

The Effect of Alcohol on Total Antioxidant Activity and Nitric Oxide Levels in the Sera and Brains of Rats

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Background: The present study investigated the effect of alcohol consumption on total antioxidant activity (AOA) and nitric oxide (NO) levels in the sera and brains of rats.

Materials and methods: The study included 24 rats that were divided into 2 groups: the control group (n = 12) and the alcohol group (n = 12). Both groups were fed regular laboratory chow and tap water for a period of 2 months; however, the alcohol group received 15% (v/v) ethanol in their drinking water. Then, the rats were decapitated, and serum and brain AOA and NO levels were measured.

Results: Both serum and brain AOA of the alcohol group were significantly lower than those of the control group. Serum NO levels of the alcohol group were significantly higher, whereas brain NO levels were lower, but not significantly, than those of the control group.

Conclusion: Our findings show that alcohol diminished both serum and brain defense mechanisms against free radical attack, which might result in many diseases. Moreover, decreased AOA levels in the alcohol group might be a significant cause of increased serum NO levels in this group or vice versa: however, the effects of alcohol on brain NO levels require further investigation.

Key Words: Alcohol, serum, brain, total antioxidant activity, nitric oxide

Alkolün Ratlarda Serum ve Beyin Antioksidan Aktivite ve Nitrik Oksit Seviyeleri Üzerine Olan Etkisi

Amaç: Biz çalışmamızda alkol tüketiminin serum ve beyinde total antioksidan aktivite (AOA) ve nitrik oksit (NO) seviyeleri üzerine etkisini araştırdık.

Materyal ve Metod: Bu amaçla 24 rat, alkol (n=12) ve kontrol (n=12) grubu olmak üzere iki gruba ayrıldı. Her iki grup çalışma süresince normal laboratuvar yemi ve musluk suyu ile beslendi fakat, alkol grubunun içme sularına % 15 (v/v) oranında etanol ilave edildi. İki aylık beslenme süresinden sonra ratlar ketamin anestezisi altında dekapite edilerek serum ve beyin dokularının AOA ve NO seviyeleri ölçüldü.

Bulgular: Alkol grubunun serum ve beyin AOA seviyeleri kontrol grubundan istatistiksel olarak anlamlı derecede düşüktü. Alkol grubunun serum NO seviyeleri kontrol grubundan istatistiksel olarak anlamlı olacak şekilde yüksek olmasına rağmen beyin NO seviyeleri kontrol grubundan düşük fakat aradaki fark istatistiksel açıdan anlamsızdı.

Sonuç: Bulgularımız, alkolün hem serum hem de beyinde birçok hastalığa sebep olan serbest radikallere karşı savunma mekanizmalarını zayıflattığını göstermektedir. Aynı zamanda, alkol grubunda total AOA'daki azalma serum NO seviyesindeki artışın önemli bir nedeni olabilir veya yüksek NO seviyeleri total AOA'deki azalmaya katkıda bulunuyor olabilir. Fakat, alkolün beyin NO seviyeleri üzerine olan etkisinin daha ileri araştırılması gerekmektedir.

Anahtar Sözcükler: Alkol, serum, beyin, total antioksidan aktivite, nitrik oksit

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Introduction

Excessive consumption of alcohol by a large proportion of the population is still a prominent medical and social problem in many countries. It leads to a fatty liver and affects physical, mental, social, and psychological activities. In addition, many neurological lesions and even cerebral atrophy may develop in alcoholics (1-5).

Alcohol is extensively metabolized in the liver, leading to the generation of acetaldehyde by the enzymatic activity in cytosol, microsomes, and peroxisomes. Acetaldehyde is further oxidized to acetate by acetaldehyde dehydrogenase in the

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mitochondria, which results in the generation of free radicals/reactive oxygen species (ROS) (1-6). Additionally, ethanol is metabolized more selectively in brain microsomes by cytochrome P-450 II E1, which may result in the generation of ROS (2,3). Oxidation of ethanol by alcohol dehydrogenase generates NADH, and NADH-dependent production of ROS by various organelles increases after chronic ethanol treatment (5,7). These ROS can cause cellular damage until they are removed by the antioxidant system. The antioxidant system includes antioxidant enzymes and antioxidant substances (2,3,5,6).

Nitric oxide (NO) is a highly diffusible, lipid-soluble, short-lived free radical gas generated from arginine by NO synthase (NOS, EC 1.14.13.39). It is associated with both physiological and pathological events in the body, including the brain (8,9). Some important interactions in biological fluids are the slow reaction of NO with O₂ to form nitrite and nitrate, and its rapid reaction with the superoxide anion (O₂^{•-}) forms the highly reactive peroxynitrite (ONOO⁻) (8,9). ONOO⁻ is a strong oxidizing agent that is capable of hydroxylating and nitrating aromatic compounds, and inducing cellular injury by lipoprotein oxidation, DNA fragmentation similar to that of apoptosis, damaging proteins and plasma lipids, depleting important plasma antioxidants, and nitration of proteins leading to cellular dysfunction that damage the endothelium and induce thickening of the intima in the artery wall (6,10).

NO has important bioregulatory functions in the immune, cardiovascular, and central nervous systems (CNS). Its role in physiological and pathological events may be modulated by alcohol (8,9,11). Kahkonen and Zvartau (12) showed that NO may modulate the cardiovascular system in alcohol withdrawal syndrome. NO synthesized in the CNS produces important effects. It is implicated in, among other things, the control of blood flow, learning and memory, neurotransmitter release, gene expression, immune responsiveness, and cell survival (9). Alcohol is lipophilic and readily crosses the blood-brain barrier, and can enter the CNS. Alcohol also produces numerous effects in the CNS, some of which overlap with those thought to be modulated or mediated by NO (8,13). The acute and chronic effects of ethanol on the CNS are complex, involving a range of cell types and signaling systems (8). Acute (9) and chronic (5,11) ethanol exposure was reported to lead to excitotoxicity, partially due to increased levels of NO.

Increased free radical formation in the brain has been shown to be one of the manifestations of acute and chronic ethanol intoxication (2,3,14). Brain mitochondria are very susceptible to oxidative injury caused by alcohol-induced free radicals (2).

Investigators have obtained variable data concerning the effects of ethanol on brain and blood total antioxidant activity (AOA), and NO levels. Therefore, the present study aimed to investigate the effect of alcohol consumption on AOA and NO levels in the sera and brains of rats.

Materials and Methods

Animals

The study included 24 inbred 4-month-old female Sprague-Dawley rats supplied by the Center for Experimental and Applied Medical Research, University of Selçuk. The rats were divided into 2 groups: the control group (n = 12) and the alcohol group (n = 12). The rats were fed regular laboratory chow consisting of 24% protein, 3.62% fat, 7% cellulose, 10% ash, and 12% water. Animals were housed in conventional wire-mesh cages in a room temperature regulated at 21 ± 1 °C, humidity 45%-50%, and light/dark cycles (12 h). The alcohol group was fed 15% (v/v) ethanol in their drinking water for a period of 2 months (13,15,16). Since the animals in this group were kept in the same cage and since they were drinking water from the same reservoir they were supposed to receive approximately similar amounts of alcohol, though not the same. Although the amount of alcohol consumed daily was not known, the indicated percentage of alcohol was expected to be high enough to manifest metabolic change during the study period.

After 2 months the rats were killed between 0900 and 1000 under ketamine anesthesia. Blood samples were collected immediately in plain tubes and serum was separated by centrifugation at 3000 rpm for 10 min at 4 °C. Brains were quickly removed, washed in cooled 0.15 M NaCl and were then homogenized in 2 ml of ice-cold homogenizing buffer (100 mM KH₂PO₄-K₂HPO₄, pH: 7.4, plus 0.1% digitonin) using an ultrasonic homogenizer (Musonix Microson Ultrasonic Cell Disruptor CML, serial no:1577). Then, they were centrifuged at 5000 rpm for 15 min at 4 °C and supernatant was removed. Serum and brain NO and AOA levels were

studied. Sera were separated without delay and investigated immediately. All procedures were performed following the *Guide for the Care and Use of Laboratory Animals*. The study was approved by the Ethics Committee of the Meram Faculty of Medicine, Selçuk University.

Determination of Total AOA

Brain and serum total AOA were measured by a commercially available kit from Randox (cat. no: NX2332). 2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) was incubated with metmyoglobin (a peroxidase) and H_2O_2 to produce the radical cation, $ABTS^+$. This has a relatively stable blue-green color measured at 600 nm. Antioxidants in an added sample cause a suppression of the color production to a degree proportional to their concentration. The degree of suppression of radical generation in the samples, indicative of the presence of antioxidant activity, is quantified by comparison with a standard.

Determination of NO

Since the half-life of NO is very short, NO levels in the sera and brains were determined by measuring levels of nitrite plus nitrate. NO values obtained by this procedure represent the sum of nitrite and nitrate. First, nitrate transforms nitrite by nitrate reductase. Then, total nitrate + nitrite concentrations were measured as NO according to the Griess method, with the use of a commercially available kit (Roche cat. no: 1 756 281).

Tissue protein levels were determined by the Biuret method (17). The protein levels of supernatants were high enough to be measured by the Biuret method, which is a sensitive method for high levels of protein.

Statistical Evaluation

Statistical analyses were performed using SPSS for Windows version 10.0. Statistical differences between the groups were evaluated using the independent t-test. The correlation between parameters was determined by Pearson's correlation test.

Results

The results are given in the Table as mean \pm SD (Figures 1-3). As can be seen from the Table, both serum ($P < 0.01$) (Figure 1) and brain ($P < 0.001$) (Figure 2) AOA levels of the alcohol group were significantly lower than those of the control group. On the other hand,

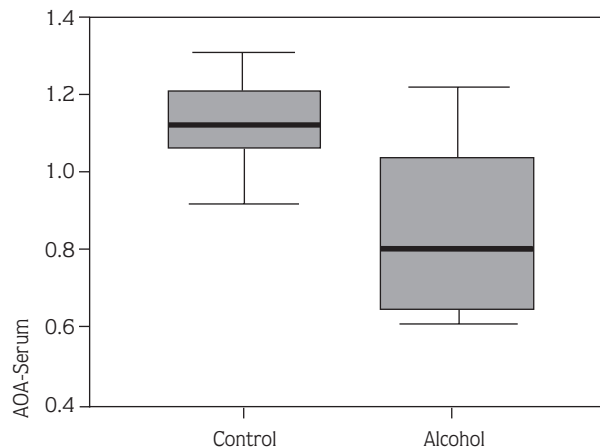


Figure 1. Serum AOA levels of the control and alcohol groups.

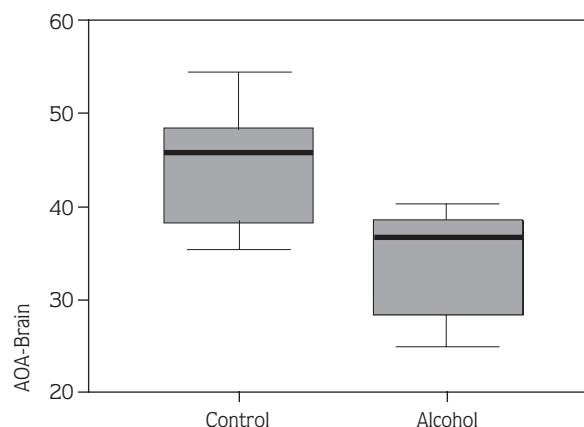


Figure 2. Brain AOA levels of the control and alcohol groups.

serum NO levels of the alcohol group were significantly higher ($P < 0.001$) (Figure 3) and brain NO levels were slightly lower, but not significantly, than those of the

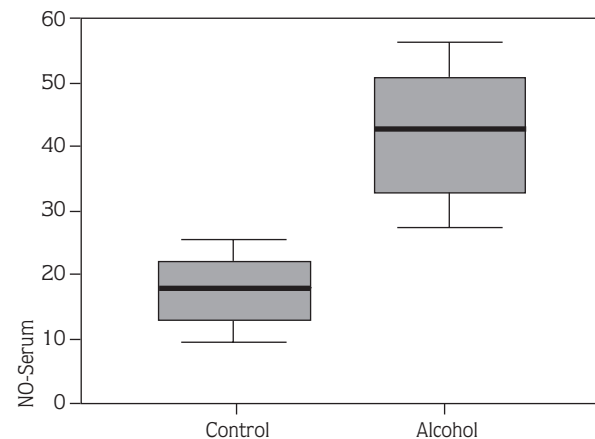


Figure 3. Serum NO levels of the control and alcohol groups.

control group (Table). There was a significant negative correlation between serum AOA and serum NO levels ($P < 0.01$), whereas the correlation between brain AOA and NO levels was not significant.

Discussion

AOA findings

Our data show that AOA levels in the alcohol group were significantly lower, both in the sera and brains, than in the control group. This is an important finding in view of the simultaneous deleterious effects of free radicals on serum constituents and the brain. It is well known that decreased AOA results in increased lipid peroxidation, which in turn plays a significant role in the pathogenesis of various diseases (2,5,14).

Many investigators have shown that alcohol decreases the activity of various antioxidant enzymes and vitamins, which results in decreased total AOA. Indeed, it has been reported that both brain (18) and serum (19,20) α -tocopherol levels, and brain (3,21,22,26) and serum (6,14,23-26) activity of some significant antioxidant enzymes were reduced after ethanol exposure. Husain et al. (6) and Aydın et al. (26) reported that chronic ethanol consumption increased plasma malondialdehyde (MDA) levels and decreased antioxidant enzymes activities.

One explanation for the observed lower level of total AOA in our ethanol-treated rats may be the increased utilization of antioxidants in the scavenging of free radicals (2). Mounting evidence points to oxidative stress as an important mechanism in alcohol toxicity, because alcohol induces peroxidation of membrane lipids, and oxidation of proteins and nucleic acids (18,22,27).

Lower levels of brain AOA in the alcohol group may have been due to a change in cerebral blood flow, lipid composition, iron content, or increased generation of ROS, and decreased antioxidant enzyme activity (3,6).

Indeed, it is reported that ROS generated in the brain due to ethanol ingestion is likely to alter the antioxidant defense system and membrane lipid peroxidation (2). Somani and Husain (3) have shown that chronic ethanol consumption significantly decreased antioxidant enzyme activity, except for that of glutathione reductase, in different regions of the brain.

Reddy et al. (2) found a significant perturbation in the antioxidant defense system and increased MDA formation, an indicator of lipid peroxidation in the brain, following graded doses of ethanol ingestion, which was thought to indicate dose-dependent oxidative stress in brain subcellular compartments in rats. They found that activity of powerful antioxidant enzymes was also reduced after ethanol exposure.

Moreover, Saravanan et al. (18) observed a disturbance in the antioxidant defense system and an increase in lipid peroxidation in the brain of ethanol-treated rats.

As seen from the above reported findings, many investigators have measured different antioxidant substances or enzymes in evaluating the effect of alcohol on the antioxidant defense system; however, separately measuring different antioxidants is time-consuming, labor-intensive, and costly. Since this is not practical and antioxidant effects are additive, the measurement of total AOA may be particularly useful for monitoring alterations in endogenous antioxidant levels (28). Thus, we think that our data provide additional, practical, and applicable information for evaluating the effect of alcohol on the antioxidant defense system, both in serum and the brain.

NO Findings

In the present study serum NO levels in the alcohol group were significantly higher than in the control group. This finding is in accordance with the findings of many other investigators (5,26,27,29,30). One mechanism

Table. Comparison of the parameters in the control and alcohol groups (mean \pm SD).

Paramaters	Control	Alcohol	P
Serum AOA mmol/l	1.13 \pm 0.12	0.86 \pm 0.23	0.01
Brain AOA (μ mol/g-prot.)	44.66 \pm 6.59	33.82 \pm 5.93	0.001
Serum NO (nmol/ml)	17.66 \pm 5.43	42.15 \pm 10.12	0.001
Brain NO (nmol/g-prot.)	180.41 \pm 77.89	155.00 \pm 42.79	0.086

proposed for this change was reported to be an increase in the level of free radicals due to alcohol consumption (1-7,18), as free radicals induced by ethanol stimulate the synthesis of some cytokines (such as interleukin (IL)-1, IL-2, IL-6, IL-8 and TNF- α), which in turn stimulate the synthesis of NO (5,27).

Two additional mechanisms have been proposed for this increase. One mechanism was reported to be the overproduction of NO in alcoholics via inducible NOS (iNOS), based on chronic inflammation and stimulation of the immune system. Increased production of NO and increased production of superoxide with vasoconstrictive effects can play a role in cardiovascular instability and the evolution of hypertension in alcoholics (5,6). The other proposed mechanism was an increase in vascular endothelial growth factor expression, as well as endothelial NOS (eNOS) activation, leading to NO generation in response to mild to moderate concentrations of ethanol *in vitro* and *in vivo* (6,31). In addition, from our results it can be postulated that since NO is also a free radical, lower AOA levels in the alcohol group might be another significant cause of increased serum NO levels, or vice versa.

Among previous reports, only Sierksma et al. (32) found that chronic moderate alcohol consumption had no effect on serum NO concentration. The present study found no significant difference between brain NO levels in the alcohol and control groups.

There are some conflicting reports about the effect of alcohol on NO production in the brain. For example, Nagatomo et al. (15) reported no difference between

brain NO levels of controls and mice that consumed ethanol for 4 weeks; however, brain NO levels of mice that consumed ethanol for 12 weeks were significantly lower than those of controls and of mice that consumed ethanol for 4 weeks.

Shih et al. (9) found that exposing cerebrovascular cells (pial) to alcohol in culture for a period of 24-48 h significantly elevated NO production. They also found that ≥ 50 mM of ethanol was required to generate significant levels of NO in cerebral vascular cells.

Neiman and Benthin (33) did not find any difference between cerebrospinal fluid NO levels in alcohol consuming subjects and healthy controls; however, Aydin et al. (26) reported that ethanol consumption for 1 month increased brain NO levels in rats. These findings suggest that ethanol may influence brain NO levels, depending on the dose, duration of consumption, and experimental conditions.

In conclusion, our findings of reduced AOA in both the sera and brains of the alcohol group are evidence that alcohol weakens the body's defense mechanism against free radical attacks. Furthermore, decreased AOA levels in the alcohol group may be another significant cause of increased levels of serum NO, or vice versa. Nonetheless, a better understanding of the effects of alcohol on brain NO levels requires additional investigation.

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References

1. Mayes PA, Lipid Transport & storage, In Harper's Biochemistry, Murray RK, Granner DK, Mayes PA, Rodwell VW (Eds), 23rd ed, London: Lange medical publication;1993. p. 260.
2. Reddy SK, Husain K, Schlorff EC, Scott RB, Somani SM. Dose response of ethanol ingestion on antioxidant defense system in rat brain subcellular fractions. *Neurotoxicology* 1999; 20: 977-87.
3. Somani SM, Husain K. Interaction of exercise training and chronic ethanol ingestion on antioxidant system of rat brain regions. *J Appl Toxicol* 1997; 7: 329-36.
4. Charness ME. Brain lesions in alcoholics. *Alcohol Clin Exp Res* 1993; 17: 2-11.
5. Zima T, Fialova L, Metsek O, Janebova M, Crkowska J, Malbohan I et al. Oxidative stress, metabolism of ethanol and alcohol-related diseases. *Journal of Biomedical Science* 2001; 8: 59-70.
6. Husain K, Mejia J, Lalla J, Kazim S. Dose response of alcohol-induced changes in BP, nitric oxide and antioxidants in rat plasma. *Pharmacol Res* 2005; 1: 337-43.
7. Kukielka E, Dicker E, Cederbaum AI. Increased production of reactive oxygen species by rat liver mitochondria after chronic ethanol treatment. *Archives Biochemistry Biophysics* 1994; 309: 377-86.
8. Davis RL, Syapin PJ. Interactions of alcohol and nitric-oxide synthase in the brain. *Brain Res Brain Res Rev* 2005; 49: 494-504.
9. Shih CL, Chi SI, Chiu TH, Sun GY, Lin TN. Ethanol effects on nitric oxide production in cerebral pial cultures. *Alcoholism, Clinical Experimental Research* 2001;25:612-8.

10. Cakatay U, Telci A, Kayali R, Tekeli F, Akcay T, Sivas A. Relation of oxidative protein damage and nitrotyrosine levels in the aging rat brain. *Experimental Gerontology* 2001; 36: 221-9.
11. Lancaster FE, Alcohol and the brain: what's NO got to do with it? *Metabolic Brain Disease* 1995; 10: 125-33.
12. Kahkonen S, Zvartau EE. Altered cardiovascular responses to nitrosorbide in alcohol withdrawal. *Neuropsychobiology* 2003; 48: 124-30.
13. Dizon ML, Brown LA, Black SM. Brain nitric oxide synthase levels increase in response to antenatal ethanol exposure. *Alcohol and Alcoholism* 2004; 39: 101-5.
14. Mihailescu I, Mohora M, Dinu V. Preliminary evidence for lipid peroxidation and changes in pro-oxidant status in brain tissue and blood in ethanol intoxicated rats. *Revue Roumaine De Physiologie* 1991; 28: 133-7.
15. Nagatomo I, Uchida M, Akasaki Y, Hashiguchi W, Tominaga M, Kuchiiwa S et al. The Effect of Alcohol on Convulsions and Nitric Oxide Levels in Seizure-Susceptible EL Mice. *Epilepsy & Behavior* 2000; 1: 176-83.
16. Evrard SG, Duhalde-Vega M, Tagliaferro P, Mirochnic S, Caltana LR, Brusco A. A low chronic ethanol exposure induces morphological changes in the adolescent rat brain that are not fully recovered even after a long abstinence: an immunohistochemical study. 2006; 200: 438-59.
17. Ahmed N, Hillman H. Preparation of an insoluble fraction of ox brain and its affinity for Na⁺ and K⁺. *Neurochem Res* 1982; 7: 911-21.
18. Saravanan R, Rajendra Prasad N, Pugalendi KV. Effect of piper betle leaf extract on alcoholic toxicity in the rat brain. *Journal of Medicinal Food* 2003; 6: 261-5.
19. Bjorneboe GA, Johnsen J, Bjorneboe A, Morland J, Drevon CA. Effect of heavy alcohol consumption on serum concentrations of fat-soluble vitamins and selenium. *Alcohol and Alcoholism* 1987; 1: 533-7.
20. Bjorneboe GE, Johnsen J, Bjorneboe A, Marklund SL, Skylyv N, Hoiseth A et al. Some aspects of antioxidant status in blood from alcoholics. *Alcoholism. Clinical Experimental Research* 1988; 12: 806-10.
21. Ledig M, M'Paria JR, Mandel P. Superoxide dismutase activity in rat brain during acute and chronic alcohol intoxication. *Neurochem Res* 1981; 6: 385-90.
22. Pushpakiran G, Mahalakshmi K, Anuradha CV. Taurine restores ethanol-induced depletion of antioxidants and attenuates oxidative stress in rat tissues. *Amino Acids* 2004; 27: 91-6.
23. Carmiel-Haggai M, Cederbaum AI, Nieto N. Binge ethanol exposure increases liver injury in obese rats. *Gastroenterology* 2003; 125: 1818-33.
24. Seo DO, Rivier C. Interaction between alcohol and nitric oxide on ACTH release in the rat. *Alcoholism. Clinical Experimental Research* 2003; 27: 989-96.
25. Schisler NJ, Singh SM. Effect of ethanol in vivo on enzymes which detoxify oxygen free radicals. *Free Radical Biology and Medicine* 1989; 7: 117-23.
26. Aydin S, Ozaras R, Uzun H, Belce A, Uslu E, Tahan V et al. N-acetylcysteine reduced the effect of ethanol on antioxidant system in rat plasma and brain tissue. *Tohoku J Exp Med.* 2002; 198: 71-7.
27. Soardo G, Donnini D, Varutti R, Moretti M, Milocco C, Basan L et al. Alcohol-induced endothelial changes are associated with oxidative stress and are rapidly reversed after withdrawal. *Alcohol Clin Exp Res* 2005; 29: 1889-98.
28. Erel O. A new automated colorimetric method for measuring total oxidant status. *Clin Biochem* 2005; 38: 1103-11.
29. Li YM, Chen SH, Yu CH, Zhang Y, Xu GY. Effect of acute alcoholism on hepatic enzymes and oxidation/antioxidation in rats. *Hepatobiliary Pancreat Dis Int.* 2004; 3: 241-4.
30. Oekonomaki E, Notas G, Mouzas IA, Valatas V, Skordilis P, Xidakis C et al. Binge drinking and nitric oxide metabolites in chronic liver disease. *Alcohol Alcohol.* 2004; 39: 106-9.
31. Bennett KR, Gu JW, Adair TH, Heath BJ. Moderate levels of ethanol induce expression of vascular endothelial growth factor and stimulate angiogenesis. *Am J Physiol Regul Integr Comp Physiol* 2001; 281: 365-72.
32. Sierksma A, van der Gaag MS, Grobbee DE, Hendriks HF. Acute and chronic effects of dinner with alcoholic beverages on nitric oxide metabolites in healthy men. *Clinical and Experimental Pharmacology and Physiology* 2003; 30: 504-6.
33. Neiman J, Benthin G. Nitric oxide is not increased in alcoholic brain. *Alcohol and Alcoholism* 1997; 32: 551-3.