Ethanol and Nicotine Interaction on Liver Oxidative Status: An Experimental Study

Etanol ve Nikotin Etkileşiminin Sıçan Karaciğerindeki Oksidatif Durum Üzerine Etkisi: Deneysel Bir Çalışma

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ABSTRACT Objective: To investigate the additive effect of ethanol and nicotine on rat liver oxidative status following either alone or combined sub-chronic administration. **Material and Methods:** Forty-eight female Sprague Dewley rats were grouped randomly into one of the protocols, which consisted of treatment for 10 days with ethanol 2 g/kg/day (ethanol group, n= 12), nicotine 0.15 mg/kg/day (nicotine group, n= 12), both drugs (ethanol plus nicotine group, n= 12) or saline solution (control group, n= 10) for 10 days. Following 10 days of administration of thesethe abovementioned agents, malondialdehyde concentration, reduced glutathione concentration and glutathione peroxidase activity were assessed in the liver. **Results:** The ethanol plus nicotine group had significantly higher malondialdehyde and lower glutathione concentrations than either the ethanol and nicotine and the control groups (both, $p< 0.05$). The ethanol or nicotine group had significantly higher malondialdehyde concentration and lower glutathione concentration and glutathione peroxidase activity than the control group (all $p< 0.05$). Malondialdehyde and glutathione concentration was not significantly higher in the ethanol group than in the nicotine group (both, $p> 0.05$). No significant difference was observed in glutathione peroxidase activity of ethanol, nicotine or ethanol plus nicotine groups (all $p> 0.05$). **Conclusions:** Co-administration of ethanol and nicotine results with significant increase in lipid peroxidation and significant decrease in glutathionelevels compared to their separate administration.

Key Words: Nicotine; alcohols; oxidative stress

ÖZET Amaç: Etanol ve nikotinin tek başına veya birlikte subkronik uygulanmasının sıçan karaciğerindeki oksidatif durum üzerine olan etkisini araştırmak. **Gereç ve Yöntemler:** Sprague Dewley türü 48 dişi sıçan tek başına etanol 2 gr/kg/gün (etanol grubu, n= 12), tek başına nikotin 0.15 mg/kg/gün (nikotin grubu, n= 12), kombine etanol 2 gr/kg/gün ve nikotin 0.15 mg/kg/gün (etanol ve nikotin grubu, n= 12) veya tek başına tuzlu su solüsyonu (kontrol grubu, n= 10) verilecek şekilde randomize edildi. Bu maddelerin 10 gün boyunca uygulanmasının ardından, sıçanların karaciğerinde malondialdehid konsantrasyonu, glutatyon konsantrasyonu ve glutatyon peroksidaz aktivitesi ölçüldü. **Bulgular:** Etanol ve nikotin grubunda diğer gruplara göre malondialdehit konsantrasyonu anlamlı ölçüde daha yüksek ve glutatyon konsantrasyonu daha düşük tespit edildi (her ikisi için $p< 0.05$). Etanol grubu ve nikotin grubu kontrol grubuna kıyasla daha yüksek malondialdehid konsantrasyonu, daha düşük glutatyon konsantrasyonu ve daha düşük glutatyon peroksidaz aktivitesine sahip idi (tümü için $p< 0.05$). Malondialdehid ve glutatyon konsantrasyonu etanol grubunda nikotin grubuna göre anlamlı şekilde yüksek değildi (her ikisi için $p> 0.05$). Etanol grubu, nikotin grubu ve etanol ve nikotin grubu arasında glutatyon peroksidaz aktivitesi açısından istatistiksel olarak anlamlı bir farklılık tespit edilmedi (tümü için $p> 0.05$). **Sonuç:** Etanol ve nikotinin birlikte uygulanması, bu maddelerin tek başına uygulanmasına kıyasla lipid peroksidasyonunda anlamlı şekilde artışa ve glutatyon düzeylerinde anlamlı şekilde azalmaya neden olmaktadır.

Anahtar Kelimeler: Nikotin; alkoller; oksidatif stres

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The pathogenesis of alcoholic liver disease and its molecular basics could be enlightened only during the last few decades.[1] In the past, it was erroneously thought that malnutrition was the main factor in

the development of alcoholic liver disease. It is well recognized today that alcohol directly causes alcoholic liver disease by its toxic effects. The metabolism of ethanol in microsomes is enzymatic specifically via cytochrome P450 II E1 isozyme. Alcohol is metabolized to acetaldehyde in the liver via alcohol dehydrogenase, and then this product is converted to acetate by acetaldehyde dehydrogenase/oxidase.[2,3] Although the cellular basics of the toxic effects of alcohol have not been completely understood yet, it is well known that ethanol leads to lipid peroxidation by increasing free oxygen radicals.[4]

Nicotine, a major toxic component of cigarette smoking, is oxidized primarily to cotinine in the liver.[5] Experimental studies have shown that chronic administration of nicotine causes oxidative stress by inducing the generation of lipid peroxidation products in serum and in various tissues.[6-8] Cigarette smoking is common among persons with alcohol dependence or abuse with as many as 80% of persons who are alcohol dependent also being smokers. Not only is smoking common in persons with heavy alcohol consumption, but also nicotine dependence appears more severe in smokers with a history of alcohol dependence.[9]

Since people consuming alcohol are usually smokers, in the present experimental study, we aimed to investigate the oxidative stress and antioxidative defense in rats' liver following sub-chronic administration of alcohol, nicotine or their combination.

MATERIAL AND METHODS

Forty-eight 60 days old female Sprague Dewley rats weighing between 180-210 g were acclimated for one week. All rats were in good health and were kept under the same physical and environmental conditions in our experiment laboratory. Rats were kept in the room with a temperature of 23 ± 2°C with a relative humidity of 55 ± 5% and 12 hours of light/dark cycle. During acclimation and study, ad libitum access to rat chow and water were supplied. All animals were weighed daily, received human care and local standards for protecting animal welfare were followed. The study protocol was carried out in accordance with the guide for the care and use of the laboratory animals and approved by the local Institutional Review Committee.

ETHANOL AND NICOTINE ADMINISTRATION

The dose of ethanol and nicotine administered to the our animals was based on the previous studies.[10,11] Rats were divided randomly into four groups and were treated as follows:

The ethanol group consisted of 12 rats receiving intraperitoneal (IP) ethanol 2 gr/kg/day, the nicotine group consisted of 12 rats receiving IP nicotine hydrogen bitartrate 0.15 mg/kg/day, the ethanol plus nicotine group consisted of 12 rats receiving IP ethanol 2 gr/kg/day plus IP nicotine hydrogen bitartrate 0.15 mg/kg/day, and the control group consisted of 10 rats receiving IP saline solution at corresponding volume for 10 days.

TISSUE EXTRACT PREPARATION

At the end of ten days, twelve hours after the administration of the last dosage, animals were executed via decapitation and their livers were rapidly extirpated and washed in cold saline solution. Liver tissues were added to 150 mM ice-cold potassium chloride and were homogenized for 3 minutes in a homogenizer (Ultra Turrax T25, Germany) to make a 10% homogenate. Then, liver tissues were immediately frozen in liquid nitrogen. Frozen livers were stored at -70°C until evaluations.[10,11]

Lipid peroxidation products and glutathione (GSH) concentration, and glutathione peroxidase (GSH-Px) activities were determined in the livers.

DETERMINATION OF LIPID PEROXIDE CONCENTRATION

The extent of lipid peroxidation was estimated as the concentration of malondialdehyde (MDA), thiobarbituric acid reactive product, using the thiobarbituric acid method.[12] For this, 750 µL 10% liver homogenate was added to 750 µL 20% trichloroacetic acid solution and the mixture was centrifuged at 4000 rpm for 20 minutes. Then, 1 mL supernatant was added to 0.8% thiobarbituric acid solution and they were incubated in boiling water for 15 minutes. Following cooling, their absorbance was read against the blank, which did not contain ho-

mogenate. 1,1,3,3, tetra-ethoxy-propane was used as an internal standard and results were expressed as nmol MDA/g-tissue.

DETERMINATION OF LIVER REDUCED GLUTATHIONE LEVEL

Non-protein free sulfhydryl groups, which are indicators of GSH, were detected in liver homogenates by Ellmann's method with a spectrophotometer.[13] Briefly, 0.75 mL of phosphate buffer and 1.5 mL of deproteinization solution were added to 0.25 mL of 10% liver homogenates prepared by 0.1 M phosphate 1 mM EDTA-Na_2 buffer (pH 7.5), all were mixed with vortex and were centrifuged for 15 minutes at 4000 rpm. Then, 0.25 mL of the supernatant was added to 1 mL of 0.3 M Na_2HPO_4 and 0.25 mL Ellman indicator. Color was read at 412 nm against the blank with LKB-Ultraspect II spectrophotometer. GSH was used as standard and results were presented as mmol GSH/g-tissue.

DETERMINATION OF LIVER GLUTATHIONE PEROXIDASE ACTIVITY

1 mL of liver homogenate was added to 1 mL of 100 mM phosphate buffer containing reduced GSH (1 mM), EDTA (4 mM), sodium azide (NaN_3; 4 mM), NADPH (0.2 mM) and GSH reductase. Following 5 minutes of pre-incubation at 37°C, the reaction was initiated by 1 mL of hydrogen peroxide (H_2O_2; 1 mM). 1 mL of reaction mixture was taken at every 3 minutes and reaction was terminated by adding 4 ml of deproteinized solution. The reaction rate was measured by a recording spectrophotometer. In order to detect non-enzymatic GSH increase, same procedures were repeated in a medium not containing the homogenate. Decrease in GSH level per minute was calculated for both enzymatic and non-enzymatic reactions. Then log (GSH)/min levels for non-enzymatic reaction were subtracted from log (GSH)/min levels for enzymatic reaction and each decrease of 0.001 unit was described as 1 enzyme unit. The enzyme activity was expressed as U/g of wet tissue.[14]

STATISTICAL ANALYSIS

Data were expressed as mean ± SD. Data distribution was assessed using the Shapiro-Wilk test. As data showed normal distribution, paired t-test was used to compare weight changes of the animals during the study. Continuous variables were compared using one-way Analysis of Variance (ANOVA), followed by post hoc Tukey test. p value of less than 0.05 was considered statistically significant.

RESULTS

Nutritional conditions of the animals were good and no significant weight change between four groups was observed throughout the study (all $p > 0.05$). In addition, according to our subjective observations, no differences were observed between groups regarding daily physical activity. Mortality was not observed during the experimental period.

The effect of sub-chronic administration of ethanol or nicotine alone or in combination on MDA and GSH concentrations, and GSH-Px activity was shown in Table 1. Sub-chronic administration of ethanol, nicotine and ethanol plus nicotine significantly increased MDA concentration to 128%,

TABLE 1: Malondialdehyde and glutathione concentrations, and glutathione peroxidase activity of the groups.

Parameters	Ethanol	Nicotine	Ethanol+Nicotine	Controls
MDA (nmol MDA/g-tissue)	246.3 ± 24.2*	226.3 ± 43.9*	373.8 ± 35.3***	192.2 ± 31.9
GSH (μmol/g-tissue	5.4 ± 0.6***	4.9 ± 0.8***	3.6 ± 0.3	7.3 ± 0.5****
GSH-Px (U/mg-tissue)	10.2 ± 1.86	11.0 ± 2.1	10.9 ± 1.44	17.7 ± 2.37****

Data are presented as mean ± standard deviation,

*p< 0.05 vs. controls,

**p< 0.05 vs. ethanol and nicotine,

***p< 0.05 vs. ethanol + nicotine,

****p< 0.05 vs. ethanol, nicotine and ethanol + nicotine,

MDA: malondialdehyde; GSH: glutathione; GSH-Px: glutathione peroxidase.

118% and 194% of control values, respectively (all p< 0.05). MDA concentration was significantly higher in the ethanol plus nicotine group than in the ethanol and the nicotine groups (both p< 0.05). MDA concentration of the ethanol group was not significantly different from that of the nicotine group (p> 0.05).

Sub-chronic administration of ethanol, nicotine and ethanol plus nicotine significantly decreased GSH concentrations to 74%, 67% and 49% of control values, respectively (all p< 0.05). The ethanol plus nicotine group had significantly more depressed GSH concentration than the ethanol and the nicotine groups (both p< 0.05). GSH concentration was not significantly different in the ethanol group from the nicotine group (p> 0.05).

Sub-chronic administration of ethanol, nicotine and ethanol plus nicotine significantly decreased GSH-Px activity to 57.6%, 62% and 61.6% of control values, respectively (all p< 0.05). No significant difference was observed in GSH-Px activity of ethanol, nicotine or ethanol plus nicotine groups (p> 0.05).

DISCUSSION

Alcohol ingestion is known to produce a variety of metabolic and pathological alterations, which are especially due to cell metabolic disturbances associated with ethanol oxidation and oxidative stress in the liver of both alcoholic patients and experimental animals exposed to alcohol.[15,16] Metabolism of ethanol via cytochrome P450 II E1 induces alpha hydroxyl ethyl radical formation.[17] MDA is an end product of lipid peroxidation and because the lipid peroxidation end products are more stable than free radicals, they provide reliable information on whether the system is under oxidative stress.[18] Therefore, in the present study, we measured MDA level to show the oxidative stress due to ethanol or nicotine alone, or their combined effects. In several studies, the individual effects of these agents on oxidative stress in liver have been separately investigated. As we showed in our study, lipid peroxides had a major role in the pathogenesis of ethanol-induced cellular injury.[10,19-21] However, in a few other studies, liver MDA level was unchanged.[22,23]

Similarly, there is increasing evidence that cellular damage that occurs with nicotine exposure is associated with an imbalance in the cellular oxidant-antioxidant system.[6,7,24,25] Despite the contradictory result of the study by Sheng et al, in which the nicotine induced oxidative stress was suggested to be the result of reduced superoxide dismutase activity rather than increased MDA level, lipid peroxidation was accepted to play an important role in the nicotine induced oxidative stress in the liver in several studies.[10,26-28] In concordance with the latter studies, in the present study, subchronic alcohol or nicotine administration resulted with increase in MDA level of animals belonging to either the ethanol or the nicotine groups.

Hepatic reduced GSH plays a crucial role in scavenging radical oxygen species (ROS).[29] GSH reacts directly with ROS and electrophilic metabolites, protects essential thiol groups from oxidation, and serves as a substrate for several enzymes, including GSH-Px. The liver is particularly susceptible to damage caused by increased ROS and GSH depletion.[11,30] Although acetaldehyde was shown to decrease the decreases the GSH pool by binding to hepatic thiol groups, unchanged, decreased and even elevated GSH levels have been reported after ethanol treatment.[10,22,23,31-34] In the present experimental study, GSH concentration was depressed in both the ethanol and the nicotine groups.

GSH-Px catalyzes the composition of a variety of substrates including hydrogen peroxide, which may be prevalent under oxidative stress.[30] There are conflicting results about the GSH-Px activity following separate administration of either ethanol or nicotine.[10,27] In our study, GSH-Px activity was significantly lower in animals administered either ethanol or nicotine alone.

The increase in MDA concentration, and decrease in GSH level and GSH-Px, which have been shown in both our ethanol and nicotine groups suggested that either ethanol or nicotine could induce oxidative stress in the liver even when they were administered alone.

The combined effect of ethanol and nicotine on oxidative stress and antioxidant defence was investigated in a few studies. Ashakumary et al investigated the additive effect of alcohol and nicotine on lipid peroxidation and antioxidant defence mechanism in rats.[35] They reported increased lipid peroxidation and decreased superoxide dismutase, catalase and GSH reductase activity, while the activity of GSH-Px and the concentration of GSH were increased. They suggested that the increase in GSH concentration and GSH-Px activity was due to increased oxidative stress and depleted other antioxidant enzyme activities caused by the additive effect of ethanol and nicotine. The combined effect of these agents was not studied in the liver in the study by Ashakumary et al.[35]

The combined effect of ethanol and nicotine on tissue oxidative stress and antioxidant system was only investigated in the study by Husain et al.[10] Similar to our study, they also investigated the effect of ethanol, nicotine or their combination on liver antioxidant system, but differently they administered these agents to the rats in a chronic for a longer period (6.5 weeks) and they investigated these parameters in the lung, kidney and testes in addition to the liver. They found that treatment with the combination of ethanol and nicotine significantly increased MDA content and GSH-Px activity to 164% and 134% of control values in the liver. They also found that the combination of ethanol plus nicotine significantly decreased GSH concentration to 62% of control values in the liver. In our study, we administered ethanol, nicotine and their combination for a subchronic period (10 days). The data in our study regarding MDA and GSH concentration was consistent with the results of Husain et al.[10] In contrast, we observed a decrease in GSH-Px activity in all treatment groups compared to controls. The equivalent activity of GSH-Px of the ethanol plus nicotine group compared to the ethanol or the nicotine groups may be suggestive of excessive oxidative stress due to excessively increased MDA concentration. The significant increase in lipid peroxidation, a marker of oxidative stress, and despite the equivalent activity of GSH-Px of the ethanol plus nicotine group compared to ethanol alone or nicotine alone groups, the significant decrease in GSH concentration suggest that ethanol and nicotine have an additive effect on liver oxidative damage. Consequently, it could be suggested that co-administration of ethanol and nicotine had an aggravated combination toxicity on the liver.

In conclusion, ethanol and nicotine had equivalent effects on hepatic oxidative status by affecting same parameters related to oxidative stress and antioxidant defence. Importantly, ethanol and nicotine have additive effect on hepatic oxidative status. Co-administration of these agents results in approximately 1.5-2-fold increase in lipid peroxidation, marker of oxidative stress, and decrease in GSH level, a marker of antioxidant defence, compared to their separate administration.

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