

Comparing the Effect of Grape Fermentative Product and Fresh Red Grape Juice on Antioxidant Biomarkers of Liver Mitochondria Isolated From Rats in Vitro



Amir Keshavarzi¹ , Akram Ranjbar² , Nejat Kheiripour³ , Ali Ghaleiha¹ , Alireza Soltaniyan , Seyyed Mostafa Hashemi^{1*} 

1. Research Center for Behavioral Disorders and Substances Abuse, Hamadan University of Medical Sciences, Hamadan, Iran.
2. Medicinal Plants and Natural Products Research Center, Hamadan University of Medical Sciences, Hamadan, Iran.
3. Research Center for Biochemistry and Nutrition in Metabolic Diseases, Kashan University of Medical Sciences, Kashan, Iran.
4. Department of Epidemiology, Faculty of Health, Hamadan University of Medical Sciences, Hamadan, Iran.



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ABSTRACT

Background: Mitochondria are a source of reactive oxygen species (ROS), and several natural compounds are used as antioxidant agents. This study aimed to investigate and compare the effects of fresh grape juice red wine on oxidative stress biomarkers in rat liver mitochondria.

Materials and Methods: In this regard, mitochondria were isolated from the liver of 27 male Wistar rats (220-250 g). The isolated mitochondria were cultured in different doses of red wine and fresh red grape juice for 24, 48, and 72 h. After treatment, total antioxidant capacity, lipid peroxidation, total thiol groups, and catalase activity were determined in the isolated mitochondria of the rat liver.

Results: The results confirmed the oxidant/antioxidant effects of red wine and fresh red grape juice at different times.

Conclusion: According to the results, the fresh red grape juice showed higher antioxidant properties than red wine in the liver mitochondrial samples.

1. Introduction

Fruits play vital roles in protecting the overall health of the body. This beneficial effect is due to various phenolic and antioxidants compounds [1]. The presence of antioxidants reduces the amount of oxidizing substances

through nitrogen donation, free oxygen fixation, and absorption of free radicals [2]. Grape (*Vitis vinifera*) and its red wine extract are among the richest sources of phenolic and antioxidant compounds. They can reduce oxidative stress and prevent oxidation of lipoproteins and accumulation of cholesterol in macrophages due to the presence of polyphenols such as resveratrol, catechin, epigallocatechin gallate, and rutin [3]. By the way, the

* Corresponding Author:

Seyyed Mostafa Hashemi, PhD.

Address: Research Center for Behavioral Disorders and Substances Abuse, Hamadan University of Medical Sciences, Hamadan, Iran.

Phone: +98 (910) 7608801

E-mail: dr.hashemi71@gmail.com

simultaneous presence of alcohol and antioxidants in red wine can reduce its antioxidant properties and can lead to irreversible complications through the formation of free radicals [4]. In general, free radicals are active and unstable compounds. They have a high affinity to react with other molecules such as DNA, proteins, and carbohydrates to compensate for their electron deficiency and stabilize them, instead make them unstable and destroy those molecules [5]. By increasing the free radicals or reducing antioxidant activity, the balance between oxidants and antioxidants collapses, which is the origin and progression of many diseases. Antioxidant systems, which include enzymatic and non-enzymatic ones, fight against oxidation stress conditions [6].

Various studies have shown that during the metabolism of ethanol, the generated free radicals can suppress the cell's antioxidant defense system [7-10]. Alcoholic beverages are considered the main oxidative stressors in the oxidative process. Long-term consumption of ethanol not only increases the production of free radicals but also reduces the levels of enzymatic and non-enzymatic antioxidants. This process can lead to oxidative stress and, ultimately, the development of diseases such as alcoholic fatty liver, liver cirrhosis, hepatocellular carcinoma, and the like [11-13]. Therefore, this study aimed to compare the effect of red grape juice with its fermentation product (red wine) on mitochondrial oxidant/antioxidant biomarkers, such as total antioxidant capacity (TAC), the concentration of thiol groups, levels of lipid peroxidation (LPO), and catalase (CAT) activities in rat liver mitochondria.

2. Materials and Methods

Reagents and chemicals were provided from Sigma-Aldrich (St. Louis, MA, USA). They comprised acetate buffer, ferric chloride, 2-thiobarbituric acid (TBA), tetraethoxypropane (TEP), 5,5-Dithiobis-2-nitrobenzoic acid (DTNB), trichloroacetic acid (TCA), 2, 4, 6-tripyridyl-S-triazine (TPTZ), Tris base, n-butanol, hydrochloric acid, ethylenediamine tetra-acetic acid (EDTA). All the other chemicals were of analytical grade.

Experimental design

In this study, after isolating the liver of 27 male Wistar rats (220-250 g), they were become homogeneous by a homogenizer. It should be noted that there were three rats in each group for 24, 48, and 72 hours and the experiments were repeated three times. Then, to separate mitochondria from the liver cells, homogenized tissues were centrifuged for 20 minutes. Finally, the specimens were stored at - 80°C to measure biochemical parameters [14]. In the

next step, 50 μ L of mitochondria samples were cultured in aerobic conditions containing a grape fermentation product such as red wine and grape juice with a concentration of 100 μ L. After 24, 48, and 72 hours, for determination of mitochondria viability, Janus green B staining was performed. Then, the biochemical parameters, including the levels of LPO, TAC, and TTM, were examined.

Measuring lipid peroxidation

Lipid peroxide levels were measured according to the Yagi method [15]. In this method, malondialdehyde (MDA), as a lipid peroxidation product, reacts with TBA as thiobarbituric acid reactive substances (TBARS) and creates a red complex absorbs maximally at 532 nm. Briefly, 100 μ L sodium dodecyl sulfate (SDS), 1.5 mL of TCA, 1 mL of TBA, and 4 mL of water were added to the 50 μ L of the sample, and the mixture was incubated in a 95°C water bath for 60 min. Then, the mixture tubes were cooled with water, and 5 mL of n-butanol was added to the mixture and shaken vigorously. Next, the mixture was centrifuged at 3000 rpm, and the upper n-butanol layer of the sample was removed. Finally, the fluorescence intensity was measured at the wavelength of 532 nm. To determine the concentrations of TBARS, we used the calibration curve of tetraethoxypropane standard dilutions.

Measurement of TAC

TAC was determined by FRAP (the ferric reducing ability of plasma) assay according to the Benzie and Strain method [16]. In this method, at low pH, the reduction of the ferric-tripyridyl triazine (Fe III-TPTZ) complex to the form of Fe II (with blue color) can be measured in a maximum absorbance at 593 nm wavelength. This reaction is non-specific, and the absorption changes directly correlate with the overall antioxidant electron production in the reaction mixture. In this method, 100 μ L of the sample was added to the 3 mL of FRAP reagent, and then the mixture was incubated at 37°C for 4 min, and absorption changes were measured at 593 nm. Eventually, the amount of light absorbed by the sample was measured again [13]. FeSO₄ solutions from 0.1 to 1 mM were used for calibration. The values were expressed as μ mol/mg protein.

Measurement of protein thiol groups

2.2 Di-nitro benzoic acid (DTNB) colorimetric methods were used for measuring protein thiol groups. DNTB, in reaction with the SH groups, produces a yellow complex that has a maximum absorption at 412 nm

[17]. Briefly, in this method, 50 μ L of homogenate sample was mixed with 150 μ L of Tris-EDTA buffer (pH=8.2), 10 μ L of DTNB reagent, and 790 μ L of absolute methanol. Then, the mixture was incubated for 15 minutes at room temperature and centrifuged at 1000 g for 10 min. Then, the mixture absorbance was measured at the maximum wavelength of 412 nm against Tris-EDTA buffer (A1). Afterward, by adding 20 μ L of the DTNB reagent (10 mM in methanol) to the mixture, the changes in absorbance were reread after 15 min (A2). Moreover, the absorbance of the DTNB reagent was also measured as blank (B), and the total concentration of thiol groups was calculated from the below equation: Total thiol concentration (mM)=(A2-A1-B) \times 1.57 mM

Quantification of catalase activity

The determination of catalase activity was carried out using a BioVision competitive ELISA kit according to its instructions [18].

Total protein

To prepare the Bradford reagent, we dissolved 100 mg of Coomassie Brilliant Blue G-250 in 50 mL of ethanol (95%) and then added 100 mL of phosphoric acid (85%) to the mixture. Next, by adding 5 mL reagent to 100 μ L of the sample and after 5 min, the changes in the absorbance were measured at 595 nm. Standard solutions of bovine serum albumin (BSA) are used to produce a standard curve of absorbance versus mass concentration [19].

Statistical analysis

The analysis was carried out in SPSS version 16.0 (SPSS Inc., Chicago-USA) and GraphPad Prism version 6.0 (GraphPad Software, San Diego-USA). In this study, due to uncertainty in the assumption of normality and the small sample size in non-parametric equivalent groups, we used 1-way ANOVA. The obtained data were expressed as Mean \pm SD, and P<0.05 was considered statistically significant.

3. Results

Mitochondrial viability

Both fresh grape juice and red wine increased the vitality of mitochondria extracted from rat liver compared to the control group after 24, 48, and 72 h (Figure 1), which this increase in grape juice was higher

than the fermentation product. However, these differences in the intervention group were not significant compared to the control group (Figure 1).

Oxidative stress parameters

The results of the present study showed that red wine significantly (P<0.05) increased the TAC (at different times), catalase (after 24 h), and TTG (after 24 and 48 h), while significantly (P<0.05) decreased MDA level in liver mitochondrial (after 24 h).

Also, grape juice increased significantly (P<0.05) TTG (after 24 h), while significantly (P<0.05) decreased MDA level in liver mitochondrial (after 24 h). On the other hand, the administration of grape juice had no significant effect on TAC and catalase activity (at different times).

Lipid peroxidation levels

The effect of grape juice and red wine on the MDA level of mitochondria extracted from rat liver is shown in Figure 2. Both fresh grape juice and red wine significantly decreased MDA levels in mitochondria than the control sample after 24 h (P<0.05). But it was not significantly different from the control group in 48 and 72 h.

Total Thiol Groups (TTG)

Both fresh grape juice and red wine increased in TTG, and this difference was significant in comparison to the control group after 24 h; (P<0.01) for grape juice and (P<0.05) for red wine (Figure 3). Also, red wine has caused a significant increase in TTG level compared to the control group in 24 and 48 h (P<0.05). Both fresh grape juice and fermentation product were not significantly different in comparison with the control group after 72 h of treatment.

Total antioxidant capacity (TAC)

As shown in Figure 4, red wine caused a significant increase in TAC of mitochondria compared to the control group after 24, 48, and 72 hours (P<0.05).

Catalase activity rate

Red wine increased catalase activity in rat liver mitochondria, which was significantly different from the control group in 24 h (P<0.05) and was not significantly different at 48 and 72 h times (Figure 5).

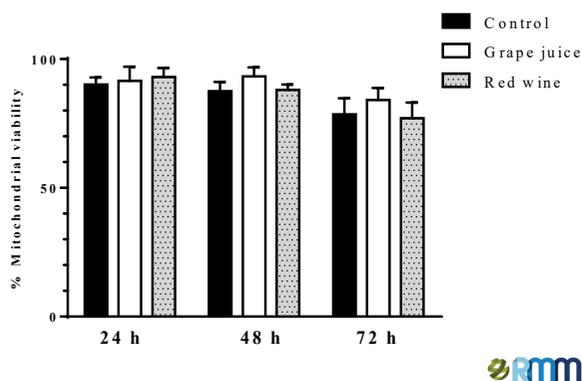


Figure 1. Effect of fresh grape juice and red wine (100 µM) on the vitality of mitochondria extracted from rat liver

4. Discussion

This study aimed to investigate and compare the anti-oxidant effects of red wine as a fermentation product of grape (*Vitis vinifera*) and fresh natural grape juice on oxidative stress biomarkers of mitochondria isolated from rat liver after 24, 48, and 72 h incubation.

Many studies have been conducted on the antioxidant properties of red wine around the world. These studies have led to the French paradox theory, which suggests the positive antioxidant results of red wine. Teissedre et al. demonstrated that phenolic compounds in grapes and red wine have inhibitory effects on the LDL oxidation process, and the antioxidant effects of red wine were more than grape juice [20]. Pazzini et al. (2015) demonstrated the effect of tannat red wine on oxidative stress induced by glucose and fructose in erythrocytes [21]. Results of this study have revealed that erythrocytes which were incubated with glucose and fructose, increased lipid peroxidation levels, while in the presence of red wine, this condition was prevented, and a significant reduction in the oxidative stress was the outcome [21]. However, the

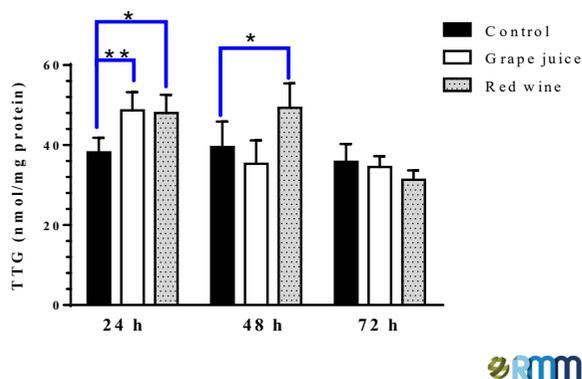


Figure 3. Effect of grape juice and red wine on the TTG levels
*P<0.05, **P<0.01.

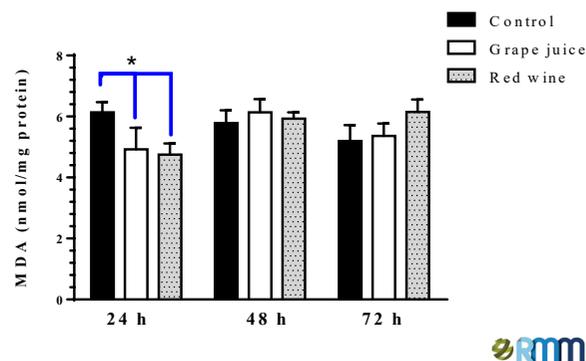


Figure 2. Effect of grape juice and red wine on the MDA level of mitochondria extracted from rat liver
*P<0.05.

presence of alcohol (ethanol) in red wine and other fruit fermentation products not only increases the production of free radicals but also induces levels of enzymatic and non-enzymatic antioxidants [22]. In this study, oxidative stress parameters such as MDA, TTG, TAC, and catalase activity in the rat liver mitochondria treated with fresh grape and red wine were investigated.

ROS formation within the mitochondria making these organelles more susceptible to oxidative damage [23, 24]. Oxidative mitochondrial damage is a recognized cause of the collapse of the mitochondrial membrane potential and the onset of mitochondria permeability transition (MPT), which also modifies mitochondrial DNA (mtDNA) [25]. Extensive MPT leads to mitochondrial swelling and precipitates hepatocyte necrosis, while, by releasing cytochrome c, transient MPT triggers apoptosis [26]. The results demonstrated that both fresh grape and red wine significantly affect mentioned oxidant/antioxidant parameters compared to the control group. Moreover, the present study results showed that both red wine and fresh grape juice have approximately the same antioxidant properties.

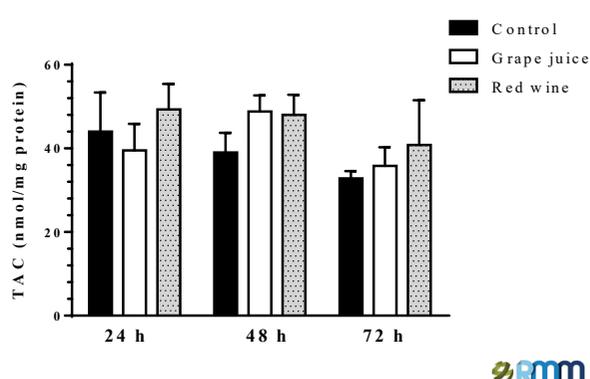


Figure 4. Effect of grape juice and red wine on the TAC level
*P<0.05.

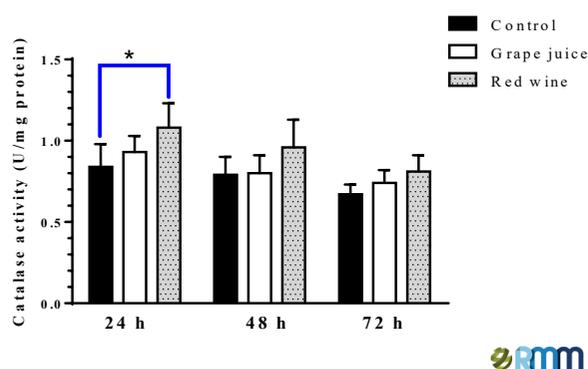


Figure 5. Effect of grape juice and red wine on the catalase activity rate

* $P < 0.05$.

In terms of lipid peroxidation, red wine and grape juice showed a significant decrease in the level of MDA compared to the control group. Comparing our results with various studies, it has been shown that fermentation products of grape such as red wine have antioxidant properties due to compounds such as resveratrol. P. Lacopini et al. indicated the antioxidant effects of ethanolic solution (red wine) extracted from 10 species of red grapes grown in the Tuscan region of Italy are because of the presence of catechin, epicatechin, quercetin, rutin, and resveratrol. These compounds decreased proxy-nitrite and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. Therefore, based on the findings of this study, the antioxidant function of the red grape extract does not depend entirely on the total phenol, but the effect of the phenolic compounds can be synergistic or antagonistic, or a combination of these compound [27]. Macedo et al. evaluated the antioxidant effects of red wine on oxidative stress induced by a high-fat diet [28]. In this study, the samples were divided into three wine-fed groups with low, medium, and high antioxidant activity. The results showed that red wine did not have the same effect on all antioxidant biomarkers. They also reported that the MDA concentration in the group with high antioxidant activity was lower than the other groups, similar to our results [28].

Findings of Tedesco et al. in relation to catalase activity showed that antioxidant effects of red wine are due to the presence of substances like anthocyanin, at least in part. They demonstrated that in human erythrocytes deprived of catalase activity due to the treatment with 4 mM sodium azide, red wine could protect human RBCs from oxidative stress through increasing catalase activity [29]. The results of our study demonstrated that treatment with red wine strongly increased TAC and TTG levels compared to the untreated control group. This observation is in line with Connor et al. showing that grape juice induces TAC, thereby suggesting antioxi-

dant's beneficial effects on the grapes [30]. This study had some limitations. First, it was better to use different doses. Second, the number of samples was low. Also, we could use the active ingredient in grapes and wine and studied the molecular pathways.

5. Conclusion

In summary, both fresh grape juice and red wine without any apparent preference increase the mitochondrial viability, antioxidant capacity, catalase activity, and thiol levels while reducing lipid peroxidation and the oxidative stress caused by mitochondria-free radicals. It is hoped that this study provides a background for future studies on the effect of red grape and its fermentation product (like red wine, vinegar, etc.) on humans through biopsy or autopsy from the human liver or other organs. Also, it seems necessary to perform a detailed analysis of the toxic dose of red wine due to the presence of alcohol and antioxidant superiority of wine over grape juice or vice versa.

Ethical Considerations

Compliance with ethical guidelines

The study was approved by the Ethics Committee Guidelines of Hamadan University of Medical Sciences (Code: IR.UMSHA.REC.1396.451).

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Authors' contributions

Investigation, writing – original draft, and approval of the final draft: All authors; Methodology: Seyed Mostafa Hashemi and Nejat Kheiripour; Data analysis and co-writing the paper: Akram Ranjbar, Nejat Kheiripour, and Ali Ghaleiha; Supervision: Amir Keshavarzi.

Conflict of interest

The authors declared no conflict of interest.

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