Middle Black Sea Journal of Health Science August 2019; 5(2):151-159

RESEARCH ARTICLE

Protective Effects of Grape Molasses and Resveratrol Against DMBA Induced Oxidative Stress in Rat Ovarian Tissues

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Received: 22 July 2019, Accepted: 20 August 2019, Published online: 28 August 2019

Abstract

Objective: The polyphenolic compound, resveratrol (3, 4', 5-trihydroxystilbene), trans-resveratrol is a natural phytoalexin that exists in many different foods such as grape peel and seed, peanut, mulberry and blueberry. Molasses is a natural food obtained by boiling and concentrating fruit juices known to be rich in minerals such as iron, phosphorus, potassium, sodium, calcium and magnesium, and phosphoric acid, formic acid, organic acids and some vitamins. Polyphenolic compounds are natural antioxidants and are known to have protective effects against tissue damage caused by reactive oxygen species (ROS). In this study, we aimed to investigate the protective effects of grape molasses and resveratrol on ovarian damage induced by 7, 12-dimethylbenz [a] anthracene (DMBA).

Methods: A total of 42 old female Wistar Albino rats, aged 18 weeks were divided into six groups. 10 mg/kg DMBA was injected in the rats in DMBA group subcutaneously on day 0 and day 7 while grape molasses feed with 20% grape molasses was given along with DMBA application to the rats in DMBA + grape molasses group. Resveratrol was administered by subcutaneous injection at 10 mg/kg/day to the DMBA + Resveratrol group, while only feed with 20% grape molasses was given to the grape molasses group. Resveratrol was administered by subcutaneous injection at 10 mg/kg/day to the resveratrol group.

Results: GSH activity between the control group and molasses group, DMBA and DMBA + molasses groups and the control group and the DMBA + grape molasses groups was found statistically significant. Regarding the NO activity, the difference between the DMBA and resveratrol groups, DMBA and DMBA + resveratrol groups and the grape molasses and resveratrol groups was found statistically significant. MDA activity between DMBA + resveratrol and DMBA + grape molasses groups, and DMBA + grape molasses and grape molasses groups was found statistically significant.

Conclusion: Molasses as one of the most important nutrient sources of Eastern and Central Anatolia was observed to decrease ovarian tissue oxidative damage induced with DMBA compared to resveratrol.

Key words: DMBA (7, 12-dimethylbenz [a] antrasen), resveratrol, grape molasses, oxidative stress, ovarian

Suggested Citation: Kiran TR, Otlu O, Karabulut E, Bay Karabulut A. Protective Effects of Grape Molasses and Resveratrol Against DMBA Induced Oxidative Stress in Rat Ovarian Tissues. Middle Black Sea Journal of Health Science, 2019; 5(2):151-159

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DOI: 10.19127/mbsjohs.595016

Introduction

Rapid developments in technology, changing lifestyles, environmental pollution and chemicals cause negative impacts on human health and natural resources (Im et al., 2019). Therefore, people are exposed to many carcinogens and mutagenic substances of exogenous origin in their daily lives. This exposure may be air, water, food and soil borne and can transform into toxic compounds as a result of biotransformation reactions as well. Increased duration of exposure to such chemicals can cause irreversible and genetic disorders (David, 1999). Recent studies have demonstrated findings that consumption of green fresh vegetables and fruits protects the organism against toxic effects (Zhao, 1999).

Polycyclic aromatic hydrocarbons (PAH) are three or more aromatic ring compounds originating from different environmental and anthropogenic sources as a result of complete or incomplete combustion of carbon and hydrogen containing organic compounds and pyrolysis processes.

PAHs are carcinogens with tumor initiator, enhancer and promoter properties. PAHs inhibit cellular and humoral immunity (Armstrong et al., 2019). PAHs with low toxicity alone show tumor and mutagenic effect after undergoing metabolic activation. DMBA (dimethylbenz [a] anthracene), a PAH-member with environmental toxic effect, is known as procarcinogen and premutagenic. It is also effective as a teratogen, especially in the adrenal gland and fetal brain, which has been reported to exhibit high cytotoxicity and atherogenic properties in vivo - in vitro. DMBA causes genetic mutation as a result of the binding of intermediates from DMBA metabolism to DNA. It also destroys DNA structure and causes lipid peroxidation. It has been reported to exert a carcinogenic effect by increasing the formation of free radicals such as intracellular hydroxyl and superoxide anion radicals (Giovanni et al., 1980; Gao et al., 2007; Zeweil et al., 2019).

Resveratrol (3, 4', 5-trihydroxystilbene), a subpopulation of stilbenes, is a polyphenolic compound found in grapes, wine, peanuts and blueberries. Resveratrol is a non-flavonoid most intensively found in black grape clusters. Resveratrol is a compound of the phytoalexin group that the grape releases to protect against environmental stress and pathogenic attacks such as thirst, cold weather conditions, fungal infections, ultraviolet rays and ozone. There are many studies reporting that resveratrol presents a number of properties with different mechanisms of action. The inhibitory effect of resveratrol has been mainly associated with anti-xenobiotic ability (De-la-Lastra and Villegas, 2005; De-la-Lastra and Villegas, 2007).

Reactive oxygen species (ROS) are produced continuously in the body as a result of various metabolic and physiological processes. When the balance between ROS production and natural antioxidant activity deteriorates, a table of "oxidative stress" emerges that can lead to serious cellular damage, premature aging, and even cancer development. As a polyphenol, resveratrol is an antioxidant and free radical scavenger that inhibits reactive oxygen species (ROS) by activating AMP-activated protein kinase. It suppresses cyclooxygenase-2 (COX-2) and lipid peroxidation. Thus, resveratrol shows different pharmacological functions such as antiangiogenic, antioxidant, antitumor and cardio-protective and anticancer (Zadi et al., 2018; Al Fatease et al., 2019; Santos et al., 2019).

Molasses, which is a rich source of energy with organic acids, carbohydrates, minerals and various vitamins, is produced mostly from grapes in our country. Grape molasses contains Thiamin (B1) and Riboflavin (B2) and Niacin (B3) vitamins, Phosphorus (P), Iron (Fe), Copper (Cu), Zinc (Zn), Potassium (K), Sodium (Na), Magnesium Mg), and Calcium (Ca) minerals (Pharm et al.,2014). It is known that resveratrol in the content of black grape molasses inhibits the formation of reactive oxygen species (ROS) triggered by tumor necrosis factor (TNF), and lipid peroxidation in cells. There are also studies showing that resveratrol reduces tumor progression through the inhibition of cyclooxygenase-2 (Cox 2) as well as antimutagenic, cancer-inhibiting effects (Krishna et al., 2002; Yu et al., 2018; Zheng et al., 2018). There are several studies in the literature about protective effects of grape seeds and grape skin on various cancer types. However, we could not found any study about the effect of grape molasses on ovarian cancer. In the light of these information, the present study was designed to investigate the protective and preventive effect of resveratrol and molasses on DMBA toxicity. The aim of this study was to investigate the effect of resveratrol with grape molasses origin as antioxidant and anti-cancerogenic on the oxidative stress parameters in resveratrol and rat ovary tissue.

Methods

Chemicals, animals and diets

Wistar albino female rats used in this study were obtained from Inonu University Experimental Animal Production and Research Center. Guidelines of Inonu University Experimental Animal Ethics Committee was complied with during the study period. 18-weeks old female rats weighing 205 ± 13 g were kept in standard cages until the day of the experiment. Throughout the experiment, the drinking water was changed daily and the standard cage cleaning was done. The rats are housed in rooms with air conditioner in 24-27 °C room temperature with 12 hours of light and 12 hours of dark. A total of 42 rats were divided into 6 groups and fed with standard pellet food during the experiment except molasses groups. In the power analysis, the number of animals in each group considering % 90 power and 0.05 error margin.

Preparation of Resveratrol DMBA

In our study, a subcutaneously injected resveratrol mixture was prepared by dissolving 110 mg resveratrol in 110 ml DMSO, which is known as the solvent of many chemicals. 65 mg of DMBA was applied after being dissolved in 65 ml of sesame oil.

Preparation of Grape Molasses

Grape picked in the harvest period is cleaned out and the acidity is removed by adding grape marl after the crushing and squeezing process. After the resting and filtering process, the sun darkening process is applied and the molasses is packed. The black grape molasses used in the study was obtained from the Arapgir district of Malatya. Feed containing 20% molasses was prepared.

Experimental Design

Control Group (n=7): The rats in this group were injected subcutaneously 1 ml each day in a mixture of 20 ml of sesame oil and 30 ml of DMSO.

DMBA Group (n=7): 10 mg/kg DMBA was injected subcutaneously on day 0 and day 7.

DMBA + Molasses Group (n=7): 10 mg/kg DMBA was injected subcutaneously on day 0 and day 7. Molasses feed with 20% molasses was given every day.

DMBA + Resveratrol Group (n=7): Resveratrol was administered by subcutaneous injection at 10 mg/kg/day. 10 mg/kg DMBA was injected subcutaneously on day 0 and day 7.

Molasses Group (n=7): Molasses feed with 20% molasses was given every day.

Resveratrol Group (n=7): Resveratrol was administered by subcutaneous injection at 10 mg/kg/day.

Obtaining Ovarian Tissues and Preparing for Analyzes

On the 10^{th} day of the study, rats were sacrificed under general anesthesia. Ovarian tissues from rats were wrapped in aluminum foil and stored at -70 °C in deep freezing until the day when biochemical tests were to be carried out.

Tissue Homogenization and Tampons

Tissues weighed approximately 200 mg were homogenized at a rate of 16000 rpm by adding 2 ml of Tris - HCl tampon (pH: 7.0). After homogenization, the tubes were centrifuged at 4000 rpm for 10 minutes at +4 °C. Supernatants formed after centrifugation were taken into eppendorf tubes and kept in the freezer until the day of operation.

Estimation of Oxidative Stress Markers

Measurement of Reduced Glutathione

The absorbance of the yellow colored product resulting from the reaction of total sulphydryl content with Ellman's Reagent (DTNB) was determined by spectrophotometrically measuring at 412 nm (Tietze, 1969).

Measurement of Lipid Peroxidation

The measurement of MDA, a lipid peroxidation indicator, is based on the spectrophotometric evaluation of the resultant pink-red color absorbance at 532 nm as a result of the reaction of MDA in the sample with TBA at 95 °C (Uchiyama and Mihara, 1978).

Measurement of Nitric Oxide

NO formed by ambient NOS activity is measured at 545 nm in a color compound spectrophotometer, which is formed after the reaction with Griess reactivity by being reduced from nitrate to nitrite with cadmium garnets (Cortas and Wakid, 1990). For the standard measurement, the standard graphic was drawn with solutions prepared by a serial dilution of the 10 mmol/L $NaNO_3$ stock solution (5-200 μM).

Statistical Analysis

Because of the clinical variables are not normally distributed within groups, non-parametric statistics were used. Descriptive statistics were calculated by median, minimum and maximum. Group comparisons according to GSH, NO and MDA were evaluated by using Kruskal-Wallis Variance Analysis. For pairwise comparisons, Bonferroni adjustment was used. Type-I error rate was taken as α=0.05 for statistically significance. SPSS 21 software was used for statistical analyses (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.)

Results

Difference between study groups according to GSH, NO and MDA was statistically significant ($p<0.001$ for overall comparison). Significance values for pairwise comparisons of these groups were given in Table 1.

Table 1. Difference between study groups according to GSH, NO and MDA ($p<0.001$). Different letters (a, b, c) on the colons indicate statistical significance ($p<0.05$).

GROUPS		GSH (µmol/wet tissue)	NO (µmol/wet tissue)	MDA (µmol/wet tissue)
CONTROL	Median	1.89 [a,b]	3.07	16.83
DMBA	Median	1.79 [c]	4.79 [a]	22.44
DMBA + RESV.	Median	1.83	4.15 [c]	19.8 [b]
DMBA + G.MOLASSES	Median	1.84 [b,c]	4.09	19.14 [a,b]
GRAPE MOLASSES	Median	1.85 [a]	3.32 [b]	18.15 [a]
RESVERATROL	Median	1.85	3.51 [a,b,c]	17.66
Overall comparison		p<0.001	p<0.001	p<0.001
Pairwise comparison		[a] p=0.008	[a] p=0.018	[a] p<0.001
		[b] p<0.001	[b] p=0.022	[b] p=0.039
		[c] p=0.027	[c] p=0.004	

GSH activity between the control group and grape molasses group was found statistically significant (p=0.008). GSH activity between the DMBA and DMBA + grape molasses groups was found statistically significant (p=0.027). GSH activity between the control group and the DMBA + grape molasses groups was found statistically significant ($p<0.001$).

NO activity between the DMBA and resveratrol groups were found statistically significant (p=0.018) and was found statistically significant between DMBA and DMBA + resveratrol groups (p=0.004). NO activity was found to be significant between the grape molasses and resveratrol groups (p=0.022).

MDA activity between DMBA + resveratrol and DMBA + grape molasses groups was found statistically significant (p=0.039). MDA activity between DMBA + grape molasses and grape molasses groups was found statistically significant ($p<0.001$).

Discussion

Various animals are used in many in vivo studies in order to produce cancer models. The most commonly used animals for this purpose are rats. In this study we also used Wistar Albino rats in ovarian toxicity model induced with DMBA.

Recently protective effects of polyphenolic compounds obtained from various diet resources, against oxidative stress resulted from cancer and cancer drugs. Polyphenolic compounds are the most important part of natural plant products with known anti-inflammatory, antimicrobial, anti-alergenic and antioxidant effects (Francischi et al., 2017).

Resveratrol, which is one of the phenolic compounds, and richly found in grapes, wine, peanut and soy, has drawn attraction of scientists

and medical doctors for many years. Resveratrol is known to have anti-oxidative properties as scavenging reactive oxygen species (ROS) such as hydroxyl, superoxide and metal-induced radicals (Leonard et al., 2003; Truong et al., 2018). Grape molasses is a popular and traditional Turkish food produced in East and Middle Anatolia for long time (Ustun and Tosun, 1997). Grape molasse is mainly produced by concentrating the fruit juice with a soluble dry substance up to 70-80% (Batu et al., 2013). Although effects of various grape products grape seed, grape skin and grape pomac on oxidative stress have been studied in the literature, there is no any study directly investigating effects of grape molasses. In our study we investigated antioxidant effects of grape molasses in comparison of resveratrol, which is among the polyphenolic compounds found in grape molasses on ovarian cancer induced by DMBA in rats.

In the study by Kim et al., it was determined that grape seed extract was chemo-preventive against DMBA-induced breast cancer in adult rats and genistein exhibited similar activity in N-methyl-N-nitrosourea (MNU) breast cancer rat model, thus, it was concluded that both grape seed and genistein effects were based on diet (Kim et al., 2004). In another study conducted on rats, the anti-tumor effect of polyphenolic fractions isolated from grape seeds was investigated by establishing a two-step carcinogenesis protocol with DMBA and TPA (12-O-tetradecanoylphorbol). Extracted polyphenolic fractions have been found to have inhibitory effect on epidermal lipid peroxidation. In the present study we also used DMBA to induce ovarian cancer in rats.

The antioxidant activities of grape and grape seed phenolics have been studied in different models in in vitro systems, and low-density lipoprotein (LDL) has been determined to protect Cu^{+2} against SIN-1-mediated oxidation producing the oxygen-based radical generator 2, 2-azobis (2-amidinopropane) dihydrochloride (AAPH) or peroxynitrite. It has been found to protect spleen cells against DNA damage induced by hydrogen peroxide (H_2O_2) and reduce oxidative stress in PC12 cells that are stimulated by the addition of Fe^{2+} and t-butyl hydroperoxide (Shafiee et al., 2003; Chanvitayapongs et al., 1997). Anticancer effects of grape antioxidants in in vitro and in vivo models have been studied and have been shown to induce cell cycle blockage and apoptosis in cancer cells, and to inhibit carcinogenesis and cancer progression in rodent models (Aggarwal et al., 2004; Garvin et al., 2006; Ebeler et al., 2002). Male Wistar rats were fed with diets containing cellulose (control) and grape fiber for 4 weeks and GSH: GSSG ratio, GSSG / 2GSH pair redox status, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), lipid peroxidation (LPO) and apoptosis level were evaluated. In the results obtained, the cytosolic GSH: GSSG ratio increased in the grape fiber diet group, and the decline in apoptosis in relation to diet has been attributed to the modulation of the glutathione redox system and endogenous antioxidant enzymes (Lopez et al., 2010). In another study, a reduction was observed in inhibition of oxidative stress, immunosuppression, reduction of tumor growth, tumor diversity and malignant transformation of papillomas to carcinomas in UVB-induced skin cancer in rats administered with grape seed pro-anthocyanidin diet supplement (Katiyar, 2008). It was found in another study that grape pomac flour alleviated hepatic oxidative stress induced in carps, by improving antioxidant defence. It was reported that this effect occurred by prevention of overproduced ROS and NOx as well as by prevention of lipid damage (Souza et al., 2019).

A study investigating the effect of grape juice consumption on 7, 12-dimethylbenz [a] anthracene (DMBA)-induced female rat breast tumorigenesis has shown that tumor mass and tumor growth was inhibited. It has been concluded that specific components or phytochemicals in purple grape juice may interfere with the initial phase of DMBA-induced rat breast tumorigenesis (Jung et al., 2006).

In MCF-7 rat cells transfected with aromatase, grape seed extract has been found to inhibit enzyme activity and formation responsible for the transformation of androgens into estrogens and suppress estrogen biosynthesis of procyanidin B2 dimer obtained from grape seed and red wine (Eng et al., 2003; Kijima et al., 2006).

The medium-term oral carcinogenesis process initiated by 4-nitroquinoline 1-oxide (4NQO) was initiated to evaluate anti-tumor activity of grape juice concentrate. As a result of the study, there was a decrease in hyperplastic and dysplastic lesions, a decrease in COX-2 and TNF-alpha and eNOS gene expression, and an increase in SOD Cu / Zn and catalase activity in the grape juice supplement group (Pacheco et al., 2014).

In another study, grape seed pro-anthocyanidins (GSP) was reported to have increased pro-apoptotic Bax protein expression, decreased anti-apoptotic Bcl2, Bcl-xl protein expression, caused degradation in mitochondrial membrane potential, and induced apoptosis in NSCLC, A549, and H1299 cells in

vitro associated with activation of 9, 3 caspases, poly (ADP- ribose) polymerase (PARP) (Singh et al.,2011).

60 healthy volunteers aged 19-57 (16 females and 51 males) were given 480 ml of grape juice daily for an additional 8 weeks to the daily diet and blood samples were obtained from these patients. DNA damage was measured using a single cell gel (comet) test with alkaline electrophoresis. A significant reduction in lymphocyte DNA damage and reduction in the number of ROS/photons by 15% were determined compared to the beginning of the study (Park et al., 2003)

It has been found that grape seed proanthocyanidins have the ability to inhibit the invasion of human cutaneous HNSCC cells by reversing the epithelial-mesenchymal transition process targeting EGFR expression, can act as free radical scavengers and help reduce reactive oxygen species (ROS) (Sun et al., 2011).

In another study, the combination of resveratrol, quercetin and catechin (0.5, 5 or 20 μM) were found to significantly reduce cell proliferation, block in vitro cell cycle continuity and reduce primary tumor growth (Schlachterman et al., 2008). In a recent study it was demonstrated that resveratrol suppressed tumor growth and inhibited leiomyoma cells in vitro (Chen et al., 2019). Again in another study, resveratrol was shown to inhibit oxidative stress induced with aflatoxin B1 in bovine mammary epithelial cells (Zhou et al., 2019).

Our findings support the hypothesis that resveratrol, of the polyphenolic compounds found in molasses, restricts DMBA-induced oxidative stress possibly through a reduction in free radical levels. In the literature review, while there were studies on anticancer in the presence of resveratrol in fruits in grape and various fruit juice concentrates, there were no studies comparing the effects of grape molasses and resveratrol on the rat reproductive organ.

Understanding the preventing and improving mechanisms of ovarian-damage of resveratrol and grape molasses which is found at higher rates in some foods suggests that it will play an active role in preventing many diseases. It is known that in recent years there has been an increase in the interest of alternative herbal or local foods in order to prevent or reduce tissue damage caused by cancer or cancer treatments. For this reason, several studies have been conducted on the antioxidant properties of green tea containing catechin and its derivatives, red onion containing quercetin, apples, tomatoes containing lycopene, foods containing isoflavones such as soybean, chickpeas and lentils, broccoli and brussels sprouts containing indol-3-carbinol, pomegranate containing polyphenol and ellagic acid, and many food products containing selenium, vitamin E and D, anthocyanin, sulforaphane, citylbin and resveratrol. In addition to the above-mentioned food products, our study results suggest grape molasses containing resveratrol, also known as black miracle, as an important antioxidant source. By increasing the number of groups specified, the effects on the metabolism damage at different doses can be supported by further clinical trials.

Conclusion

It was supported by the findings that the molasses as one of the most important nutrient sources of Eastern and Central Anatolia played an effective role as an effective agent in both the protection and the healing process when its effect on the reproductive system was compared to its active ingredient, resveratrol.

Acknowledgements

We thanks for all collaboration and contribution with laboratory personnel and Research Center of Experimental Animal Laboratory in Inonu University.

Ethics Committee Approval: Ethics committee approval was received for this study from Clinical Research Ethics Committee of Inonu University (2011/A-106).

Peer-review: Externally peer-reviewed.

Author Contributions: Externally peer-reviewed.

Author Contributions: TRK, ABK Design TRK, ABK Supervision TRK, OO; Materials –TRK, ABK; Data Collection and/or Processing – TRK, OO, EK, ABK; Analysis and/or Interpretation - TRK, OO, EK, ABK; Literature Review - TRK, OO, EK, ABK; Writing - TRK, OO, EK, ABK; Critical Review - TRK, OO, EK, ABK.

Conflict of Interest: No conflict of interest was declared by the author.

Financial Disclosure: The authors declared that this study hasn't received no financial support.

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