

## HOMOCYSTEINE LEVELS OF RATS CHRONICALLY TREATED WITH NICOTINE

### KRONİK OLARAK NİKOTİN UYGULANMIŞ SIÇANLARDAKİ HOMOSİSTEİN DÜZEYLERİ

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**Anahtar Sözcükler:** nikotin, homosistein, sıçan, sigara içimi

**Key Words:** nicotine, homocysteine, rat, smoking

### SUMMARY

*Cigarette smoking has detrimental effects on the cardiovascular and cerebrovascular systems and among the contents of tobacco, nicotine shows considerable medical significance because of its toxicity. Homocysteine is an intermediate metabolite of methionine and increased levels of homocysteine may underlie atherosclerosis. In the present study, we investigated the relationship between the levels of homocysteine and nicotine in female and male rats which were treated with nicotine (0.35, 1.05 and 2.1 mg/kg, subcutaneously) for 20 days. Blood was obtained by cardiac puncture and serum cotinine levels, as an index of nicotine exposed, and homocysteine levels were measured by "Enzyme Immunoassay (EIA)" and "Fluorescence Polarization Immunoassay (FPIA)" respectively. Nicotine administration resulted in a dose dependent increase in the serum cotinine levels of the rats. Homocysteine levels were higher in female rats than male rats both in control and nicotine-administered groups, however nicotine treatment did not significantly change homocysteine levels in either sex. These results suggest that there is no direct correlation between plasma homocysteine levels and chronic nicotine exposure.*

### ÖZET

*Sigara içilmesi kardiyovasküler ve serebrovasküler sistemlerde zararlı etkilere neden olur ve toksisitesinden ötürü sigaradaki nikotin tıbben önemli bir bileşiktir. Homosistein, metioninin metabolizması sırasında oluşan bir ara üründür ve homosisteinin artışı aterosklerozun patogeneğinde rol oynar. Bu çalışmada 20 gün boyunca nikotin (0.35, 1.05 ve 2.1 mg/kg, subkütan) uygulanan dişi ve erkek sıçanlarda nikotinin homosistein düzeylerine etkisi araştırılmıştır. Hayvanlardan intrakardiyak olarak kan alınmış ve serum homosistein düzeyleri immünolojik yöntem ("enzyme immunoassay", EIA) ile, maruz kalınan nikotin miktarının bir göstergesi olarak kotinin düzeyleri ise immünolojik floresans polarizasyon yöntemi ("fluorescence polarization immunoassay", FPIA) ile ölçülmüştür. Sıçanlara nikotin uygulaması serum kotinin düzeylerinde doza bağımlı bir artışa neden olmuştur. Kontrol ve nikotin tedavi gruplarında dişi sıçanların homosistein düzeyleri erkek sıçanlardan yüksek bulunmuştur, ancak nikotin uygulaması her iki cinsten de homosistein düzeylerinde anlamlı bir değişiklik yapmamıştır. Bu bulgular, kronik olarak nikotine maruz kalış ile plazma homosistein düzeyleri arasında direkt bir ilişki olmadığını göstermektedir.*

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### INTRODUCTION

Cigarette smoking is one of the important causes of death and disability seen worldwide and tobacco use has

been shown to cause several effects on various systems such as respiratory, cardiovascular and cerebrovascular. Homocysteine is also a significant risk factor for atherosclerosis (1,2) cerebrovascular disease (3), and

myocardial infarction (4) and it is an intermediate compound formed during metabolism of methionine. It is known to produce endothelial cell injury in both experimental animal and cell culture studies (5,6). Plasma homocysteine concentrations are regulated by a number of enzymes, essential cofactors and the availability of the important co-substrate methyltetrahydrofolate. The causes of hyperhomocysteinemia are multifactorial such as genetics, age, gender, renal function, nutrition, disease states and drugs (7).

Some studies indicated that smoking was associated with elevated serum/plasma homocysteine (8-12) while some investigations were controversial (13,14). Pagan et al. (2001) suggested that smoking did not affect homocysteine concentrations significantly in the pregnant population, but it was essential to consider the nutritional status in evaluating the effect of smoking on homocysteine metabolism (15). Female smokers have lower and decreasing folate levels during pregnancy, which can lead to hyperhomocysteinemia (16). It has been suggested that chronic cigarette smoking seems to adversely affect plasma homocysteine levels in young adults with type 1 diabetes (17). Most of these studies on nicotine-homocysteine interrelation are performed in pathological conditions. However in normal Guinea-pigs chronically exposed to nicotine, the lung tissue was found to be depleted of S-adenosyl-L-homocysteine and adenosyl-L-methionine (18). Nicotine is the chief alkaloid found in tobacco and cotinine is the primary proximate metabolite of nicotine. Cotinine has a much longer half-life than nicotine and cotinine levels are on average 15-fold than levels of nicotine (1).

We haven't encountered any study in the literature showing the relationship between nicotine treatment and serum homocysteine levels in normal subjects. Therefore in our study, we aimed to investigate the correlation between the serum cotinine and homocysteine levels in the adult female and male rats chronically treated with nicotine.

## MATERIALS AND METHODS

Wistar rats of each sex weighing  $200\pm 30$ g were used in the study. Prior to and during the experiments, the animals were housed (at  $22\pm 1^\circ\text{C}$  with a 12 h light/12 h dark cycle) in plastic cages with pellet food (YEMTA-TARİŞ Yem Sanayii Ltd. Şti., İzmir, Turkey) and water *ad libitum* for 10-15 days.

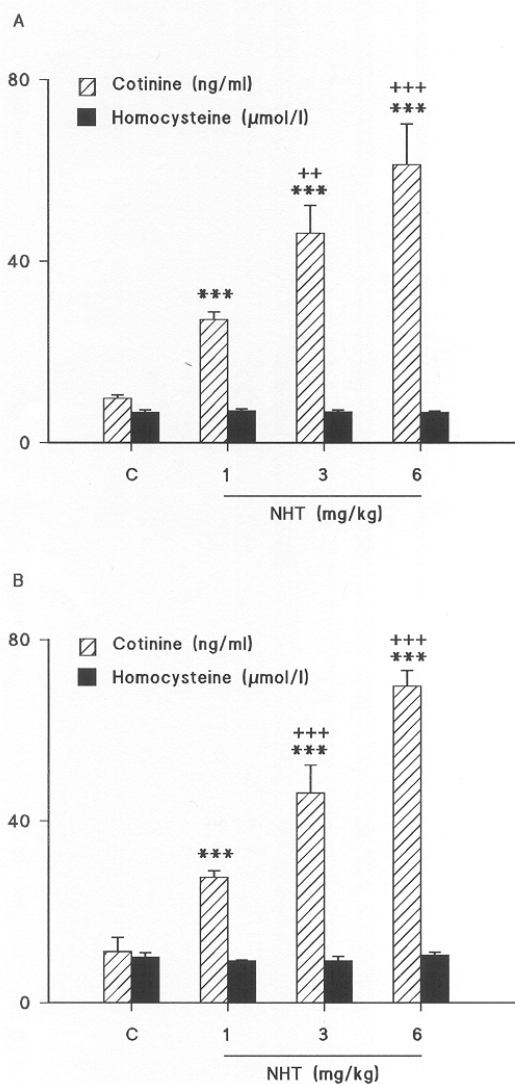
Nicotine hydrogen tartrate (Sigma, USA) was given subcutaneously (s.c.) to the animals for 20 days at 1 mg/kg (eight males, seven females), 3 mg/kg (eight males, seven females) and 6 mg/kg (eight males, nine

females). Base nicotine equivalences are 0.35, 1.05 and 2.1 mg/kg respectively. Control animals (eight males, five females) were injected with saline. Injections were performed at 3.00-4.00 p.m. in the afternoon. At the end of the treatment period, blood was obtained by cardiac puncture under ether anaesthesia 17 hours after the last nicotine injection and serum was separated by centrifugation. Serum samples were stored at  $-70^\circ\text{C}$  until analysis. The measurements were performed after at least five samples in each group were collected.

Serum homocysteine levels were measured with "Fluorescence Polarization Immunoassay (IMx, Abbott Laboratories, Diagnostic Division, Abbott Park, IL, USA). The sensitivity of the assay was  $<0.50\ \mu\text{mol/l}$  which corresponded to the upper limit of the 95% confidence intervals and the results of the within-run and day-to-day precision tests showed coefficient of variation (CV) values between 1.9% and 4.1%. Serum cotinine levels were measured with "Enzyme Immunoassay (Cozart Bioscience Lt., UK) (19) and the precision test for this assay showed a CV value of 9.4% for the lowest sample tested (5 ng/ml) and 4.4% for the highest sample tested (50 ng/ml). Serum concentration measured in our study were within the analytical range of both assays. Data were analyzed using two-way analysis of variance (ANOVA) followed by post-hoc analysis with Bonferroni test. If  $p<0.05$ , then the differences were considered significant.

## RESULTS

Treatment of the rats with 1, 3 and 6 mg/kg nicotine hydrogen tartrate for 20 days resulted in a dose-dependent increase in the plasma cotinine levels of both male and female rats [Fig. 1A;  $F(3,43)=66.15$ ,  $p<0.001$ ], indicating that a reasonable amount of s.c. nicotine reached the systemic circulation. There was no significant difference between males and females [ $F(1,43)=1.12$ ] and no influence of gender difference on the nicotine-induced increase in the plasma cotinine levels was observed [ $F(3,43)=0.38$ ]. Homocysteine levels of control female rats are significantly higher than male rats [Fig 1B,  $p<0.01$ ]. Treatment of the rats with nicotine did not significantly change the homocysteine levels in both sex. The higher homocysteine levels of female rats persisted significantly after nicotine exposure [ $F(1,50)=64.25$ ,  $p<0.01$ ], however, there was no influence of nicotine treatment on the gender difference in the homocysteine levels [ $F(3,50)=0.33$ ].



**Figure 1.** Serum cotinine and homocysteine levels of the male (A) and female (B) rats chronically treated with nicotine hydrogen tartrate (NHT). Data are presented as mean  $\pm$  SEM. \*\*\* $p < 0.001$ , when compared to control (C); ++ $p < 0.01$ , +++ $p < 0.001$  when compared to 1mg/kg nicotine-administered group, ## $p < 0.01$ , when compared to males.

## DISCUSSION

Homocysteine is an intermediate compound formed during the metabolism of methionine, an essential sulfur-containing amino acid supplied from dietary proteins (2). Homocysteine serum concentrations were studied in nicotine exposed male and female rats in the current study. S.c administration of nicotine induced a significant dose-dependent elevation in serum cotinine levels, showing that s.c nicotine treatment was quite sufficient to maintain stable, high nicotine levels in the blood. Despite this high nicotine exposure and elevation in the levels of nicotine metabolite, serum homocysteine levels of nicotine-treated rats were not significantly different from the control rats. This finding has suggested that chronic

exposure to nicotine does not affect the homocysteine levels.

Intraperitoneal injection of 2 mg/kg nicotine hydrogen tartrate (0.7 mg/kg base equivalent) would lead to an approximate concentration of 7  $\mu$ M in a rat weighing 250 g assuming a body volume of 150 ml and such a concentration is highly compatible with the low micromolar concentrations of nicotine achieved with smoking (20). Smoking of one cigarette results in an approximate nicotine exposure of 6-8 mg in humans and the nicotine concentration of a 60-70 kg woman who smokes 20 unfiltered cigarettes per day reaches to 126 mg or 2.1 mg/kg/day (21). Therefore, daily s.c. administration of the maximum dose in our study (6 mg/kg, equivalent to 2.1 mg/kg base) would achieve a plasma nicotine concentration equivalent to a heavy smoker. Indeed, serum nicotine levels of an active smoker would be 20-60 ng/ml and administration of the highest dose in our study resulted in a cotinine concentration of 65 ng/ml (see Fig 1). Cotinine concentrations are normally higher than nicotine concentrations and nicotine metabolism may differ from one species to another, however we suggest that nicotine doses used in this study yield reasonable plasma nicotine concentrations within the ranges obtained by usual cigarette smoking in the society.

Previous studies have shown that serum homocysteine levels were almost always higher in men than in women (22, 23). Knowing that there may be a gender difference in homocysteine levels, the possible correlation between nicotine exposure and serum homocysteine levels was investigated in both sex in our study. Contrary to previous data, homocysteine levels were found higher in female rats both in the control group. Most of the articles on sex-related differences in homocysteine levels are performed on human subjects. The discrepancy of our results with the previous data may be explained by differences in homocysteine metabolism among species. Nevertheless, our primary goal was to determine any correlation between nicotine administration and homocysteine levels and treatment of the rats with nicotine did not significantly change the homocysteine levels in either sex. Based on our data, it seems unlikely that exposure to nicotine, the active ingredient of tobacco, significantly affects homocysteine levels; a finding which is independent of gender difference.

There is a limited number of investigations studying the relationship between smoking and homocysteine levels. In human studies, some of the investigators have proposed that smoking yields to elevated serum homocysteine concentrations in adults; although some controversy also exists (13,14). Stein et al. (2002) have shown that homocysteine levels decrease in subjects who stop

smoking; but significant changes in homocysteine levels were not observed in subjects who reduced smoking or continued to smoke (24). Pagan et al. (2001) have shown that pregnancy and nonpregnancy serum homocysteine concentrations of the smokers and nonsmokers were not significantly different; but the concentrations of folate and vitamin B<sub>12</sub> were generally found low in smokers than nonsmokers (15). Similarly Wersch et al. (2002) have shown that folate plasma concentrations were decreased in smoking pregnant women (16). Serum folate concentrations were found to be lower in smokers compared to non-smokers not only in pregnancy but also in a gender mixed healthy population as well (25). Serum homocysteine levels were found to be elevated in >95 % of patients with folate or vitamin B<sub>12</sub> deficiency (16) and it has been proposed that smoking affects the nutritional status of folate, vitamin B<sub>12</sub> and vitamin B<sub>6</sub> (26,27), each of which regulates homocysteine metabolism (28). It is not clear whether it is nicotine *per se* that causes the deficiency of folate and vitamins B among the contents of cigarette or not. Rats in the current study are fed with commercially available pellet food. There is no information about folate and vitamin B<sub>12</sub> content of the food; however the pellets contain caryopses, i.e. wheat, barley, corn and oat, which are rich of these vitamins. Since both control and nicotine-treated rats received the same standard food, an effect of nutritional status on the homocysteine levels obtained in this study can easily be ruled out; yet nicotine treatment failed to increase homocysteine levels in the study.

As an endogenous intermediate formed during methionine cycle, almost 50% of intracellular homocysteine is metabolized via transsulfuration to cystathionine and the rest is remethylated back to methionine (7). Approximately 80% of homocysteine in blood is protein bound. Normally, the intracellular concentration of homocysteine is kept within narrow bounds. There is limited information about the half life of homocysteine formed during normal metabolism and most of the studies on the estimation of homocysteine half life refers to methionine loading test

which is used to evaluate impaired homocysteine metabolism (7). After administration of an oral dose of methionine, 0.1 g/kg body weight, plasma total homocysteine (free+protein bound) level peaks at 4-6 hours, there is still an almost 2.5 fold increase at the end of the first day and returns to its preload value 3 days later (7). A loading test has not been performed in our study, homocysteine measured is the total form and homocysteine levels were determined 17 hours after the last injection of nicotine. We suggest that there is an ongoing "turnover" of homocysteine in the system and should there be a significant nicotine-induced elevation in plasma total homocysteine levels, this would easily be determined within the first day of nicotine injection. However, this was not the case and we failed to determine the expected increase in homocysteine levels. There may be an element of nicotine half-life, since bolus nicotine injection of nicotine was performed in the study. Nevertheless, cotinine levels were found high 17 hours after nicotine injection, indicating that reasonable amount of nicotine was available in the system when blood was withdrawn for homocysteine measurement. The effect of anaesthesia should also be questioned, however rats were exposed to light ether anaesthesia for 1-2 minutes. We suggest that such short duration of exposure would hardly affect the homocysteine level which is involved in rather chronic processes such as atherosclerosis and, yet, both control and nicotine-treated rats received the similar anaesthesia procedure.

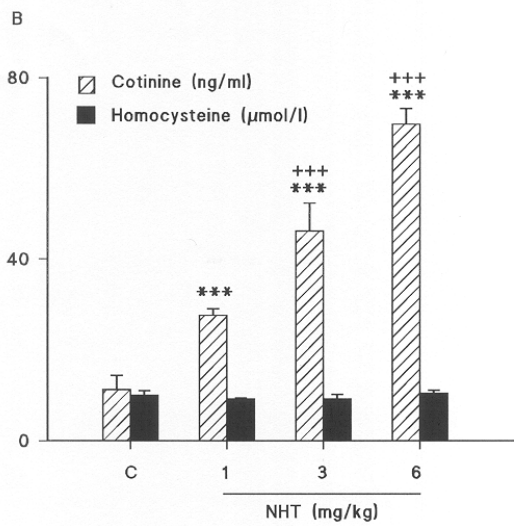
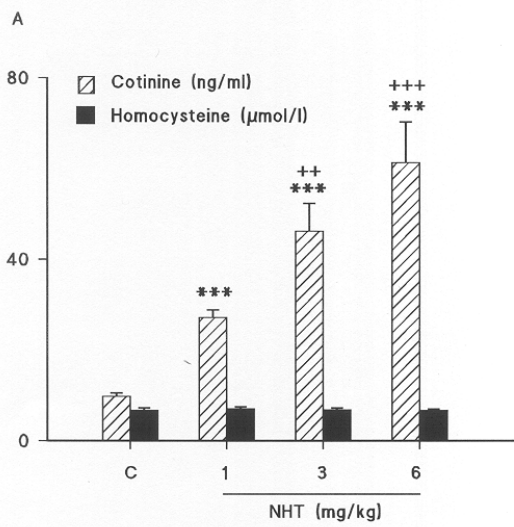
In conclusion; we have observed that nicotine which has been administered to rats chronically through 20 days does not change the plasma total homocysteine levels, suggesting that nicotine directly or indirectly does not affect serum homocysteine levels and, therefore, nicotine is not a candidate ingredient of cigarette mediating smoking-hyperhomocysteinemia interaction. As it has been suggested in previous reports (15,16,26,27) the effects of smoking on the levels of homocysteine should be investigated for the components of cigarette other than nicotine.

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## LEGENDS TO FIGURES

**Figure 1.** Serum cotinine and homocysteine levels of the male (A) and female (B) rats chronically treated with nicotine hydrogen tartrate (NHT). Data are presented as mean  $\pm$  SEM.\*\*\* $p$ <0.001, when compared to control (C); ++ $p$ <0.01, +++ $p$ <0.001 when compared to 1mg/kg nicotine-administered group, ## $p$ <0.01, when compared to males.