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THE EFFECTS OF L-ASCORBIC ACID ON ISONIAZID-INDUCED PROTEIN OXIDATION AND HEPATOTOXICITY IN RATS

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ABSTRACT

Isoniazid (INH) is hepatotoxic drug. Oxidative stress has been reported as one of the mechanisms of INH induced hepatotoxicity. In the current study, our aim was to evaluate the redox status of plasma and liver proteins as well as the protective role and dose of ascorbic acid (AA) in INH-induced hepatotoxicity in rats. Protein oxidation parameters such as protein carbonyl (PCO), total thiol (T-SH) levels and paraoxonase-1 (PON-1) activity was determined in the liver and the plasma of rats. Rats under study were randomly divided into four groups: (1) control; (2) INH (50mg/kg/day); (3) INH (50mg/kg/day) + AA (100 mg/kg/day); and (4) INH + AA (1000 mg/kg/day). INH administration resulted in abnormal elevation of plasma and hepatic PCO levels. On the other hand, the levels of the plasma and hepatic T-SH and PON1 activity significantly decreased. Supplementation of AA (100 mg/kg/day) dose partially reverted these abnormalities in the redox status of the proteins and activities of PON1 after the administration of INH. Changes in oxidative stress are likely involved in the pathogenesis of INH-induced hepatoxicity in rats.

Keywords: Ascorbic acid, Isoniazid, Paraoxonase, Protein carbonyl, Total thiol, Protein oxidation

SIÇANLARDA L-ASKORBİK ASİDİN İZONİAZİD KAYNAKLI PROTEİN OKSİDASYONU VE HEPATOTOKSİSİTE ÜZERİNE ETKİLERİ

ÖZET

İzoniazid (INH) hepatotoksik bir ilaçtır. Oksidatif stres, INH kaynaklı hepatotoksisitenin mekanizmalarından biri olarak rapor edilmiştir. Çalışmada amacımız, sıçanlarda INH kaynaklı hepatotoksisitede askorbik asid (AA)'in koruyucu rolü ve dozunun yanı sıra plazma ve karaciğer proteinlerinin redoks durumunu değerlendirmekti. Sıçanların karaciğer ve plazmalarında protein karbonil (PCO), total tiol (T-SH) seviyeleri ve paraoksonaz-1 (PON-1) aktivitesi gibi protein oksidasyon parametreleri belirlendi. Sıçanlar rastgele dört gruba ayrıldı: (1) kontrol; (2) INH (50mg/kg/gün); (3) INH (50mg/kg/gün) + AA (100 mg/kg/gün); ve (4) INH + AA (1000 mg/kg/gün). INH uygulaması, plazma ve hepatik PCO düzeylerinde anormal yükselme ile sonuçlanmıştır. Aynı zamanda, plazma ve hepatik T-SH seviyeleri ve PON1 aktivitesi önemli ölçüde azaldı. AA (100 mg/kg/gün) dozu takviyesi, proteinlerin redoks durumundaki ve INH uygulamasından sonra PON1 aktivitelerindeki bu anormallikleri kısmen geri döndürdü. Oksidatif stresteki değişiklikler muhtemelen sıçanlarda INH kaynaklı hepatoksisitenin patogenezinde yer almaktadır.

Anahtar Kelimeler: Askorbik asit, İzoniazid, Paraoksonaz, Protein karbonil, Total tiyol, Protein oksidasyonu

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INTRODUCTION

Tuberculosis (TB) is still a leading cause of death worldwide. The three groups of medications isoniazid (INH), rifampicin (RMP), and pyrazinamide (PZA) have been effective therapeutic agents for the treatment of TB because of high therapeutic efficacy and good patient tolerance (1). However, a variety of adverse reactions have been reported; one of the well-known toxic effects is hepatotoxicity. The formation of hydrazine, the key intermediate product of INH metabolism which is a potent acylating agent that is capable of leading to liver necrosis (2).

The molecular mechanism of hydrazine-induced cytotoxicity was attributed to oxidative stress as reactive oxygen species (ROS) and protein carbonyl (PCO) formation occurred before the onset of hepatocyte toxicity (2). In view of the fact that the formation of PCO groups is of a magnitude greater than other oxidative modifications, the level of PCO groups has become the most widely used marker for protein oxidation during oxidative stress and various diseases (3). One or more reduced thiol groups are essential for the fuction of many proteins. The thiol (-SH) group on the side chain of the amino acid cysteine is particularly sensitive to redox reactions and is an established redox sensor. Proteins containing Cys thiol groups are particularly susceptible to oxidation by free radicals (4). Paraoxonase (PON1)'s free thiol group is supposed to be the active site for its antioxidant activity (5). Human PON1 (aryldialkylphosphatase, EC 3.1.8.1) is a 43-kDA esterase associated with apolipoprotein A-I (apoA-I) and clusterin (apolipoprotein J) in HDL (5, 6). PON1 functions in preventing lipid oxidation not only of LDL, but also of HDL itself. This protection is most probably related to the PON1 hydrolyzing activity of some activated phospholipids and/or lipid peroxide products (7).

Ascorbic acid (AA) is an ideal antioxidant that directly queches free radicals, inhibits ROS and regenerates other antioxidants (8). Treatment of rats with AA may slow hepatic protein oxidation, opening new avenues for the pharmacological prevention of INH-induced hepatotoxicity. In the current study, our aim was to evaluate the redox status of plasma and liver proteins as well as the protective role and dose of AA in INH-induced hepatotoxicity in rats.

Materials and methods

Chemicals and apparatuses

Chemicals and solvents used were of highest purity and analytical grade. Deionized water was used in analytical procedures. All reagents were stored at +4 °C. The reagents were maintained in equilibrium at room temperature for 0.5 hour before use.

All centrifugation procedures were performed with a Jouan G 412 centrifuge. Protein oxidation and PON1 activity were measured by a spectrophotometer (Heraeus 400, Kendro Laboratory Product, Osterode, Germany).

Experimental procedure

Male Wistar albino rats (4 months old, weighing 190-220 g) supplied from Cerrahpasa Medical Faculty's Animal Research Laboratory were used. These animals were kept in conventional wire-mesh cages, four rats per cage, in a room with the temperature regulated at 21 ±1 °C, humidity 45-50%, and light/dark cycles (12h). All efforts were made to minimize the number of animals used in the experiments. Food and fresh tap water were supplied ad libidum throughout the experiment "The Guide for the Care and Use of Laboratory Animals" Interventions concerning experimental animals were performed according to published by the US National Institutes of Health (NIH Publication 85, 23, revised 1996).

The INH and AA solutions were freshly prepared in sterile distilled water and given orally as described below for two weeks (9). Forty rats were randomly divided into four groups: (1) Control (n=10) (2) INH (50mg/kg/day) (n=10); (3) INH (50mg/kg/day) + AA (100 mg/kg/day as a moderate dose) (n=10); and (4) INH + AA (1000 mg/kg/day as a high dose) (n=10). A successful model of hepatotoxicity was created by giving 50 mg/kg/day INH (9). The rationale behind the use of moderate AA dose (100 mg/kg/day) and the way of administration have been described previously (9), and the single oral dose of AA has been shown to reduce oxidative stress and restore redox-balance in acetaminophen-induced hepatotoxicity in rats (10). All animals were anesthetized by a mixture of ketamine and xylazine (i.p. 75 and 10 mg/kg. respectively) and sacrified by decapitation on the 21st day of experimental period.

Blood-sample collection and processing

All samples were taken in the morning. Blood samples were drawn in the fasting state and processed within 1 hour of collection. Samples were collected in tubes containing lithium heparin, EDTA or no additive depending on the nature of the analysis. For protein- oxidation parameters and PON1, plasma samples containing lithium heparin were stored at –70 °C until the actual analysis. All other routine clinical-chemistry parameters such as AST, ALT, ALP, and GGT were determined on the same day of collection by using the Hitachi 704 autoanalyzer (Boehringer Mannheim, Tokyo, Japan).

Preparation of liver samples

Liver tissue from the rats was quickly removed, washed in cooled 0.15 M NaCl, and placed on ice-cold plate. Tissue samples were then immediately frozen in liquid N_2 until experimentation. Liver tissue (200 mg) samples were homogenized manualy in 2 ml of homogenizing buffer (100 mM KH_2PO_4-K_2HPO_4, pH 7.4, plus 0.1% (w/v) digitonin) in a glass homogenizer to avoid disruptions of nuclear membranes. In this way, contamination by nucleic acids was minimized. Liver homogenates obtained from rats were centrifuged at 5000 x g for 10 minutes, and determination of various analytes was performed in the supernatant fraction.

Determination of protein carbonyl levels

Protein carbonyls were measured spectrophotometrically by using the method of Reznick and Packer (11). PCO groups react with 2,4-dinitrophenylhydrazine (DNPH) to generate chromophoric dinitrophenylhydrazones. DNPH was dissolved in HCl, and after the DNPH reaction, proteins were precipitated with an equal volume of 20% (w/v) trichloroacetic acid and washed three times with 4 ml of an ethanol/ethyl acetate mixture (1:1). Washings were achieved by mechanical disruption of pellets in the washing solution using a small spatula, and re-pelleting by centrifugation at 6000 g for 5 min. Finally, the precipitates were dissolved in 6 M-guanidine-HCl solution and the absorbances were measured at 360 nm, using the molar extinction coefficient of DNPH, 2.2 x 10^4 L mol^{-1}. cm^{-1}. Protein contents were determined on the HCl blank pellets spectrophotometrically using a Folin kit (Sigma Diagnostics, St.Louis, MO, USA). The coefficients of intra- and inter- assay variations for carbonyl assay were 5.2% (n=12) and 9.3% (n=10), respectively.

Determination of plasma total thiol (T-SH) levels

T-SH concentration was determined by using 5,5`-dithiobis (2-nitrobenzoic acid) (DTNB) as described by Hu (12). The coefficients of intra- and inter-assay variations were 1.8% (n=10), and 4.6% (n=9), respectively.

Determination of liver total thiol levels

T-SH groups were measured spectrophotometrically by using the method of Sedlak and Lindsay (13). Aliquots of 250 µl of the supernatant fraction of the liver homogenate were mixed in 5 ml test tubes with 750 µl of 0.2 M Tris buffer, pH 8.2, and 50 µl of 0.0l M 5,5`-dithiobis(2-nitrobenzoic acid) (DTNB). The mixture was brought to 5 ml with 3950 µl of absolute methanol. A reagent blank (without sample) and a sample blank (without DTNB) were prepared in a similar manner. The test tubes were stoppered with rubber caps, color was developed for 15 min and the reaction mixtures were centrifuged at approximately 3000g at room temperature for 15 min. The absorbance of supernatant fractions was read in a spectrophotometer at 412 nm. Molar extinction coefficient at 412 nm was 13100 L. $mol^{-1}cm^{-1}$.

Determination of paraoxonase-1 (PON-1) activity

PON1 activity was assayed using paraoxon (diethyl-p-nitrophenyl phosphate) as substrate (14, 15). PON1 activity was determined by measuring the initial rate of substrate hydrolysis to p- nitrophenol, the absorbance of which was monitored at 412 nm in the assay mixture containing 2.0 mM paraoxon, 2.0 mM $CaCl_2$ and 20 µL of plasma in 100 mM tris-HCl buffer (pH 8.0). The blank sample containing incubation mixture without sample was run simultaneously to correct for spontaneous substrate breakdown. Enzymatic activity was calculated from the molar extinction coefficient 18.290 L. $mol^{-1}cm^{-1}$ and is expressed as units per milliliter; One unit of paraoxonase activity is defined as 1 nmol of 4-nitrophenol formed per minute under the above assay conditions.

The intra-assay and inter-assay coefficients of variation were 5.7% (n=20) and 3.8% (n=26), respectively.

Statistical analyses

Data are expressed as mean±SEM for ten animals in each group. Differences between groups were assessed by one way analysis of variance (ANOVA) using the SPSS (version 16) software package for Windows. Post-hoc testing was performed for inter-group comparisons using the least significance difference (LSD) test. A probability value of less than 0,05 was considered statistically significant for all comparisons.

Results

Routine clinical-chemistry parameters of the rat groups subjected to INH alone, in combination with AA administered at either 100 or 1000 mg/kg/day, and respective controls are summarized in Table 1. The treatment with INH progressively and significantly increased the serum levels of AST, ALT, GGT, and ALP. The trend of progressively increased enzyme activities in the serum of the rats treated with isoniazid alone occurred only in diminished rates, or exhibited partial effect, when treated with AA and INH combined.

The parameters used to determine oxidative protein damage in plasma and liver in all supplemented groups and those of the controls are given in Figure 1. Plasma and liver PCO levels of isoniazid-induced hepatotoxicity rats increased significantly compared with AA supplemented rats and the control group. Plasma and liver T-SH levels in rats with isoniazid-induced hepatotoxicity decreased significantly compared with the control group while T-SH levels increased significantly with AA supplementation.

The PON1 activity in plasma and the liver in all studied groups are given in Figure 2. AA partially restored the activities of PON1 to near normalcy. Plasma and liver PON1 levels decreased significantly in the INH-treated group compared with the control group. INH induced decreases in PON1 activities were partially blocked in those animals treated with AA.

Discussion

The preventative and therapeutic strategy against INH-induced hepatotoxicity is still unresolved. The original point of this study is to examine the INH-affected liver tissue and the plasma and the consequent hepatotoxicity of INH for the changes in the levels of protein oxidation and the changes in the activity of PON1, as well as the hepatoprotective effect of AA.

The treatment with INH significantly increased the transaminase activity and co- administration of AA (100 mg/kg/day) blocked the induction of the increased transaminase activity, which is caused by INH treatment in our study. On the other hand, our study failed to reveal why AA does not protect against the ALP increase as another biomarker of hepatotoxicity. Most cases of anti-TB drugs-induced hepatotoxicity are mild (i.e. asymptomatic with <3-fold elevation of serum ALT and AST). However, some patients taking anti-TB drugs develop severe hepatitis that may progress to liver failure and death if drug administration is not stopped promptly (1).

Table 1: The effects of isoniazid and ascorbic acid on plasma markers (AST, ALT, GGT and ALP) in all groups.

	Control Group (I) (n=10)	INH (50 mg/kg) Group (II) (n=10)	INH + AA (100 mg/kg) Group (III) (n=10)	INH + AA (1000 g/kg). Group (IV) (n=10)
AST (U/l)	34,2±5,22	71,5±30,18***	41,9±7,84**	49,1±6,26***
ALT (U/l)	153,8±33,99	552,0±100,39***	149,4±29,49	141,9±26,89
GGT (U/l)	2,9±1,37	5,1±1,45***	3,9±1,73**	3,2±1,14*
ALP (U/l)	189,5±41,35	224,7±93,54***	255,5±123,63**	223,6±85,63***

*Values are represented as means ± SEM. Comparisons are made between group I and groups II, III, IV. Statistical significance levels of the analysed parameters in the study groups: *p<0.05; **p<0.01; ***p<0.001.*

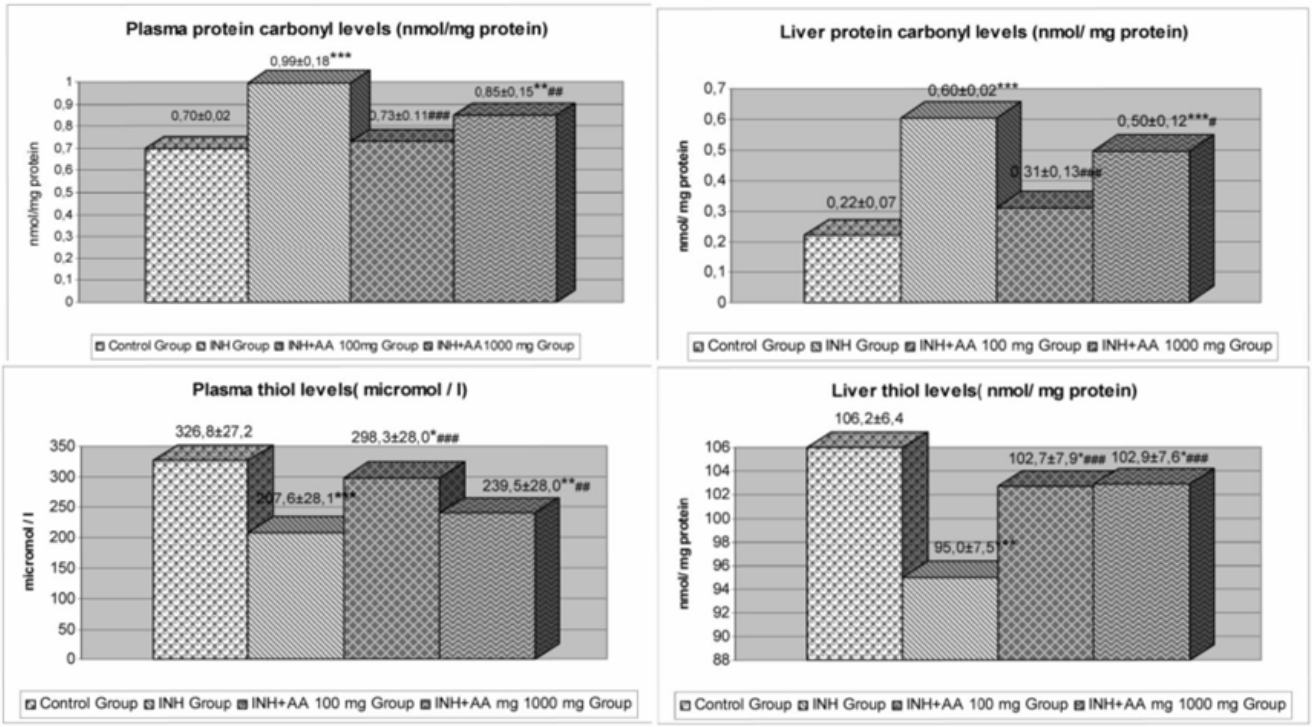

Figure 1. The effects of isoniazid and ascorbic acid on protein carbonyl (PCO) and thiol (T- SH) levels in plasma and liver. Results are presented as arithmetic mean ± SEM for ten animals in each group. Comparisons are made between:--group I and groups II, III, IV; # -- group II and groups III, IV. Values are statistically significant at p<0,05.*

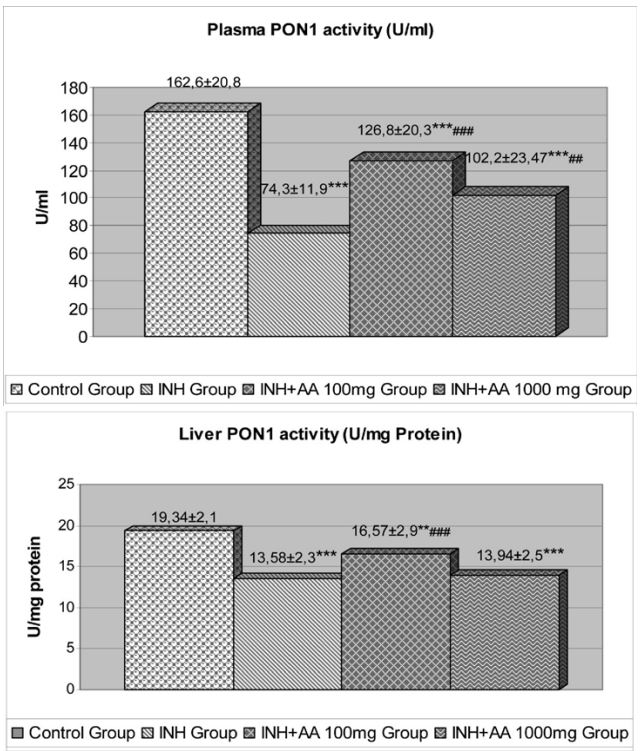

Figure 2. The effects of isoniazid and ascorbic acid on paraoxanase (PON 1) activities in plasma and liver. Results are presented as arithmetic mean ± SEM for ten animals in each group. Comparisons are made between:--group I and groups II, III, IV; # -- group II and groups III, IV. Values are statistically significant at p<0,05.*

Several studies have suggested that ROS play an important role in the pathogenesis of INH- induced hepatotoxicity (2,16,17) . The molecular mechanism of drug-induced cytotoxicity was attributed to oxidative stress as ROS and PCO formation occurred before the onset of hepatocyte toxicity (2,16,17). The steady-state concentration of PCOs and other oxidative modifications in the plasma or in other biological specimens may thus reflect the accumulated oxidative stress in the compartment in question during the lifetime of the protein, thereby providing a potentially valuable biomarker for low-level oxidative stress (8). The increased hepatocyte susceptibility to hydrazine by the H2O2 generating system could result from catalase and superoxide dismutase inactivation by hydrazine as there was a significant increase in PCO levels (16). There are no studies currently available on the relationship between protein oxidation markers and ascorbic acid. On the other hand, Tafazoli et al. showed that the hepatotoxic mechanism also involved extensive oxidative stress as endogenous ROS and H2O2 as well as protein carbonylation were markedly increased and ROS scavengers that prevented ROS formation also prevented the ensuing hepatoxicity (2,17). Most interestingly, our current study shows that moderate dose of AA supplementation is associated with lower PCO and higher T-SH levels in plasma and liver in rats with isoniazid hepatotoxicity.

Liver synthesis of PON1 may be reduced as a part of a general sign of hepatic toxicity. An antioxidant hepatoprotective role may be readily hypothesized for hepatic PON1 because liver microsomes are the major sites for the catabolism of xenobiotic compounds, and their reactions may cause increased production of ROS (2). The results of this study confirm previous studies that experimentally induced liver injury decreases plasma PON1 activity (18, 19). In addition, treatment with AA (100mg/kg/day) decreased INH-induced hepatic injury and normalized PON1 activity but not in plasma. The induction of the synthesis of PON1 in liver cells may be reduced as a part of a general sign of hepatic toxicity. On the other hand, increased ROS production due to INH-induced hepatotoxicity may result in decreased PON1 activity.

Selection of appropriate pharmacological doses of antioxidants for use in oxygen-related diseases is critical. On the other hand, much of the discussion in experimental studies has been devoted to the prooxidant role of exogenous antioxidants. Our observations indicate that AA seemed to reduce the hepatotoxic effects of isoniazid at some doses. The mechanism by which AA protects rats from hepatotoxicity may be due to the moderate dose (e.g. 100 mg/kg/day) of AA but its exact mechanism is not completely understood. High doses of AA (e.g. 1000 mg/kg/day) afford no protection. AA has been reported to have prooxidant effects when given in various concentrations, thus making its antioxidant effects controversial (20).

Oxidative stress may be one of the mechanisms (or the most important one) responsible for INH-induced hepatic injury. The results of the current study clearly show that AA supplementation causes some antioxidant therapeutic and protective effects on INH-induced hepatotoxicity. In conclusion, AA was found to inhibit INH-induced reduction in the activity of PON1 and to have beneficial effect by preventing increased oxidative protein damage. More studies are needed to investigate the most effective dose of AA as well as to bring out the antioxidant mechanism on protein oxidation to prevent INH induced hepatotoxicity in human beings.

Conflict of interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

Ethics Committee Approval

Ethics Committee Approval of the study Maltepe Üniversitesi from Hayvan Deneyleri Yerel Etik Kurulu (MÜ-HADYEK) taken on 22.08.2023 (Decision No: 2023.08.02).

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