

# Energy Drink Induced Lipid Peroxidation and Oxidative Damage in Rat Liver and Brain When Used Alone or Combined with Alcohol

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**Abstract:** Energy drinks (ED) are containing large doses of metabolic stimulants and its use with ethanol has increased dramatically among young adults. In this study, we examined the effects of ED exposure either alone or in combination with ethanol on oxidative stress parameters including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and lipid peroxidation parameter malondialdehyde (MDA) in rat. Some histopathological findings were also evaluated. ED exposure led to a dose-dependent increase in liver MDA compared to the control indicating oxidative damage. Histopathological findings also revealed that ED alone may generate liver damage. Ethanol exposure increased MDA level and SOD, CAT, and GSH-Px activity in both the brain and the liver. The combination of ethanol and ED produced greater damage which is considered by further increases in SOD and GSH-Px activity in the brain. Similar results for MDA were observed in both the liver and brain as well. Our findings suggest that ED consumption alone or combination with ethanol may represent a significant public health concern.

**Keywords:** alcohol, energy drink, ethanol and energy drink mixture, lipid peroxidation, oxidative stress

**Practical Application:** ED consumption among young adults, especially combined with alcoholic beverages, has increased over the last decade. There is not enough research in the literature to determine the total effects of co-administrated alcoholic beverage and ED. This study aimed to provide awareness of the deleterious effects from the consumption of EDs and alcohol both together and separately. Results of this study are expected to be a reference for public service broadcasting and manufacturer of ED in terms of producing a deterrent packaging in case of incorrect self-consumption or mixture preparation.

## Introduction

Energy drinks (EDs) are beverages which contain large doses of caffeine and other metabolic stimulants such as taurine, carbohydrates, glucuronolactone, and B-complex vitamins (Attila and Çakır 2011). The energy boosting capacity of EDs comes mainly from sugar and the stimulating effect from its caffeine content, which is comparable to 1 cup of coffee containing a minimum 80 mg caffeine (Miller 2008). Over the last decade, ED use among young adults, especially its combination with alcohol, has increased dramatically (Franklin and others 2013; Marczynski 2014). These drinks are considered the most popular dietary supplement besides multivitamins among American adolescents and they are reported to be the most popular supplement among British athletes due to improved performance with mental function and alertness (Campbell and others 2013). A report in 2011 indicates that emergency department visits in the United States because of ED and alcohol mixed ED-related problems has increased 10-fold in the previous 5 years (Marczynski and others 2013). France and Denmark have banned the sale of these kinds of EDs and the gov-

ernments of Sweden and Canada published their concerns about limited safety data on caffeinated EDs (Howland and others 2010).

In addition to their limited safety data, there is not enough information about the effects of EDs and the mixture of EDs with ethanol on oxidative stress and lipid peroxidation in the literature. Oxidative stress is attributed to the etiology of several diseases such as diabetes, cancer, and cardiovascular disorders. However, reactive oxygen species (ROS) may have beneficial functions in terms of cellular signaling, and combating with microorganism in cells. Enhanced production of ROS or depletion of antioxidant defense system can cause an imbalance between oxidants and antioxidants (Ilaiyaraja and others 2011). Overexpression of ROS causes damages to lipids, proteins, and DNA structure (Bloomer and others 2013). To prevent oxidative consequences, cells are surrounded by a defense mechanism which consists of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px; Ilaiyaraja and others 2011).

Lipid peroxidation is an autocatalytic process which can be described as an attack of ROS unsaturated bonds of membrane fatty acids due to increased generation of these oxidant species (Souza and others 2013). Physical breakage of cell membrane caused by lipid peroxidation leads to an influx of ions and body fluids into the cell which results in cell membrane rupture and death. Among lipid peroxidation products, malondialdehyde (MDA) is a very reactive one and serves as a marker of lipid peroxidation. MDA can also bind to cellular proteins and forms protein-aldehyde adducts which act as neo-antigens (Everitt and others 2007; Ilaiyaraja and others 2011).

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In this study we aimed to investigate the effects of EDs and the mixture of EDs with ethanol on lipid peroxidation parameter MDA and oxidative stress parameters SOD, CAT, and GSH-Px in liver and brain tissue. Histopathological evaluations were also examined in this study.

## Materials and Methods

### Chemicals

Hematoxylin and eosin were supplied from Bio Optica and DDK, Italy, respectively. Formalin was purchased from J.T. Baker, Netherlands. Potassium chloride was purchased from Riedel-de Haen, Germany. All other chemicals were supplied from Sigma (St. Louis, Mo., U.S.A.).

### Instrument

The centrifuge used for the preparation of samples was a 3–16PK laboratory centrifuge (Sigma, D-37520 Osterode am Harz, Germany). The spectrophotometer used for the analysis of oxidative stress parameters was an EVO300 PC UV-Vis (Thermo Fischer Scientific, Madison, WI, U.S.A.).

### Animals and housing

Male Sprague–Dawley rats, weighing 200 to 250 g, were obtained from the Yeditepe Univ. Medical School Experimental Research Center (YUDETAM) and housed at controlled room temperature ( $21 \pm 1$  °C) with 12:12 h light–dark cycle. They were allowed unrestricted access to water and rodent chow. The experimental protocol was approved by the Ethic Committee of Yeditepe Univ. (protocol #414).

### Experimental design

A total of 36 Sprague–Dawley rats were divided into control and 5 treatment groups. Control group received tap water (C). Treatment groups are as follows: low-dose ED (L): 3.5 g ED/kg/d, high-dose ED (H): 7 g ED/kg/d, ethanol (E): 1 g ethanol/kg/d, ethanol mixed with low-dose ED (EL): 1 g ethanol/kg/d + 3.5 g ED/kg/d, ethanol mixed with high-dose ED (EH): 1 g ethanol/kg/d + 7 g ED/kg/d. All administrations were done by oral gavage for 14 d.

We used a brand of marketed ED, Rockstar (250 mL per can). Ingredients of Rockstar were sucrose, glucose, dextrose, acid (citric acid), carbon dioxide, taurine (4 g/L), caffeine, sodium citrate, glucuronolactone (240 mg/L), colors (ammonia caramel and riboflavins), inositol (100 mg/L), niacin, pantothenic acid, vitamin B6, vitamin B12. Energy content was indicated as 114 kilocalories/250 mL (calorie breakdown is 2% fat, 96% carbohydrates, and 2% protein) according to the information on its label. Designing of the dose of ED was based on the consumption of one container of ED (250 mL) per day by an adult weighing 65 kg for low-dose ED and 2 containers (500 mL) per day for high-dose ED exposed group. Dose of ethanol was determined according to a previous study (Novier and others 2012). On day 15, animals were weighed and sacrificed by decapitation. Brain and liver tissues were dissected and weighed. Tissues from each group which were separated for histopathological evaluation were fixed in 10% formalin for 24 h. Other portions of the liver and brain tissue were frozen immediately on dry ice and stored at  $-80$  °C until analysis of MDA and oxidative stress parameters.

### Sample preparation

Brain and liver samples were minced and homogenized with a glass homogenizer in a cold potassium chloride solution (1.15%)

and centrifuged at 3722 g and 4 °C for 15 min. The obtained supernatants were then used to analyze MDA, SOD, CAT, GSH-Px, and protein content.

### Determination of protein content in samples

The protein content in tissue homogenates was determined according to the method published by Lowry and others (1951). Briefly, main solutions including reagent A (2% sodium carbonate in 0.1 N sodium hydroxide), reagent B (0.5% copper sulfate), and reagent C (1% potassium sodium tartrate) were prepared. Then 50 mL of reagent A, 0.5 mL of reagent B, and 1 mL of reagent C were mixed to obtain alkaline copper reagent. Working standard solutions were prepared from bovine serum albumin in the concentration range of 0.625 to 20 mg/mL. For measurement, 0.01 mL of standard solutions or samples were mixed with 1 mL distilled water and 4.5 mL of alkaline copper reagent and allowed to stand for 10 min at room temperature. Then 0.5 mL of Folin's reagent (1:2 v/v) was added to this solution, mixed and allowed to stand for 20 min at room temperature. Finally, the optical density of standard and sample solutions was measured at 640 nm.

### Determination of SOD activity

Each tissue homogenate was diluted 1:40 with 10 mM phosphate buffer (pH 7.0). Twenty-five microliters of diluted sample was then mixed with 850  $\mu$ L of substrate solution containing 0.05 mmol/L xanthine sodium and 0.025 mmol/L INT (2-[4-iodophenyl]-3-[4-nitrophenol]-5-phenyltetrazolium chloride) in a buffer solution containing 50 mmol/L CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) and 0.94 mmol/L EDTA disodium dihydrate (pH 10.2). Then, 125  $\mu$ L of xanthine oxidase (80 U/L) was added to the mixture and absorbance increase was followed at 505 nm for 3 min against air. Standard solutions were prepared from stock solution of SOD from bovine erythrocytes in the concentration range of 0.217 and 5.2 kU/L and phosphate buffer was used as the blank solution (Aydin and others 2001).

### Determination of CAT activity

For determination of the CAT activity, the reaction mixture was composed of 50 mM phosphate buffer (pH 7.0), 10 mM H<sub>2</sub>O<sub>2</sub>, and tissue homogenate sample. The reduction rate of H<sub>2</sub>O<sub>2</sub> was followed at 240 nm for 45 s against air at room temperature (Park and others 2010). CAT activity was expressed as kU/g protein. Phosphate buffer was used as the blank solution and the calibration curve was plotted with standard solutions (1.25 to 15 U/mL) prepared from CAT stock lyophilized powder.

### Determination of GSH-Px activity

To measure the GSH-Px activity, the reaction mixture was composed of 50 mmol/L tris buffer (pH 7.6) containing 1 mmol/L of EDTA disodium dihydrate, 2 mmol/L of reduced glutathione, 0.2 mmol/L of NADPH, 4 mmol/L of sodium azide, and 1000 U of glutathione reductase. The reaction mixture (990  $\mu$ L) and 10  $\mu$ L of tissue homogenate (diluted 17 times) were pipetted into a quartz spectrophotometer cuvette and incubated for 5 min at room temperature. Then, the reaction was initiated with the addition of 10  $\mu$ L of *tert*-butyl hydroperoxide solution (1: 1000 dilution). The decrease in NADPH absorbance was followed at 340 nm against air for 3 min and absorbance change per min was calculated. GSH-Px activity was expressed as kU/g protein (Aydin and others 2001; Park and others 2010).

### Measurement of MDA level

Level of MDA was determined as a thiobarbituric acid reactive substance (TBARS) in the liver and brain homogenates according to the method described by Jamall and Smith (1985). Tetramethoxypropane solution was used as the standard for plotting the calibration curve and the results in the sample were expressed as nmol/g protein. Briefly, 200  $\mu$ L of sample or standard solution, 200  $\mu$ L of sodium dodecyl sulfate (8.1%), 1500  $\mu$ L of acetic acid solution (20%), and 1500  $\mu$ L of thiobarbituric acid solution (0.8%) were mixed. The volume was completed to 4000  $\mu$ L with distilled water. The tubes were kept at 95 °C for 1 h. Then, they were cooled under tap water and 2000  $\mu$ L of the mixture was added to 2000  $\mu$ L of trichloroacetic acid. They were centrifuged at  $1017 \times g$  for 10 min. The absorbance of the supernatant was measured at 532 nm.

### Histopathology

The brain and liver tissues from each group were fixed in 10% formalin for 24 h. After routine tissue processing, the tissues were embedded in paraffin. Sections at 4  $\mu$ m thickness obtained from each paraffin block were stained with hematoxylin and eosin for histopathological evaluation under light microscope (Olympus BX53, Tokyo, Japan). Micro photos were taken by Olympus DP 73 (Japan) micro camera. All biopsies at baseline and at follow-up were read by the same pathologist of Yeditepe Univ. Hospital

who was blinded to details of the study. Histopathological variables were scored according to the NASH Clinical Research Network Scoring System (Brunt and others 1999).

### Statistical analysis

SPSS version 20 was used for all statistical analyses. MDA, SOD, CAT, and GSH-Px data were analyzed using 1-way ANOVA. When significant interactions were present, follow-up analyses were performed by separate 1-way ANOVAs between variables. For histopathological data, continuous variables were presented as mean (standard deviation) and categorical variables were presented as number (percentage). Differences between several groups were tested with one-way ANOVA, and *post hoc* tests were performed using Tukey. Differences were considered significant at  $P < 0.05$ .

## Results and Discussion

### Body and organ weight changes

Figure 1 presents the body and relative organ weight changes. No significant differences were found for either body or relative brain and liver weights after 14-d of oral exposure to ED alone and the mixture of EDs with ethanol.

### Change in SOD activity

SOD activity is presented in Figure 2(A). In the brain tissue of the ethanol exposed group, SOD activity exceeded in all treated

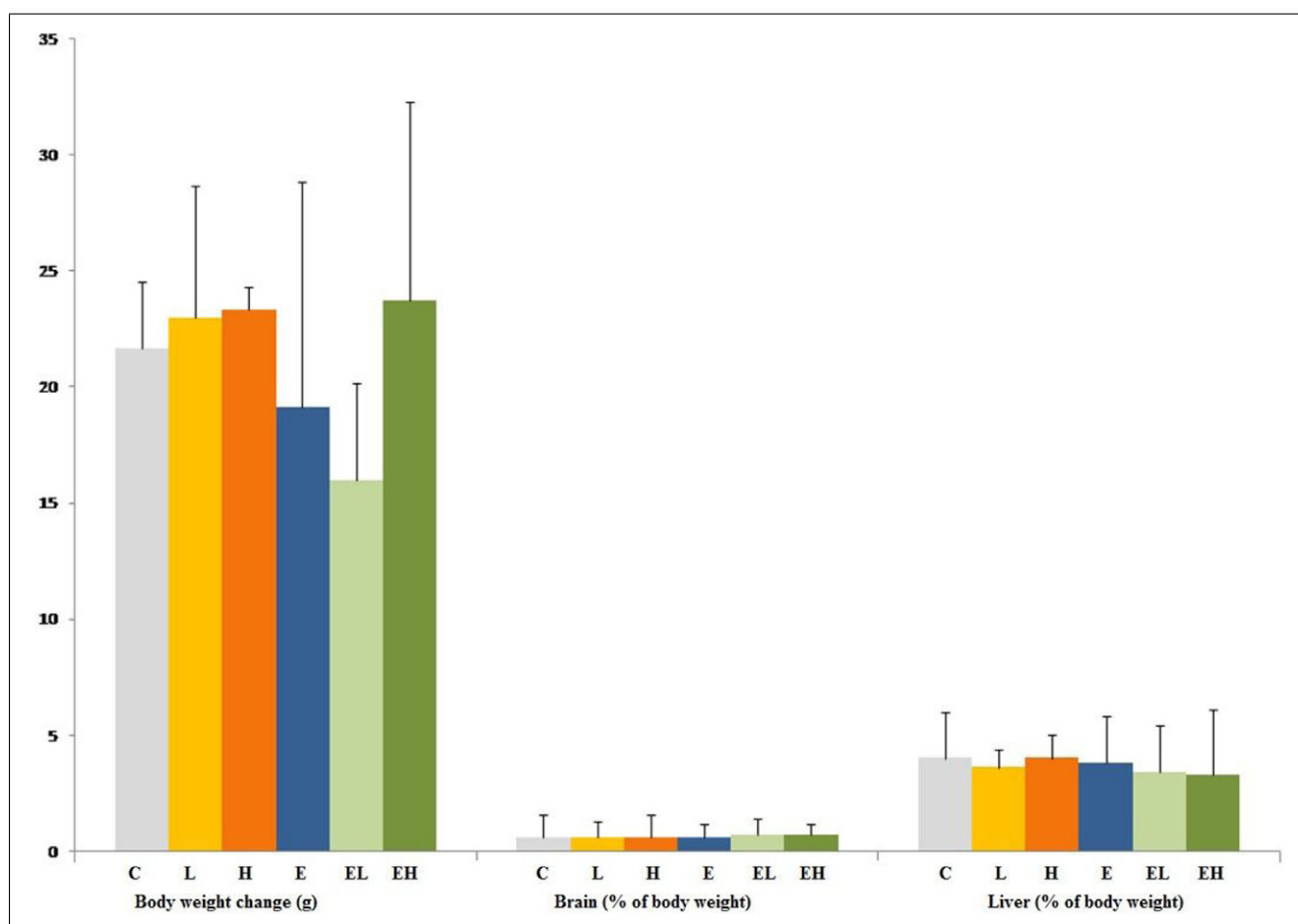


Figure 1—Body weight (g) and relative organ weight (%) changes in rats exposed to energy drink and energy drink-ethanol mixture. C: control, L: low-dose energy drink group, H: high-dose energy drink (3.5 g/kg/d) group, E: ethanol (1 g/kg/d) exposed group, EL: ethanol (1 g/kg/d) plus low-dose energy drink (3.5 g/kg/d) group and EH: ethanol (1 g/kg/d) plus high-dose energy drink (7 g/kg/d) group.

groups, but that was statistically significant when compared to the control ( $P < 0.05$ ). Administration of low-dose ED led to an increase in SOD activity when is compared to the exposure of high-dose ED ( $P < 0.05$ ). Also, addition of ethanol to high dose of ED showed a significant increase in SOD activity compared to the high-dose ED alone ( $P < 0.05$ ). In liver tissues, difference on the SOD activity between groups was negligible. Only ethanol exposed group has higher SOD activity when compared to the other groups, but that was not significant.

### Change in CAT activity

CAT activity is presented in Figure 2(B). Both in the liver and brain tissues, there is no significant difference between groups.

### Change in GSH-Px activity

GSH-Px activity is presented in Figure 2(C). GSH-Px activities were increased significantly in brain tissue of ethanol and ethanol plus low-dose ED exposed group when we compared to the control ( $P < 0.05$ ). Addition of ethanol to low-dose ED increased this enzyme activity in comparison to low-dose ED alone ( $P < 0.05$ ). In contrast to the results seen in brain tissue, GSH-Px activity differences were negligible in the liver tissue of all groups. Only ethanol exposed group in liver had higher GSH-Px activity when

compared to the other groups, but that failed to reach statistical significance.

### Change in MDA level

MDA content in brain and liver tissues is presented in Figure 2(D). Liver MDA levels in all treated groups exceeded that of the control. Administration of ED alone led to dose dependently higher MDA content in the liver. This induction was significant ( $P < 0.05$ ) for high-dose ED group, which suggests that ED exposure at this level exerts oxidative damage in the liver. Co-administration of high-dose ED and ethanol caused greater increment of liver MDA level ( $P < 0.05$ ). Similarly, in brain tissue, MDA levels were increased in a dose dependent manner in both ethanol plus ED groups compared to the ethanol alone, ED alone, and control groups ( $P < 0.05$ ).

### Histopathology

Histopathological findings and mean scores of variables for liver tissue are shown in Figure 3 and Table 1. Only 2 histopathological variables, lobular inflammation, and ballooning degeneration, were evaluated in liver tissues according to nonalcoholic fatty liver disease scoring (NAS; Brunt and others 1999). Histopathological

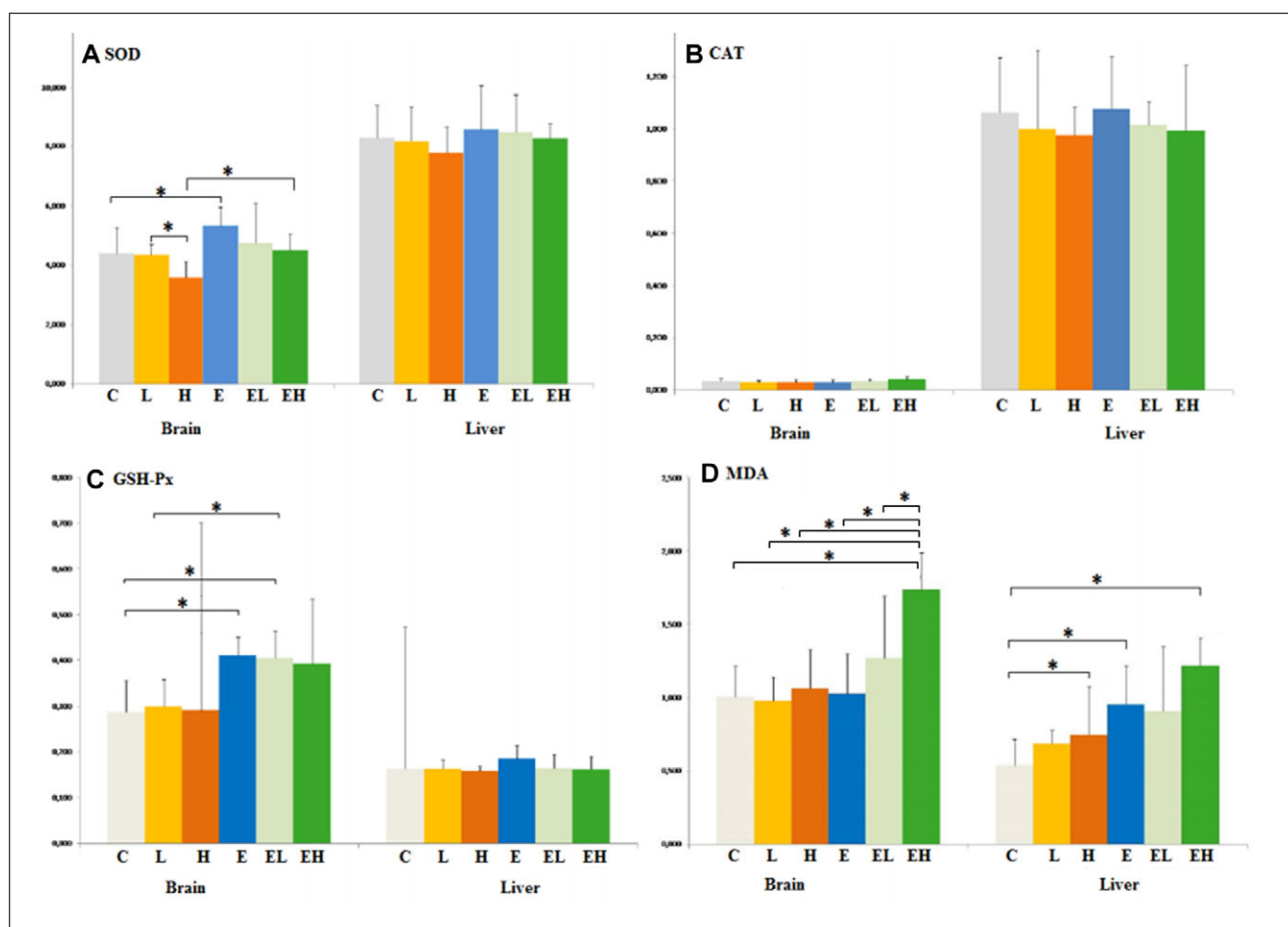


Figure 2—SOD, CAT, and GSH-Px enzyme activity and MDA level in liver and brain tissues of rats exposed to energy drink, ethanol and energy drink-ethanol mixture. SOD activity is expressed as U/mg protein, CAT activity is expressed as kU/mg protein, GSH-Px activity is expressed as U/mg protein and MDA level is expressed as nmol/g protein. C: Control, L: low-dose energy drink group, H: high-dose energy drink (3.5 g/kg/d) group, E: ethanol (1 g/kg/d) exposed group, EL: ethanol (1 g/kg/d) plus low-dose energy drink (3.5 g/kg/d) group and EH: ethanol (1 g/kg/d) plus high-dose energy drink (7 g/kg/d) group. \* $P < 0.05$ .

**Table 1—Mean scores of histopathological variables for liver tissue.**

	Control (n = 6)	L (n = 6)	H (n = 6)	E (n = 6)	EL (n = 6)	EH (n = 5)
<b>Lobular inflammation</b>						
None (0)	6 (100%)	1 (17%)	1 (17%)	1 (17%)	3 (50%)	0
Mild (1)	0	5 (83%)	4 (66%)	5 (83%)	3 (50%)	3 (60%)
Moderate (2)	0	0	1 (17%)	0	0	2 (40%)
Mean score	0 ± 0.00	0.83 ± 0.41	1.00 ± 0.63	0.83 ± 0.41	0.50 ± 0.55	1.40 ± 0.55
<b>Ballooning degeneration</b>						
None (0)	0	0	0	0	2 (33%)	0
Few (1)	0	3 (50%)	5 (83%)	5 (83%)	3 (50%)	5 (100%)
Many (2)	0	3 (50%)	1 (17%)	1 (17%)	1 (17%)	0
Mean score	0 ± 0.00	1.50 ± 0.55	1.16 ± 0.41	1.17 ± 0.41	0.83 ± 0.75	1.00 ± 0.00

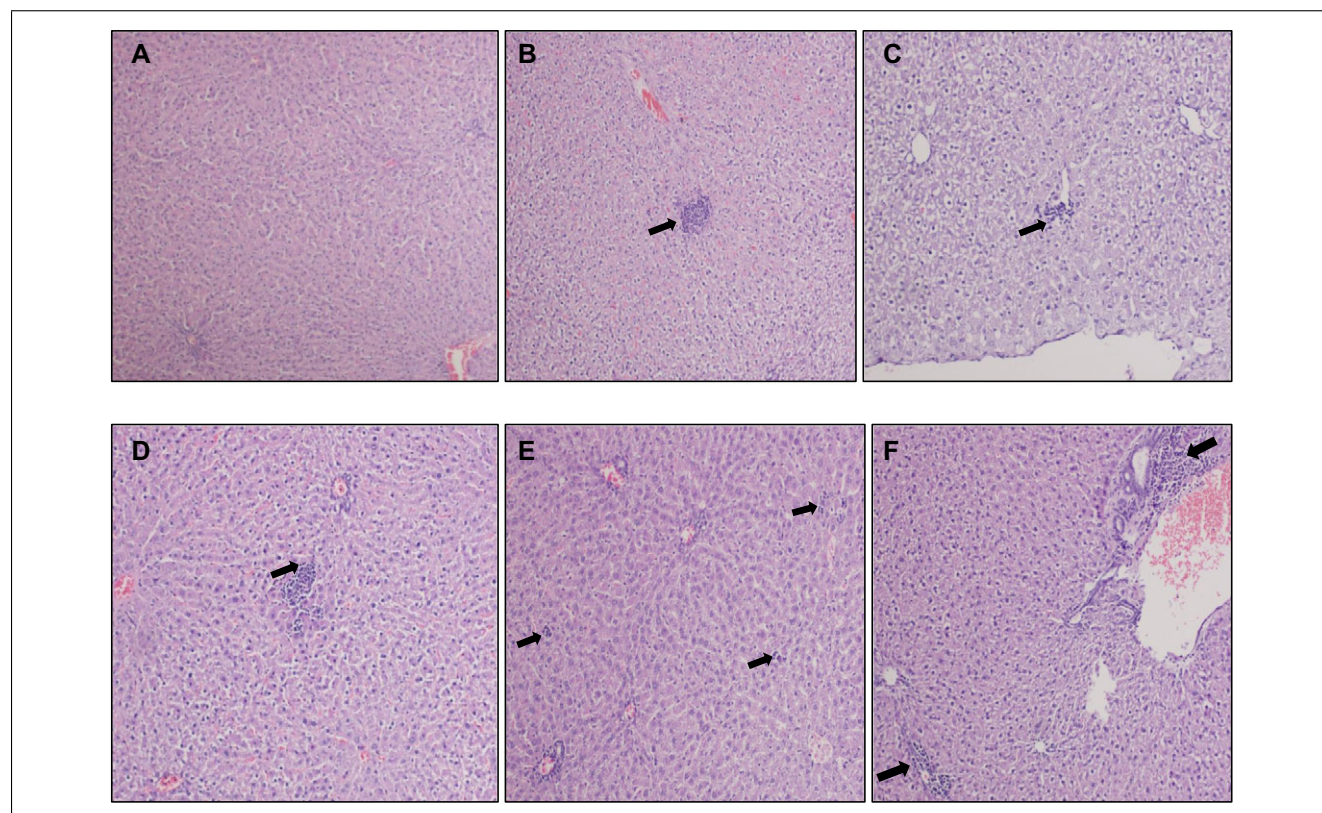
Results were expressed as the mean ± standard deviation (SD). Lobular inflammation was graded according to the number of inflammatory foci per 200 field (0 is no foci; 1 is <2 foci per 200 field, 2 is 2 to 4 foci per 200 field, 3 is >4 foci per 200 field); hepatocellular ballooning was graded as none, few balloon cells, and many cells/prominent ballooning (0 to 2). L, low-dose energy drink group; H, high-dose energy drink group; E, ethanol group; EL, ethanol plus low-dose energy drink group; EH, ethanol plus high-dose energy drink group.

variables such as steatosis and portal fibrosis were not evaluated for any of the liver tissue due to the short administration period.

Histological characteristics showed the high incidence of lobular inflammation and ballooning degeneration in treated animals compared to controls. The lobular inflammation was observed in 5/6 of low-dose ED, high-dose ED, and ethanol-treated groups as well as 3/6 of ethanol plus low-dose ED exposed animals but not in controls. The lobular inflammation rates were highest in ethanol plus high-dose ED (6/6) group compared to other groups and 40% of them were categorized as having moderate inflammation. These differences were significant in all treated groups for ballooning degeneration compared to the control group (Table 2).

Also, 33% of ethanol plus low-dose ED and 100% of all other treated groups showed the ballooning degeneration incidence with no such observation in controls (Table 2).

Most of the previous studies investigated the cognitive effects of EDs or their major components (for example, caffeine or taurine), alone or in combination with alcohol (Giles and others 2012; Franklin and others 2013; Alford and others 2001). This study was undertaken to assess the effect of ED alone and in combination with ethanol on oxidative stress and lipid peroxidation status in rat. In this study, because of having high sensitivity to ROS, consuming approximately 20% of the body's total oxygen and being wealthy of polyunsaturated fatty acids, the brain was selected as one of our



**Figure 3—Hepatocyte ballooning and lobular inflammation in liver tissues of rats exposed to energy drink and energy drink mixed with ethanol. (A) Control (×40), (B) low-dose energy drink (L) group (×100), (C) high-dose energy drink (H) group (×100); (D) ethanol (E) group (×100), (E) ethanol plus low-dose energy drink (EL) group (×100), and (F) ethanol plus high-dose energy drink (EH) group (×100). Marked areas show lobular inflammation.**

**Table 2—Statistical significance of ballooning degeneration and lobular inflammation of liver between groups.**

	<i>P</i> <sup>a</sup>	<i>P</i> <sup>b</sup> 1-2	<i>P</i> <sup>b</sup> 1-3	<i>P</i> <sup>b</sup> 1-4	<i>P</i> <sup>b</sup> 1-5	<i>P</i> <sup>b</sup> 1-6	<i>P</i> <sup>b</sup> 2-3	<i>P</i> <sup>b</sup> 2-5	<i>P</i> <sup>b</sup> 3-6	<i>P</i> <sup>b</sup> 4-5	<i>P</i> <sup>b</sup> 4-6	<i>P</i> <sup>b</sup> 5-6
Lobular inflammation	0.001	0.047	0.011	0.047	0.452	0.000	0.989	0.818	0.721	0.818	0.368	0.038
Ballooning degeneration	0.000	0.000	0.002	0.002	0.038	0.012	0.799	0.150	0.100	0.800	0.100	0.012

Control: 1, L (low-dose energy drink): 2, H (high-dose energy drink): 3, E (ethanol): 4, EL (ethanol plus low-dose energy drink group): 5, EH (ethanol plus high-dose energy drink group): 6.

<sup>a</sup>Differences between the 6 groups were evaluated with one-way ANOVA.

<sup>b</sup>*Post hoc* tests (Tukey) were performed when the primary analysis was <0.05.

target organ. Also, it has lower levels of endogenous antioxidants compared to the other tissues (Abreu and others 2011). Having a special defense mechanism against ROS and being the main organ that metabolizes ethanol via alcohol dehydrogenase, aldehyde dehydrogenase, and cytochrome enzymes, the liver was selected as other target. The liver exposed to excessive amounts of alcohol undergoes numerous changes as a consequence of oxidative stress and inflammation (Ilaiyaraja and others 2011; Cichoz-Lach and Michalak 2014).

There are many ingredients in EDs which may be responsible for oxidative consequences and liver toxicity, but these effects are probably not attributable to a single ingredient of EDs. It might be caused by the mixture of many ingredients having high antioxidant capacity, or due to an augmented oxidative effect through synergistic interaction of them. Ingredients of ED used in this study were carbohydrates (10.8 g sucrose, 12.4 g glucose), sodium (as sodium citrate), antioxidants such as glucuronolactone (240 mg), inositol B<sub>8</sub> (only listed), caffeine (32 mg), taurine (400 mg), pyridoxine hydrochloride (B<sub>6</sub>; 2 mg, 100% of its recommended daily allowance [RDA]), niacin (B<sub>3</sub>; 8 mg, 40% of its RDA), and cyanocobalamin (B<sub>12</sub>; 2 µg, 32% of its RDA) per 100 mL (Higgins and others 2010).

As suggested by Higgins and others (2010), a great majority of the ingredients in one can of these beverages (250 mL) are above the RDA values. According to The American Academy of Pediatrics, caffeine and other stimulants such as taurine, guarana, and so on, contained by EDs have no place in the diet of children and adolescents. Therefore, there is no reported RDA value for these stimulants. Some studies have suggested that caffeine is responsible for major toxicity (Seifert and others 2013) and conclusions about glucuronolactone are not clear due to limited human data (Abreu and others 2011). In our study, we cannot attribute the toxicological findings to any of the ingredient specifically.

ROS have some physiological functions including cellular defense against microorganisms, and adaptive responses. During these physiological functions, some of the radicals can escape from antioxidant defense system. Therefore, exogenous antioxidant supplementation can protect cells against ROS. However, overconsumption of them could lead to a state of “antioxidative stress,” where antioxidants might attenuate or block adaptive cellular stress responses. In addition, antioxidative stress may interfere with endogenous physiological defense mechanisms decreasing the level of free radicals which are necessary for cellular protection (Poljsak and Milisav 2010). In this study we suggest that the combination of antioxidant substances through ED intake results in an “antioxidative stress.” This status may be explained by decreased SOD activity in brain tissue of the low-dose ED and high-dose ED exposed group in our study. This reduction was significant in high-dose ED exposed rats’ brain tissue. Zeidán-Chuliá and others (2013) studied the cellular response to ED and its components in Human Neuronal SH-SY5Y cell line. They identified that ED components alone or together induced a significant decrease in

cellular SOD and CAT activities. These authors suggested that the decrease of basal free radical generation due to the strong antioxidant effect of ED downregulates the cellular enzymatic antioxidant defense which subsequently drives a cytotoxic response.

It is well known that ethanol consumption increases the production of ROS and enhances oxidative damage in many tissues (Jing and others 2012; Reedy and others 2013). However, data on the effects of alcohol exposure on SOD, CAT, and GSH-Px activity are discordant, with results having been reported as increase, no change, or decrease (Grasselli and others 2014). In this study, the ethanol treated group exhibited significantly increased SOD and GSH-Px activity in brain tissue compared to the control group. Although not statistically significant, slight increases in SOD, CAT, and GSH-Px activity were observed in liver tissue of the ethanol exposed group. These findings may indicate increased production of SOD to combat ROS production from ethanol exposure.

Increased liver MDA levels in the ED exposed groups of this study may be similarly explained by decreased cellular defense mechanisms. As explained by Montoliu and others (1994), alcohol apart from being a source of free radicals also disturbs the antioxidant defense system. The increased lipid peroxidation level observed in ED-ethanol co-exposed rats might be a consequence of increased free radical formation as well as the disruption of cellular antioxidant defense status. Findings on liver histopathology revealed that EDs may generate liver damage and the combined effect of ethanol and ED may produce greater damage than either treatment alone which is indicated by increased MDA level. Histopathological examination of brain tissue did not reveal any abnormality related to treatment. This may be due to the short period of exposure (14-d) which is not enough to produce brain damage.

Results of this study clearly revealed that oral administration of EDs and ethanol alone caused significant increases in oxidative stress and lipid peroxidation which was exaggerated after co-exposure. But, it is important to recognize that there are many ingredients in EDs, and these effects are difficult to assign to one specific ingredient. Also, it may be due to the combination of ingredients that augments the observed toxicity. These findings should cause major concerns about the impact of these beverages on public health. Further experiments are warranted to explain the effects of the other non-caffeine components in ED (including glucuronolactone, niacin and so on) when combined with ethanol or through interactions with each other on oxidative stress and lipid peroxidation.

## Conclusion

These data suggest that ED consumption especially in exaggerated amounts might be a risk factor for antioxidative stress, as indicated by decreased brain SOD activity. Also, the particular combination of EDs’ ingredients may be responsible for the augmentation of oxidative stress parameters and histopathological abnormalities in liver and to some extent in brain tissue. Thus,

consumption of EDs especially with alcoholic beverages appears to represent a significant public health concern due to the increased toxicological consequences both peripherally and centrally. On account of these toxicological findings, further researches and regulations on marketed EDs and their combination with alcoholic beverages are needed.

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## Authors' Contributions

R. Reis, M. Charehsaz, Ç. Macit, and H. Akkaya designed the study. R. Reis, M. Charehsaz, and H. Sipahi interpreted the results. A.I. Doğan Ekici performed the histopathological evaluation. R. Reis, M. Charehsaz, and A. Aydın collected test data and drafted the manuscript.

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