully elucidated. The concentrated grape juice (ENT) is particularly rich in polyphenols and is actually considered as an additive food with antioxidant properties. The present study investigates the eventual effects of ENT on cell proliferation and NO production within mammary carcinomas experimentally induced by a subcutaneous injection of $2 \times 10^6$ EAC (Ehrlich Ascite Carcinoma) cells in mice. For that, 28 female balb/c mice were randomly divided into 2 equal groups: mice were daily orally treated with ENT (0.5 mL/day) (ENT group) or with 0.9% NaCl (0.5 mL/day) for 7 consecutive days before carcinoma induction and 22 days after. On day 30, animals were slaughtered under ether anaesthesia and PCNA, eNOS and iNOS expressions in tumour tissues were evaluated by immunohistochemistry: proliferation indexes (percentages of PCNA positive cells in 1 000 cells) and the respective eNOS and iNOS HSCORE values (cumulated percentages of positive stained cells according to the staining intensity X staining intensity) were then calculated. The ENT treatment has significantly reduced the proliferation indexes of the tumours. Moreover, tumour iNOS expression was dramatically lowered compared to controls whereas the eNOS expression was weakly affected. These results suggest that carcinoma growth was restricted in mice orally treated with ENT because of the marked reduction of iNOS expression.

Keywords: ENT, INOS, eNOS, PCNA, Ehrlich Ascite Carcinoma cells, mice.

RéSUMÉ

Effets du jus de raisin concentré (ENT) administré oralement sur l’expression des enzymes eNOS et iNOS et sur la prolifération cellulaire au sein de carcinomes expérimentalement induits chez la souris

Le rôle exact joué par l’oxyde nitreux (NO) dans le développement tumoral n’est pas encore complètement élucidé. Le jus de raisin concentré (ENT) est particulièrement riche en polyphénols et est actuellement considéré comme un additif alimentaire doué de propriétés anti-oxydantes. Le but de cette étude est de rechercher les effets éventuels de l’ENT sur la prolifération cellulaire et la production de NO de carcinomes mammaires induits expérimen-talement chez la souris par une injection sous-cutanée de $2 \times 10^6$ cellules EAC (Ehrlich Ascite Carcinoma). Pour cela, 28 souris Balb/c femelles ont été aléatoirement réparties en 2 groupes égaux : les souris ont reçu par voie orale une dose quotidienne d’ENT (0.5 mL/jour) (groupe ENT) ou de NaCl 0.9% (0.5 mL/jour) (groupe contrôle) 7 jours avant et 22 jours après l’induction du carcinome. À J30, les animaux ont été sacrifiés par anesthésie profonde à l’éther et l’expression du PCNA, et des enzymes eNOS et iNOS dans les tissus tumoraux ont été quantifiées par immunohistochimie : les index de prolifération (pourcentage de cellules exprimant le PCNA déterminé sur 1000 cellules) et les scores d’expression des enzymes (pourcentages cumulés de cellules positives en fonction de l’intensité du marquage x intensité du marquage) ont été calculés. Le traitement par l’ENT a réduit significativement l’index de prolifération des tumeurs. En outre, l’expression tumurale de iNOS a été considérablement réduite par rapport aux contrôles alors que celle de eNOS n’a été que faiblement modifiée. Ces résultats suggèrent que la croissance tumurale a été limitée chez les souris traitées oralement par l’ENT en raison d’un effondrement de l’expression de iNOS.

Mots-clés : ENT, iNOS, eNOS, PCNA, cellules d’Ehrlich Ascite Carcinoma, souris.

Introduction

The exact role of nitric oxide (NO) in tumour biology has not been fully elucidated, because NO has been implicated in many different aspects of cancer biology with both pro and anti-tumour functions. NO may have a role in carcinogenesis by inducing DNA strand breaks [34, 59] and by impairing the tumour suppressor function of P53 [9]. It has also been implicated within signalling cascades for neovascularization [29] and it can increase tumour blood flow [45]. The presence of inducible Nitric Oxide Synthease (iNOS) has been correlated...
with metastatic disease [14] and a NOS inhibitor (L-NAME) significantly reduces bone metastasis [28]. NO has also been shown to induce apoptosis [43, 49] in tumour cells. The balance between these opposing roles may depend on NO concentrations: high concentrations may exert anti-proliferative effects while low concentrations may facilitate tumour growth [29].

Some antioxidant, anticancer, anti-proliferative, apoptotic, antibacterial, antiviral properties have been reported for black grape. Moreover, it partially regulates cholesterol metabolism and it may induce detoxification enzymes [4, 6, 16, 28, 55]. Recently, products of grapes such as red wine, grape juice and grape seed extracts have acquired a great importance due to their polyphenol components which have strong antioxidant effects [3, 28]. In the cancer studies the most attractive and the most studied polyphenols are resveratrol, quercetin, catechin, epicatechin and their dimmers and polymers called proanthocyanidines [3, 6, 28].

Resveratrol is a phytoalexin polyphenol and this natural antioxidant compound is found in various plants, including grapes, berries and peanuts. One of the most striking biological activities of resveratrol largely investigated during the late years has been its cancer chemopreventive potential. In fact, it has been demonstrated recently that it blocks the multistep process of carcinogenesis at various stages, such as tumour initiation, promotion, and progression. One of the possible mechanisms involves inhibition of the iNOS [10, 27] and WALLERATH et al. [57] suggested that resveratrol increased the activity of the endothelial NOS (eNOS) promoter in human endothelial cells.

Catechin, which is also isolated from the green tea and epigallocatechin gallate (EGCG) exhibit the most potent antioxidative and chemo preventive properties in different in vitro and in vivo animal experiments [47, 58]. The EGCG was also found to block LPS or IFN-a-activated iNOS expression and enzyme activity in vitro [8, 34]. In parallel, it inhibits the induction of iNOS in mouse peritoneal macrophages which is stimulated with LPS produced via down-regulation of NF-κB [34]. The EGCG was reported to selectively induce apoptotic death in several cancer cell lines [1, 39, 47].

The protective components in red wine and grapes include the polyphenol compounds such as quercetin, a flavonoid, and resveratrol, a stilbene. Molecular mechanisms of these compounds are current topics of interest [25, 33]. They inhibit LPS-stimulated NO production and iNOS gene expression in rat astrocytes, Kupffer cells, and murine macrophages [30, 31], but these compounds also enhance endothelium-dependent loosening of pre-contracted rabbit and rat aortic rings, through an increase of NO production via eNOS [18, 19]. Thus, grape components may alter the eNOS and iNOS expressions in opposite ways. Furthermore, an inverse relation between iNOS and eNOS has been shown. In endothelial cells, inflammatory agents (i.e. LPS and cytokines) induce expression of iNOS but reduce the expression of eNOS [35].

ENT is a concentrated grape juice derivate from grape’s stems, peels and seeds and it is particularly rich in polyphenol content, such as flavonoids (quercetin, catechin, epicatechin, tannin, proanthocyanidins and anthocyanidins) and non flavonoids (resveratrol and its acid derivatives). It is obtained from Cabernet Sauvignon genus black grapes, which are grown especially in 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 [50]. Besides its polyphenols content, it contains some trace elements such as iron, potassium, magnesium, organic acids, and vitamins B. Because of its high polyphenol content, ENT is a nutritive reinforcement used especially because of its antioxidant properties. Besides, the various antioxidant compounds might have many different biological activities for avoidance of cancer.

This study investigates the effects of an oral ENT treatment on proliferative cell density of solid carcinoma experimentally induced in mice by injection of Ehrlich Ascites Carcinoma cells. Furthermore, the iNOS and eNOS activities in tumour cells were evaluated.

### Material and Methods

#### 1. ANIMALS AND EXPERIMENTAL PROTOCOL

The protocol used in this study was approved by the Istanbul University’s Ethic Board. Female Balb/c mice weighing 30g in average, bred in Department of Veterinary Pre-science were used in this study. The animals 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.

Mice were divided into 2 equal groups. In the first group (control group, n = 14), animals daily received an oral dose of 0.5 mL 0.9% NaCl whereas in the second group (ENT group, n = 14) mice were orally treated by ENT (0.5 mL/day) for 7 first days. On day 8, 2.10⁶ EAC cells diluted in 0.5 mL of 0.9% NaCl were subcutaneously injected in the napes of all animals for inducing solid carcinoma, and oral respective treatments with ENT or NaCl were continued for 22 days (day 8 – day 30).

The tumour development was evaluated by palpation method. The tumour was under the skin of the nape. On day 30, animals were slaughtered after deep ether anaesthesia and tumour tissues were harvested and fixed in 10% formaldehyde solution, embedded in paraffin and sectioned at 5 µ thickness and collected on Poly-L-Lysine coated glasses. Sections were stained with the Haematoxylin-Eosin (HE) stain and then examined under Bs200Doc digital monitoring system.

The ENT composition was determined by HPLC analysis and was the following: resveratrol 23 µg/mL, quercetin 130 mg/mL, catechin 1.47 mg/mL and epicatechin 0.88 mg/mL.

#### 2. HISTOLOGICAL INVESTIGATIONS

**Proliferation indexes:**

The density of proliferative tumour cells was evaluated with proliferating cell nuclear antigen (PCNA) immunohis-
tochemistry, because the PCNA expression was enhanced during the G1/S phase of the cell cycle [13].

The evaluation of PCNA expression was 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 graded alcohol series and washed with 0.01 mol/L of PBS (phosphate-buffered saline). Endogenous peroxidase activity was quenched by application of 0.3 % H2O2 in PBS for 10 minutes and sections were placed in antigen retrieval solution (0.01 M of citrate buffer, pH 6.0) and heated in a microwave oven at 700 watts for 10 minutes. The tissue samples were incubated with 1:100 dilution of primer anti-PCNA antibody (Santa Cruz-7907-rabbit polyclonal) 50 minutes at room temperature. After rinsing three times with PBS, sections were incubated first in biotinylated secondary antibody, then in HRP (Horse Radish Peroxidase) Streptavidin at room temperature during 15 minutes for each step. Finally, sections were incubated 5 minutes in DAB (diaminobenzidine; Zymed) for the development of product reaction and counterstained with haematoxylin. The negative control sections were incubated with PBS instead of being incubated with the primer antibody PBS.

Immunohistochemical staining was 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) [38].

Determination of iNOS and eNOS expressions:

Five-micrometer formalin-fixed paraffin embedded tissue sections were mounted on poly-L-lysine slides. The slides were air-dried and the tissue sections were then deparaffinized. Specimens were washed in 0.01 mol/L phosphate-buffered saline (PBS). After three washes, tissues sections were incubated in an antigen retrieval solution (0.01 M citrate buffer, pH 6.0) for 15 min at 100°C in a microwave oven and endogenous peroxidase were blocked by 3 % hydrogen peroxide in distilled water for 15 min at room temperature. Subsequently, eNOS (1:100) and iNOS (1:100) rabbit polyclonal antibodies (NeoMarkers) were applied to sections and incubated at 4 ºC overnight. The sections were washed three times with PBS, and then incubated with biotinylated secondary antibody at room temperature for 30 minutes. Finally, immunohistochemical staining was performed using the avidin-biotin-peroxidase complex. The AEC (3-amino, 9 ethyl-carbazole) was used as a chromogen, and the sections were counterstained with haematoxylin.

The evaluation of the iNOS and eNOS immunohistochemical staining was done using HSCORE [44]. Briefly, tumour tissues stained with antibodies against eNOS or iNOS were evaluated using an Olympus microscope with a special ocular grid on 5 different fields at x 400 magnification by 2 blind observers. Positive stained cells were counted and graded according to the staining intensity: 0 (no staining), 1+ (weak but detectable above control), 2+ (distinct), 3+ (intense). For each tissue, the HSCORE value was given by the following formula:

\[ HSCORE = S \sum i (i + 1) \]

where “i” is the intensity score and “Pi” the corresponding percentage of cells presenting a given staining.

3. STATISTICAL ANALYSIS

For statistical analysis, software SPSS was used (SPSS 8.0, SPSS for Windows Advanced Statistics Release 8.0, 1997) and paired t-test and Mann–Whitney rank sum test were used for the group comparisons. The threshold value of p<0.05 was accepted as statistically significant.

Results

PROLIFERATION INDEXES:

The PCNA-positive cells in ENT and control groups exhibited a brown staining nucleus (figures 1 and 2). The proliferation index was significantly lower in the ENT group (59.0 ± 4.5%) than in the control group (77.0 ± 2.9%) (p < 0.05) (figure 3).

![Figure 1: PCNA staining (arrow) of tumour experimentally induced by a subcutaneous injection of 2.10^6 EAC cells in a mouse not treated by ENT (control group) x 800.](image1)

![Figure 2: PCNA staining (arrow) of tumour experimentally induced by a subcutaneous injection of 2.106 EAC cells in a mouse orally treated by ENT (0.5 mL / day) during 7 consecutive days before and 22 days after the carcinoma induction (ENT group) x 800.](image2)
In controls, the eNOS expression in the tumour cells was significantly lower than the iNOS expression as revealed by the respective HSCORE values presented in Table I (eNOS: 232.00 ± 30.60, iNOS: 310.40 ± 53.17). By contrast, when mice were previously treated with ENT (ENT group), the iNOS activity of tumour cells was dramatically depressed. Compared to the control group, the mean HSCORE value was significantly diminished (p < 0.05). On the other hand, the mean HSCORE value of eNOS activity was slightly increased but the difference with the control value was not statistically significant. Consequently, the expression of eNOS was significantly greater than the iNOS expression in tumours bearing by mice receiving the oral ENT treatment.

The iNOS and eNOS HSCORE values:

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Discussion

Cell proliferation is considered to play an important role in cancer prevention as well as in cancer development. It has been reported that nitric oxide synthase (NOS) isofoms are present in human tumour cell lines and solid tumour tissues [7, 53]. Since a Ca2+/calmodulin-dependent constitutive NO synthase (cNOS) activity in human epithelial type tumour cells was characterized [15], increased NOS expression was observed in lung [11, 20] and in colon cancer tissues [7, 40] when compared to the corresponding normal tissues. The increased expression was inversely correlated with the degree of differentiation in human gynaecological cancer [51]. Recent reports have shown that NO induces endothelial cell growth [29], mediates neovascularization and reduces blood flow in tumour tissues [7, 54]. Actually, NO is considered as a potential carcinogen. For example, high concentrations of NO and its metabolites such as peroxinitrite and NO2 cause DNA damage in the course of nitration, nitrosation and deamination [2, 37, 52]. Nitric oxide inhibits DNA ligase activity resulting in the accumulation of DNA breaks [23]. It has been suggested that elevated NO production enhances the growth of some tumours through the suppression of anti-
tumour immune responses [32]. These observations suggest that NO may have some roles in growth, progression, maintenance and/or metastasis of tumours.

The activated macrophage has been implicated in the destruction of tumour cells. Specifically, nitric oxide (NO) production seems to be a major effector mechanism by which macrophages destroy tumour cells [12, 26]. The NO production by activated macrophages is regulated by the inducible nitric oxide synthase isoform (iNOS) [36]. Polyphenol compounds such as resveratrol, quercetin, catechin... can decrease its expression [5, 17, 46] whereas they increase eNOS synthesis [48, 56, 57]. In the present study, treatment of mice with ENT (which contains polyphenols) induced a significant marked decrease of iNOS expression whereas the eNOS content in tumours was slightly increased.

In pathological conditions, the evaluation for a possible role of NO in the development of cancer has notably evolved. Many authors have examined iNOS expression in various neoplasms and have characterized the relationship between the extent of iNOS positivity and the degree of malignancy. However quite contradictory results have been reported. In extracutaneous malignancies, some groups have demonstrated a positive correlation between the over expression of iNOS and the nature of tumours, such as pancreatic cancer [55] gynaecological cancer [24] and gastric cancer linked to the Helicobacter pylori infection [22]. By contrast, REVENEAU et al. [42] showed that breast cancers with high iNOS activity had low proliferation rates and low neoplasm grading. Contrary to REVENEAU et al. [42], proliferation rates and iNOS activities were both increased in mammary tumour tissues of control mice in the present study. When mice were orally treated with ENT, the proliferation index of EAC cell induced carcinoma was markedly decreased and iNOS expression was dramatically depressed in parallel; the extent of the intensity of iNOS staining correlated with the degree of proliferation, suggesting that the decrease of iNOS activity would contribute to the reduction of cell proliferation. Although eNOS content was slightly enhanced in tumours from ENT treated mice, this modification would be too weak for directly altering proliferate cell density. Consequently, the antioxidant compounds of ENT suppress cell proliferation essentially by down regulating iNOS expression.

The treatment of mice with ENT has significantly reduced cell proliferation in tumours induced by injection of EAC cells, and the iNOS expression in the experimental tumours was also markedly depressed. As iNOS expression is usually positively correlated with high degrees of malignancy, its down regulation would delay the tumour proliferation and consequently, the process of tumorgenesis. In a word, ENT can suppress iNOS expression causing a delay in the development of tumours. However, cancer progression is regulated by many genes and the exact mechanism by which ENT induces the tumour cell suppression is unknown [21, 41]. It is clear that the relationship between ENT and inhibition of EAC needs further study. Additionally, it must be considered that the various ENT compounds can differently alter the growth of several cancer lines and their effects on those different cancer types must also be investigated. As a conclusion, our results showed protective effects of ENT on experimentally induced mammary tumours and the proliferation rate was decreased by NO dependent pathway in tumour tissues, but further experiments are required for elucidating molecular mechanisms in order to include ENT into therapeutic indications.

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References


50. - TASKIN E. I. AND COLLABORATORS


