Effects of grape wine and apple cider vinegar on oxidative and antioxidative status in high cholesterol-fed rats

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ABSTRACT

Background: Oxidative stress is the result of an imbalance between the rates of free radical production and elimination via endogenous antioxidant mechanisms such as antioxidant enzymes, which include glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT). There are mainly two vinegar production methods. The first is the surface method which is also known as the traditional method. The second method is known as the industrial method or submerged method which involves the use of a submerged culture with supplemented aeration.

Objective: The aim of this study is to determine the effects of grape and apple cider vinegar consumption against oxidative stress in rats fed a high cholesterol diet.

Methods: Fifty-four male, adult Wistar albino rats were included in this study. Rats were fed for 7 weeks by oral gavage as given in the experimental procedure. Rats were sacrificed at the end of the experiment and blood samples were collected. Catalase (CAT) activity, malondialdehyde level (MDA), glutathione peroxidase (GSH-Px) activity, superoxide dismutase (SOD) activity were analyzed. Grape and apple vinegar fermentation products prepared using both the surface culture method and submerged methods were prepared. The total antioxidant activity of vinegar samples were measured by Oxygen Radical Absorbance Capacity (ORAC) and 2,2'-azinobis (3-ethlybenzthiazoline)-6-sulfonic acid (ABTS) methods.

Results: Levels of CAT, GSH-Px, SOD in high cholesterol diet group (CHCNT) were significantly decreased while MDA levels were significantly increased in control rats fed with the high-cholesterol diet (CHCNT) when compared to the Control group (CNT) (P<0.05). Levels of MDA, which is the end-product of lipid peroxidation, were significantly decreased in the apple cider vinegar administered groups when compared to the CHCNT (P<0.05). GSH-Px levels were significantly increased in rat groups, which were fed with the vinegars produced by traditional surface methods (P=0.03, P=0.001 respectively) as compared to the CHCNT. SOD levels of rat groups which were fed with all the vinegars were significantly increased as compared to CHCNT group (p<0.05).

Conclusions: This study indicated that a high cholesterol diet increased lipid peroxidation and consumed the antioxidant enzymes. Although the impact of vinegars on antioxidant enzyme activity differs, the use of vinegar and especially vinegars produced by surface culture methods seem to have favorable effect *in vivo*.

Keywords: Oxidative stress, grape vinegar, apple cider vinegar, glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT)

BACKGROUND

Humans are exposed to a large number of chemical, biological and physical agents, as well as environmental determinants that can impinge on health. The ability of humans to fight against these factors is important for maintenance of their health and productivity [1]. Oxygen free radicals and other reactive oxygen species are produced in the human body as by-products through numerous physiological and biochemical processes. As a result of aerobic metabolism, oxygen related free radicals (superoxide and hydroxyl radicals) and reactive species (hydrogen peroxide, nitric oxide, peroxynitrile and hypochlorous acid) are produced in the body [2, 3]. An increase in oxidative stress is the result of an imbalance between the rates of free radical production and elimination via endogenous antioxidant mechanisms such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) [4].

In humans, free radicals have been associated with over one hundred diseases which include arthritis, hemorrhagic shock, atherosclerosis, early aging, ischemia and reperfusion injury of many organs, Alzheimer's and Parkinson's disease, gastrointestinal dysfunctions, tumor promotion and carcinogenesis, and more. Consequently, endogenous and exogenous antioxidants are believed to be important for preventing such diseases. The exogenous antioxidants that are widely available in fruits, vegetables, nuts and seeds have a broad spectrum of biological, pharmacological and therapeutic activities against free radicals and oxidative stress [5]. Although the bioavailability and therapeutic efficacy of antioxidants differ vastly, the antioxidants have been demonstrated to improve human health [5]. The exogenous antioxidants are further supported with the endogenous antioxidant enzymes, such as SOD, CAT, glutathione reductase (GSH-R) and GSH-Px that exert synergistic actions in removing free radicals from cells [6].

Many fruits and vegetables have high amounts of polyphenolic compounds which are known antioxidants [7]. Polyphenolics have been reported to play an important role in human

health and in modification or prevention of several diseases including cancer, hypertension, heart attack and diabetes [2]. Additionally, foods derived from fruits and vegetables such as fruit juices, wines and vinegars also contain significant amount of polyphenolic compounds. Vinegar is produced from different varieties of fruits by a two fermentation step; ethanol and acetic acid fermentations. There are two main vinegar production methods. The first is known as the "surface method." In this method, following wine production, a culture of acetic acid bacteria is inoculated on the surface of the vat and incubated. The surface method is also known as the traditional method. The second method is known as the industrial method or submerged method which involves use of a submerged culture with supplemented aeration [8].

Budak and Guzel-Seydim [9] reported the antioxidant activity of traditional wine vinegar was higher than industrial wine vinegar. ORAC and TEAC levels of traditional wine vinegar were 10.50 μmol/mL TE and 13.50 mmol/L TEAC, while ORAC and TEAC levels of the industrial wine vinegar were 8.84 μmol/mL TE and 10.37 mmol/L TEAC [9]. In another study of traditional vinegar samples, the highest level of total phenolic and flavonoid compounds and the levels of anti-radical activity were obtained in grape vinegars [10]. Chou et al. [11] demonstrated that Chinese black vinegar contained polyphenolic substances, especially catechin and chlorogenic acid. Budak et al. [12] summarized the functional properties of vinegar; in particular, health effects of vinegar arising from consuming the inherent bioactive components such as acetic acid, gallic acid, catechin, epicatechin, chlorogenic acid, caffeic acid, and p-coumaric acid, in addition to ferulic acid cause antioxidative, antidiabetic, antimicrobial, antitumor, antiobesity, antihypertensive, and cholesterol-lowering responses.

The aim of the present study was to determine the effects of grape and apple cider vinegar consumption against oxidative stress in rats fed a high cholesterol diet. Furthermore, vinegars produced by both the traditional and industrial techniques were compared by evaluating parameters that reflected antioxidant and oxidant status of the rats.

METHODS
Vinegar Production
"*Ulugbey karasi*" grapes and "*Red delicious*" apples were used to make vinegar by traditional surface and industrial submerged methods. Vinegars were produced in the facilities of Süleyman Demirel University Department of Food Engineering in cooperation with Carl Kuhne Vinegar Plant (Afyon, Turkey).

Antioxidant Activity of Vinegar Samples
ABTS (2,2'-azinobis (3-ethlybenzthiazoline)-6-sulfonic acid) Assay
A solution of 2, 2'-azinobis (3-ethlybenzthiazolin-6-sulfonic acid) diammonium salt ($ABTS^+$) radical cation was prepared by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate [13]. $ABTS^+$ inhibition against Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was spectrophotometrically measured. The concentration of the resulting blue-green ABTS radical solution was adjusted to an absorbance of 0.700 ± 0.020 at 734 nm in a spectrophotometer (Shimadzu Scientific Instruments, Inc., Tokyo, Japan). TEAC values were calculated from a Trolox standard curve and expressed as Trolox equivalents (in mM).

ORAC (Oxygen Radical Absorbance Capacity) Assay

All samples were analyzed by Oxygen Radical Absorbance Capacity (ORAC) assay described previously [14]. The samples were diluted with phosphate buffer (pH 7.4) for ORAC analysis. An aliquot (25 µL) of the diluted sample, blank (phosphate buffer) or Trolox calibration solutions were added to a black, clear-bottom well in a 96 well plate; samples were conducted in triplicate. After the addition of 150 µM fluorescein stock solution (0.004 µM) to each well, the microplate was incubated at 37 °C for 30 min. The 25 µL 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) solution (153 mM) was then added to start the reaction. The bottom reading microplate reader was programmed to record the fluorescence reading with an excitation-emission wavelength of 485 – 520 nm using Gen 5^{TM} software. Antioxidant activity was kinetically measured with a Biotek Synergy™ HT Multi-Detection Microplate Reader (Winooski, Vermont, USA). ORAC values were expressed as µmol of Trolox equivalents (TE) per mL of sample (µmol TE/ml).

Experimental Design

This study was approved by the Animal Care Ethical Committee at Süleyman Demirel University. Research and was carried out at Animal Production and Research Center in Süleyman Demirel University, Isparta, Turkey. Fifty-four male Wistar albino rats weighing between 200-250 g were used in the study. The rats were kept in a room with 12 hours light-dark cycle. The room temperature was controlled at 24°C and the rats were given ad libitum access to food and water. Rats were divided into six treatment groups (Table 1). One mL of 2.5% cholesterol and 1 mL of each vinegar sample was administered daily by oral gavage [15] for 7 weeks; vinegars were orally gavaged at 9 am and cholesterol solution was orally gavaged at 5 pm during the experiment. The control-diet (CNT) group received 1 mL of normal saline solution concurrently with the experiment groups. Rats were anesthetized with intraperitoneal 2% xylazine (10 mg/kg) and 10% ketamine (80 mg/kg) at the end of the experiment. Blood samples were taken from the inferior vena cava and rats were sacrificed by exsanguination. The anticoagulated blood was separated into plasma and erythrocytes by centrifugation at 1500g for 10 min at +4°C. The erythrocyte samples were washed three times in normal saline (0.9% w/v) and then hemolyzed with 2 mL of cold distilled water. All samples were portioned and kept -80°C until the day of analysis.

Table 1. Experimental Design

Group	Treatment (N=54, n=9)	Code
1	Rat chow (control)	CNT
2	High cholesterol diet (cholesterol control)	CHCNT
3	High cholesterol diet + grape vinegar produced using surface technique	TGV
4	High cholesterol diet + grape vinegar produced using submerged technique	IGV
5	High cholesterol diet + apple cider vinegar produced using surface technique	TAV

| 6 | High cholesterol diet + apple cider vinegar produced using submerged technique | IAV |

Biochemical Analyses of Blood Samples

CAT, GSH-Px, SOD, MDA

CAT, GSH-Px, and SOD were analyzed in order to assess the antioxidant status. MDA was analyzed for assessing the oxidant status. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240 nm [16]. The principle of the assay is based on the determination of the rate constant of decomposition of hydrogen peroxide by catalase. The activity of CAT is expressed as U/g hemoglobin. Hemoglobin concentration was determined by the cyanomethemoglobin method from the hemolyzed erythrocytes.

The determination of GSH-Px activity was based on the method of Paglia and Valentine [17]. The principle of the method is as follows: GSH-Px catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance of NADPH was measured at 340 nm.

The measurement of SOD is based on the principle that xanthine reacts with xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity is then measured by the degree of inhibition of this reaction [18]. The enzyme activities are expressed as U/g hemoglobin or U/g Hb.

SOD and GSH-Px levels were measured by using RANDOX and RANSOD assay kits (Randox Laboratories Ltd. Ardmore, Crumlin, UK), an autoanalyzer (Model 5400-Beckmann Coulter, Brea, California, USA), and a spectrophotometer (Model UV-1601 Shimadzu, Kyoto, Japan) was used to estimate CAT and MDA enzyme activity. MDA was determined by the double heating method of Draper and Hadley [19]. The principle of the method is spectrophotometric measurement of the color developed during reaction of thiobarbituric acid (TBA) with MDA. The concentration of MDA is expressed as nmol/g hemoglobin.

Statistical Analysis

All data were reported as the means with standard deviation and were analyzed by using SPSS for Windows (version 17.0, SPSS Inc.). The statistical analysis among the groups was assessed for homogeneity by the Levene test. Where the data were homogeneous, one-way ANOVA was used for comparison of groups for the oxidative stress parameters. A *P* value of less than 0.05 was considered statistically significant. For the significance values used, Bonferroni correction as post-hoc tests were run to confirm where the differences occurred between groups but this time a *P* value of less than 0.01 was considered statistically significant.

RESULTS AND DISCUSSION

This study was comprised of two parts. The first part was production of apple cider vinegar and grape wine vinegar by two different production methods (traditional and industrial) and the second part was the oral administration of these vinegars to rats fed a high cholesterol diet and subsequent analysis of the vinegars as antioxidants. The results of first part indicated that the

total antioxidant activity of vinegar samples (TGV and TAV) produced by the surface method (traditional) was higher than the samples produced by the submerged method (industrial) (Table 2). TEAC values of TGV and IGV samples were 11.82 mM and 10.44 mM, respectively. These findings were in concordance with previous studies [9, 20]. Seeram et al. [21] reported that TEAC and ORAC value of apple juice samples ranged between 2.5-6.2 μmol of TE/mL and 2.7-4.3 μmol/mL respectively. It was reported that apple vinegar contained gallic acid, catechin, epicatechin, caffeic acid, chlorogenic acid and p-coumaric acid [19].

Table 2. Antioxidant Activities of Vinegar Samples

Vinegar Samples	TEAC (mM)	ORAC (μmol TE/ml)
TGV	11.82±0.97 [a]	10.5±1.18 [A]
IGV	10.44±0.72 [a]	7.62±0.61 [A, B]
TAV	5.5±0.67 [b]	5.89±0.88 [B, C]
IAV	4.1±0.46 [b]	2.74±0.56 [C]

Different letters in superscript following values indicate statistical significance within each column (P < 0.05). Data are expressed as ± standard errors of mean (SEM)

The second part was the experimental animal study. Vinegar samples were administered to the high cholesterol-fed rats regularly for 7 weeks. The levels of CAT, MDA, GSH-Px, and SOD in blood samples of all groups are shown in Table 3.

Table 3. CAT, MDA, GSH-Px, and SOD levels in rats

	CAT (kU/g)	MDA (nmol/g)	GSH-Px (U/g)	SOD (U/g)
CNT	83.81±17.64	394.66±49.17	148.86±16.43	3513.34±536.51
CHCNT	48.29±15.52[a]	468.54±45.70[a]	109.47±5.21[a]	1058.59±333.69[a]
TGV	65.84±16.21	473.37±23.04	122.83±7.16[x]	1628.01±231.53[x]
IGV	59.16±20.87	444.79±28.75	116.93±6.65	1610.17±279.02[x]
TAV	55.88±6.45	337.08±62.58[x]	125.61±4.56[x]	1761.72±431.97[x]
IAV	49.15±8.55	377.46±40.49[x]	118.66±5.03	1856.80±363.02[x]

[a]means significance when compared to CNT (P < 0.05); [x] means significance when compared to CHCNT (P < 0.05). Data are expressed as ± standart errors of mean (SEM)

Levels of CAT, GSH-Px, and SOD of the CNT group were significantly decreased while the level of MDA of the CNT group were significantly increased when compared to control rats fed with the high-cholesterol diet (CHCNT) (P<0.05). Although the CAT enzyme activity was increased in all groups fed with vinegar and cholesterol (TGV, IGV, TAV, and IAV) when compared to only high cholesterol-fed animals (CHCNT), it was not statistically significant. Malondialdehyde (MDA) is the end products of lipid peroxidation. MDA level was significantly

decreased in groups administered apple cider vinegar (TAV and IAV) when compared to the high-cholesterol-fed animals (CHCNT) (P < 0.05). MDA level was not significantly changed in groups given grape vinegar (P > 0.05). GSH-Px levels of TGV and TAV groups were significantly increased (P < 0.05, P < 0.001) when compared to the high-cholesterol-fed animals (CHCNT). It should be emphasized that TGV and TAV were produced by using traditional surface method. Budak et al. [20] reported that total phenolic content and phenolic compounds of apple vinegars produced with traditional surface methods were higher than industrial submerged method.

GSH-Px level of rats fed with vinegars produced by the industrial submerged method (IGV, IAV) were not significantly changed when compared to the high-cholesterol-fed animals (CHCNT). Our findings indicated that while MDA levels were increased, the antioxidant enzyme (i.e. CAT, SOD, and GSH-Px) levels were consumed in order to lower the high lipid peroxidation products in cholesterol-fed rats (CHCNT). The consumption of vinegar decreased MDA levels and prevented the depletion in antioxidant enzymes. The decrease in MDA level was observed especially in the apple cider vinegar samples. An increase in GSH-Px was observed with both grape and apple cider vinegar which was produced by surface method (traditional). Since GSH-Px is the main antioxidant enzyme for the erythrocytes, this finding is valuable and well-matched with the the total antioxidant capacity results of the vinegars. SOD levels of the groups increased due to all four consumed vinegar samples. CAT levels of vinegar administered groups were increased (P>0.05). Levels of SOD were significantly increased in all groups fed with vinegar and cholesterol (TGV, IGV, TAV and IAV) when compared to only high cholesterol-fed animals (CHCNT) (P < 0.05). The SOD level of the rats fed with apple cider vinegar was higher than those fed with grape vinegar (P < 0.05). Chlorogenic acid was the dominant phenolic substance in apple cider vinegar [19]. A protective effect of chlorogenic acid against cardiovascular diseases with inhibition of LDL oxidation was previously reported [22]. Yang et al [23] investigated the hepatoprotective effects of apple polyphenols (AP) against CCl4-induced acute liver damage in mice. They showed that the extent of MDA formation was reduced, the SOD activity was enhanced, and the GSH concentration was increased in the hepatic homogenate in AP-treated groups compared with the CCl4-intoxicated group. There are few studies in this field. Denis et al. [24] provided evidence of the capacity of polyphenols of dried apple peels to reduce oxidative stress and inflammation in gastrointestinal mucosa of intestine. Although the target tissue was different, their results revealed the antioxidant effect of polyphenols in apple was in agreement with our study. The consumption of apples was shown to lead to significant increases in the activities of some antioxidant enzymes and in the antioxidant potential values of the blood, also decreasing oxidation reactions in the body in significant amount in elderly subjects [25]. Yuan et al. [26] reported that two weeks of apple and grape juice consumption increased the plasma T-AOC and decreased the concentration of MDA in healthy subjects. Our study examines the effect of two kinds of vinegars made by two different production methods on oxidative and antioxidative status in vivo. This will be the first report regarding the effect of apple cider and grape wine vinegar consumption on blood antioxidant levels.

CONCLUSION

This research supports that both grape and apple cider vinegars, especially the ones which were produced by traditional methods, provided benefit on antioxidant status. When the traditional method was used for vinegar production, fermentation was completed in approximately 60 days. This length of time enables the production of bioactive components and a complex polysaccharide structure, called mother of vinegar. In industrial productions, vinegar is produced within one day. It was concluded that the selection of the vinegar production method is significant for the level of functional properties. Future studies should address the effect of the individual bioactive substances (phenolic substances, etc.) that result in positive effects on antioxidant status.

Competing Interests: The authors have no financial interests or conflicts of interest.

Author's Contributions: ACS, NB, ZBGS, ÇS and DKD designed the research; NB, ACS, ZBGS produced vinegars and carried out the antioxidant assays of vinegar samples. NG, ÇS and DKD performed the animal test; ACS, ÇS and NB analyzed the data; NB, DKD, ACS, ZBGS prepared the manuscript, figures, references.

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