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RESEARCH ARTICLE

Ellagic acid could prevent experimentally induced oxidative stress in rats

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Elagik asit, sıçanlarda deneysel olarak indüklenen oksidatif stresi önleyebilir

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Öz

Amaç: Dietilnitrozamin (DEN) ve fenobarbital (PB), metabolizması sırasında reaktif oksijen türlerini (ROS) üretilmektedir. Ellagik asit (EA), antioksidan aktiviteye sahip, biyolojik olarak aktif, doğal bir polifenoldür. Bu çalışma, sıçanlarda DEN-PB'nin neden olduğu karaciğer hasarı üzerinde EA'nın önleyici ve koruyucu etkilerini değerlendirmek için yapılmıştır.

Gereç ve Yöntem: 60 yetişkin Sprague-Dawley sıçan 6 gruba ayrıldı: Negatif kontrol, DEN, EA, DEN-PB (pozitif kontrol), DEN-PB-EA (tedavi) ve EA-DEN-PB (ön tedavi). DEN, pozitif kontrol, tedavi ve ön tedavi gruplarına 150 mg/kg DEN verildi. İki hafta sonra pozitif kontrol ve tedavi gruplarına 500 ppm PB uygulandı. EA ve ön tedavi gruplarına 50 mg/kg/gün EA oral yolla sekiz hafta ve tedavi grubuna ise dört hafta boyunca günaşırı verildi. Sekiz hafta sonra sıçanlar dekapite edildi, karaciğer ve kan biyokimyasal değerlendirmeye tabi tutuldu.

Bulgular: DEN'in tek başına veya PB ile birlikte karaciğer MDA düzeylerini önemli ölçüde arttırdığı; ancak plazma MDA seviyelerini değiştirmediği görüldü ($p<0,001$). EA ön tedavisi, karaciğer MDA seviyelerini azalttı. CAT aktivitesinin, pozitif kontrol grubunun karaciğer ve eritrositlerinde değişmediği belirlendi. Karaciğer CAT aktivitesi, ön tedavide önemsiz ve tedavide ise anlamlı olarak arttı ($p<0,05$). SOD aktivitesi, DEN ve pozitif kontrol gruplarının karaciğer ve eritrositlerinde değişkenlik göstermedi. EA, karaciğer SOD aktivitesini önemli ölçüde arttırdı ($p<0,001$). Tek başına veya PB ile birlikte DEN uygulaması hem karaciğer hem de kan GSH düzeylerini önemli ölçüde düşürürken ($p<0,05$), EA uygulaması tedavi grubunda karaciğer GSH düzeylerini arttırdı ($p<0,05$). Tedavi grubunda kan GSH seviyeleri önemli ölçüde azaldı ($p<0,001$).

Öneri: EA koruyucu tedavisinin, sıçanlarda DEN ve PB kaynaklı karaciğer oksidatif stresinde EA tedavisinden daha etkili olabileceği düşünülmektedir.

Anahtar kelimeler: Dietilnitrozamin, ellagik asit, fenobarbital, oksidatif stress

Abstract

Aim: Reactive oxygen species (ROS) are produced during the metabolism of diethylnitrosamine (DEN) and phenobarbital (PB). Ellagic acid (EA) is a natural biologically active polyphenol with antioxidant activity. The present study was conducted to assess the preventive and protective capability of EA on DEN-PB-induced oxidative damage in rats.

Materials and Methods: 60 Sprague-Dawley rats were assigned into 6 groups: Negative control, DEN, EA, DEN-PB (positive control), DEN-PB-EA (treatment), and EA-DEN-PB (pretreatment). 150 mg/kg DEN were administered to DEN, positive control, treatment and pretreatment groups. After two weeks, 500 ppm PB applied to positive control and treatment groups. 50 mg/kg/day EA were orally given to EA, and pretreatment groups for 8 weeks and treatment group was given for four weeks. After 8 weeks rats were sacrificed, liver and blood were subjected to biochemical evaluation.

Results: DEN alone or with PB increased liver MDA levels significantly; however plasma MDA levels didn't change. EA pretreatment decreased liver MDA levels ($p<0.001$). CAT activity did not change in the liver and erythrocytes of the positive control group. Liver CAT activity increased insignificantly in pretreatment and significantly in treatment group ($p<0.05$). SOD activity did not change in liver and erythrocytes of DEN and positive control groups. EA increased liver SOD activity significantly ($p<0.001$). DEN application alone or with PB decreased liver and blood GSH levels significantly ($p<0.05$), while EA application increased liver GSH levels in treatment ($p<0.05$). Blood GSH levels decreased significantly in treatment ($p<0.001$).

Conclusion: It is considered that EA pretreatment could be more effective than EA treatment in DEN, PB induced oxidative stress in rats.

Keywords: Diethylnitrosamine, ellagic acid, oxidative stress, phenobarbital

Introduction

Diethylnitrosamine which is hazardous compound found in tobacco, processed and smoked foods could be formed either during drug metabolism or with an interaction between amine and nitrite (Shaban et al 2013, Gokuladhas et al 2016). Nitrite is a highly used as preservative in food industry. Therefore, increased amount of DEN might found in processed food (Park et al 2015). Oxidative stress is proven to increase during metabolism of DEN and its derivative (Gokuladhas et al 2016). Increased reactive oxygen species (ROS) formation due to oxidative stress is responsible for cell damage and regarded as one of the causes of liver diseases (Bai et al 2017). Reactive oxygen species forming capacity of DEN, especially in liver, causes liver damage through increasing ROS and might use as liver damage initiator (Shang et al 2018). Although Phenobarbital (PB) is not a genotoxic agent like DEN, it activates cytochrome p450 and might promote liver tumors by increasing liver cell proliferation (Zhao et al 2008). Similarly to DEN, PB is capable to increase oxidative stress when administrated following DEN through activation of cytochrome P450 (Zhao et al 2008). As a central organ in the metabolism, the liver is prone to ROS exposure and produces antioxidants to reduce or delay the effects of ROS (Arauz et al 2016). Furthermore, the effects of ROS might be incapacitated not only by controlling its generation but also via exogenous antioxidants supplementation (Shaban et al 2013). Aforementioned reasons indicate that antioxidant supplementation might be beneficial to protect liver from the hazardous effects of oxidants.

The growing interest has been directed to the investigation of phytochemicals on deleterious effects of hepatotoxic agents (Zhao et al 2008, Shaban et al 2013). Ellagic acid is a natural biologically active polyphenol with antioxidant activity (Srigopalram et al 2014). It is largely found in woody plants, berries, grapes and pomegranates (Ceci et al 2018). Radical scavenging activity and chelating capacity makes it a promising compound against liver damage (Srigopalram et al 2014). Although there are studies indicating antioxidant activity of EA on HEPG2 liver cells (Ding et al 2019), and liver toxicity (Girish et al 2009, Girish and Pradhan 2012), yet there is lack of knowledge about the effects of EA on DEN and PB induced liver damage. Moreover, accumulated evidence indicated the protective effects of EA on liver toxicity (Girish et al 2009, Girish and Pradhan 2012), however curative effects of EA requires further investigation. In this concept, the present study was conducted to evaluate the effect of EA on DEN-PB-induced liver damage in rats.

Material and Methods

Chemicals and reagents

DEN (CAS No: 55-18-5), PB (CAS No: 50-06-6), Trichloroacetic acid (CAS No: 76-03-9), 2-Thiobarbituric acid (CAS No: 504-17-6), 5,5′-Dithiobis-2-nitrobenzoic acid (CAS No: 69-78-3), nitrobluetetrazolium (NBT) (CAS No: 298-83-9), Hydrogen Peroxide (CAS No: 7722-84-1) were purchased from Sigma-Aldrich (St . Louis MO, USA). EA was purchased from Alfa Aesar, Germany (A15722 Lot: 10176718, Ellagic acid hydrate).

Study design

All animal experiments were done according to the approval of Animal Experimentation Ethics Committee of Firat University (Elazig, Turkey) (Approval number 2017/03-34). Sixty male weighing 220 ± 20 g. Sprague-Dawley strain rats were placed in cages in a temperature and humidity controlled environment. Following the adaptation period rats were assigned into 6 groups as negative control, DEN, EA, DEN-PB (positive control), DEN-PB-EA (treatment), and EA-DEN-PB (pretreatment). Tap water and standard diet were distributed to the control group for 8 weeks. DEN, positive control, pretreatment and treatment groups were received 150 mg/kg intraperitoneal (i.p.) DEN injection (Karaca and Sözbilir 2007). Then, PB (500 ppm) was dissolved in tap water and applied to positive control, pretreatment and treatment groups for 6 weeks (Kakehashi et al 2009). Pretreatment and EA groups were given 50 mg/kg/day EA orally every other day for 8 weeks (Alirezaei et al 2012). Treatment group on the other hand, was given 50 mg/kg/day EA every other day orally for four weeks. After eight weeks, blood was collected under ether anesthesia then rats were sacrificed, liver tissues were removed and washed in 0.9% NaCl .

Preparation of blood samples

Blood samples were collected into two anticoagulant containing tubes. The first tube was maintained until the whole blood assays. The latter tube was centrifuged at 3500 rpm (Nuve NF800R) for 5 min; the plasma was separated and frozen. The remained red blood cell suspension were adequately washed in 0.9% NaCl and frozen. MDA, GSH, CAT and SOD levels were determined calorimetrically with spectrophotometer.

Preparation of liver homogenates

Liver samples cut into small pieces and weighed. Subsequently, samples homogenized in 1.15% KCl buffer 1/10 (weight/volume) final volume by using Potter-elvehjem homogenizer. Obtained homogenates centrifuged for fifteen minutes at 3500 rpm for measurement of Malondialdehyde (MDA), Glutathione (GSH) levels together with Catalase (CAT) and SOD activity.

Determination of malondialdehyde levels

Plasma and liver MDA level were measured according to the method described by Placer et al (1966). In summary, the method is determined MDA level through a reaction between thiobarbituric acid (TBA) and MDA. The pink color generated after the reaction between MDA and TBA, were measured at 532 nm wavelength. MDA levels presented as nmol/mL in plasma and nmol/g in liver. A standard curve was prepared with the serial dilution of standard solution (1,1,3,3-tetramethoxypropane). Obtained values were calculated from the standard curve and expressed as nmol/ml plasma and nmol/g tissue in plasma and liver tissues respectively (Placer et al 1966).

Evaluation of catalase activity

CAT activity was measured in red blood cell suspension and liver homogenates as described by Aebi (1984). This method is measured CAT activity according to consumed H2O2 levels during the first 30 seconds at 240 nm wavelength. The results were presented as katal (k)/g Hb respectively, in red blood cells and k/g protein in liver. k refers to rate constant of a first-order kinetic and the rate constant of hemoglobin or tissue content attributed to specific erythrocyte or tissue catalase activity.

Determination of glutathione levels

The whole blood and liver tissue supernatant were used in determination of GSH according to the Chavan et al(2005) method. The principle of this method is based on yellow color formation subsequently to 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) addition. GSH levels were given as µmol/g Hb in the blood and µmol/g protein in the liver tissues. GSH standard curve was drowned by plotting the different amount of GSH standard (37.5, 75, 186, 372, 930, 1490 µmol/dl) to calculate the GSH concentration of blood and liver samples. GSH concentrations were expressed as µmol/g Hb and µmol/g protein in blood and liver tissues respectively (Chavan et al 2005).

Superoxide dismutase activity measurement

SOD activity was assessed in the liver and erythrocytes samples. This experiment is based on the activity of the xanthine-xanthine oxidase system to generate blue color through the conversion of nitroblue tetrazolium (NBT) to formazan. Formazan derived blue color was determined at 560 nm wavelength and SOD enzyme activity was calculated from the prepared standart curve as defined earlier (Sun et al 1988). SOD activity was given as U/g Hb in red blood cells and U/g protein in liver.

Statistical analyzes

Results were given as mean and standard error for continuous dependent variables. The homogeneity was controlled with Levene test and the normality of the data was checked with Shapiro-Wilks test along with the coefficient of variation. As all of the continuous dependent variables are violated the homogeneity and normality assumptions, Kruskal Wallis test was performed in order to indicate the differences among the six experimental groups. In case of any statistical difference, as post-hoc test, Mann-Whitney U test together with Bonferroni correction were performed. $p<0.05$ are used as statistical significance value. IBM SPSS Statistics for Windows, Version 22.0 was used for the statistical analyses.

Results

DEN when administrated alone or with PB increased liver MDA levels significantly compared to negative control (Table 1, $p<0.001$), however plasma MDA levels remained same (Table 2, $p>0.05$). Compared to DEN group, PB distribution in drinking water increased both liver ($p<0.001$) and plasma MDA levels ($p<0.01$) significantly. EA group liver MDA levels were found to be same compared to negative control ($p>0.05$), on the other hand it decreased significantly in plasma ($p<0.001$). Compared to positive control, pretreatment with EA decreased liver MDA levels significantly ($p<0.001$), while treatment did not changed liver MDA levels (Table 1, $p>0.05$). Although EA pretreatment and treatment decreased plasma MDA levels significantly compared to positive control ($p<0.001$), the decrease in treatment group was higher.

CAT activity was determined to be same both in the liver and erythrocytes of positive control group compared to negative control (Table 1, 2, $p>0.05$). DEN administration alone was found to decrease CAT activity in liver (Table 1, $p<0.001$),

Table 1. Effects of EA on liver antioxidant enzymes and malondialdehyde (MDA) levels in DEN-PB induced liver damage in rats

Items	Groups					
	Control	DEN	DEN-PB	EA	EA-DEN-PB	DEN-PB-EA
Liver MDA(nmol/g)	20.17 ± 0.60^{cd}	40.00 ± 3.43^{b}	69.64 ± 3.38^{a}	18.65 ± 1.62^{d}	31.33 ± 2.26^{bc}	68.48 ± 3.01^{a}
Liver CAT (k/g protein)	223.31 ± 9.55^{c}	148.32 ± 4.29^{d}	196.17 ± 2.51^{c}	304.61 ± 9.13^{a}	214.19 ± 11.24^{c}	262.37 ± 8.54^{b}
Liver GSH (µmol/g protein)	623.31 ± 11.17^{c}	475.61 ± 38.55^{d}	465.99 ± 12.86^{d}	1086.18 ± 38.96^{a}	691.70 ± 26.43^{bc}	784.93 ± 44.04^{b}
Liver SOD (U/g protein)	117.50 ± 2.98^{c}	107.39 ± 3.33^{c}	113.05 ± 2.39^{c}	162.01 ± 2.84^{a}	144.32 ± 4.38^{b}	146.67 ± 3.98^{b}

The data presented as means and standard error.
The different superscripts (^{a,b,c,d}) in the lines indicate group means differences ($p<0.05$).

Table 2. Effects of EA on blood/erythrocytes/plasma antioxidants enzymes, glutathione (GSH) and malondialdehyde (MDA) levels in DEN-PB induced liver damage in rats

Items	Groups					
	Control	DEN	DEN-PB	EA	EA-DEN-PB	DEN-PB-EA
MDA (nmol/ml plasma)	10.94±0.65^{ab}	9.43±0.54^{bc}	12.39±0.72^{a}	2.76±0.24^{d}	8.01±0.47^{c}	3.98±0.11^{d}
CAT (k/g Hb)	60.54±3.19^{bc}	70.03±5.57^{bc}	43.12±3.80^{c}	53.70±8.95^{bc}	122.06±5.28^{a}	48.21±4.93^{bc}
GSH (µmol/g Hb)	63.13±4.98^{ab}	41.45±3.74^{cd}	45.37±7.46^{c}	71.20±3.36^{a}	49.79±2.36^{bc}	28.76±1.91^{d}
SOD (U/g Hb)	70.85±7.05^{bc}	74.67±6.73^{bc}	55.05±3.90^{c}	74.37±6.73^{bc}	156.93±5.93^{a}	91.84±4.21^{bc}

The data presented as means and standard error.

The different superscripts (^{a,b,c,d}) in the lines indicate group means differences ($p<0.05$).

whereas it was unchanged in erythrocytes (Table 2, $p>0.05$). EA application increased liver CAT activity significantly (Table 1, $p<0.001$); however erythrocyte CAT activity remained unchanged (Table 2, $p>0.05$). Although liver CAT activity increased both in pretreatment ($p>0.05$) and treatment groups ($p<0.05$), the increase in pretreatment wasn't statistically significant (Table 1). Oppositely to liver findings, erythrocyte CAT activity increased in both pretreatment ($p<0.001$) and treatment groups ($p>0.05$) compared to EA group, yet the increase was only significant in the pretreatment group (Table 2).

DEN application either alone or in combination with PB decreased GSH levels significantly not only in liver, but also in blood, in compared with control group (Table 1, 2, $p<0.05$). Compared to DEN group, PB addition didn't change GSH levels (Table 1, 2, $p>0.05$). Compared to control, EA administration alone increased liver ($p<0.001$) and blood GSH levels ($p>0.05$), however the increase found significant only in liver (Table 1). EA application increased liver GSH level in pretreatment and treatment groups compared to positive control (Table 1, $p<0.001$). Blood GSH level was comparable in pretreatment group ($p>0.05$), while it significantly decreased in treatment group (Table 2, $p<0.05$).

Compared to negative control, SOD activity was found to be same in liver and erythrocytes in DEN alone and DEN supplemented with PB groups (Table 1, 2, $p>0.05$). PB application didn't affect SOD activity in positive control compared to DEN alone ($p>0.05$). EA administration increased liver SOD activity significantly in EA group in compare with negative control (Table 1, $p<0.001$), yet the increase in erythrocytes was not significant (Table 2, $p>0.05$). EA pretreatment and treatment to positive control increased SOD enzyme activity significantly in liver and red blood cells compared to control group (Table 1, 2, $p<0.05$).

Discussion

Malondialdehyde (MDA), the end product of lipid peroxidation, regarded as an important lipid peroxidation indicator (Shaban et al 2013). Polyunsaturated fatty acid oxidation resulted in enhanced MDA content (Hamouda et al 2015). DEN and PB are stated to cause lipid peroxidation through increasing MDA levels in the liver and eryhtrocytes (Yadav and Bhatnagar 2007, Shaban et al 2013). Our previous studies showed increased liver and plasma MDA levels following DEN and PB application (Özeren et al 2019, Kisacam et al 2022). Although in our study liver MDA levels increased, plasma MDA levels didn't change significantly suggested that DEN and PB increased liver ROS levels, yet blood has the capacity to compensate for ROS formation. EA pretreatment also demonstrated to decrease MDA levels in mice liver toxicity either with paracetamol or carbon tetrachloride (Girish et al 2009, Girish and Pradhan 2012). In a study assessing the antioxidant activity of EA, it was shown that EA decreased MDA and ROS levels in HEPG2 cells exposed to high glucose (Ding et al 2019). In agreement with these studies (Girish et al 2009, Ding et al 2019, Girish and Pradhan 2012), in present study EA pretreatment decreased both liver and plasma MDA levels. On the other hand, EA treatment demonstrated to decrease MDA levels in cisplatin and valproic acid induced liver damage in rats (Yüce et al 2007, Abdelkader et al 2020). However, in the present study liver MDA levels remained unchanged following EA treatment, still plasma MDA levels decreased. These results might be speculated as the EA concentration increases in plasma and subsequently adequate amount of EA and its derivatives reach to liver in EA pretreatment group and exert antioxidant effects. However, EA treatment might be speculated as, when ROS levels increased EA distribution might be not enough for exerting effective antioxidant potential.

Gani (2019) reported decreased CAT activity in diethylnitrosamine and phenobarbital induced hepatocarcinoma in mice. Decreased CAT activity was reported in DEN and PB induced hepatocarcinoma in rats (Saraswati et al 2013). Solely DEN administration to young rats decreased CAT activity (Owumi et al 2019). Moreover, in our previous study we found that DEN-PB administration decreased erythrocyte CAT activity. In parallel to mentioned studies, in the current study liver CAT activity decreased significantly in DEN application and insignificantly both

in liver and erythrocyte after DEN and PB application. Abdelkader (2020) demonstrated that CAT activity remained unchanged with 50 mg/kg/body weight EA pretreatment. Similarly in our study liver CAT activity remained unchanged in rats administrated 50 mg/kg/body weight EA. Although there are studies reported decreased SOD activity following solely DEN or DEN-PB combine application (Fathima et al 2018, Owumi et al 2019), unchanged SOD activity was also reported by Hussein (2015). Similarly to Hussein (2015), in the present study SOD levels remained unchanged. CAT and SOD are crucial anti-oxidant defense enzymes. Decrease or unchanged CAT and SOD activity might cause to vulnerability to ROS and membrane phospholipid oxidation in liver and erythrocyte. Increased lipid peroxidation end product MDA levels in the current study validated this interpretation. EA is reported to induce SOD and CAT anti-oxidant enzymes activity (Han et al 2006). EA treatment was found to increase liver SOD activity in rats (Srigopalram et al 2012). Moreover different amounts of EA pretreatment was indicated to induce SOD activity in rats exposed to cerebral ischemia/reperfusion (Ahmed et al 2014). In accordance with these studies, in present study EA pretreatment and treatment increased SOD activity, however blood SOD increased in pretreatment, and remained same in treatment. These results suggested that pretreatment with EA could be more effective. However unchanged blood SOD levels might probably related to EA concentration distributed to rats.

Thiol containing tripeptide GSH is an important non-enzymatic antioxidant participates in detoxification of ROS. DEN and PB administration is reported to decrease GSH levels in rats (Shahjahan et al 2005, Shaban et al 2013). Our previous studies also showed decreased blood and liver GSH levels after DEN-PB exposure (Özeren et al 2019, Kisacam et al 2022). Consistently with these reports (Shahjahan et al 2005, Shaban et al 2013) in the present study, liver and blood GSH levels decreased following both DEN and DEN-PB administration. Decreased GSH levels may be related to the increased requirement of GSH for coupling with DEN or its reactive products as it has been reported by Sherif (2018). Moreover, the lack of GSH might also contribute to the LPO generation and oxidative stress (Shahjahan et al 2005). EA is reported to induce endogenous GSH synthesis and activates antioxidant defense (Abdelkader et al 2020). EA is also indicated to activate antioxidant mechanism through increasing GSH levels against cisplatin derived liver oxidants (Yüce et al 2007). In agreement with these studies in present study EA enhanced liver GSH levels suggested that both pretreatment and treatment with EA might show strong anti-oxidant potential against DEN-PB induced liver damage. Although hepatic GSH is the primary source of blood GSH supply, in present study blood GSH levels remained same which might speculated as liver GSH is used in the liver to detoxifying internal ROS production. However, decreased plasma MDA levels brought the idea that blood ROS scavenged by antioxidant enzyme activity such as CAT and SOD regardless of GSH.

Conclusion

In conclusion, the results obtained from this study can be explained as, DEN and PB induced liver injury in rats by increasing MDA levels and decreasing GSH levels, on the other hand EA acts as an antioxidant agent. Especially acquired results indicated that EA pretreatment could be more effective than EA treatment in DEN and PB induced liver oxidative stress in rats. Besides, preventive utilization of EA also could be more effective than treatment by enhancing antioxidant defense in blood.

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Conflict of Interest

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During this study, any pharmaceutical company which has a direct connection with the research subject, a company that provides and / or manufactures medical instruments, equipment and materials or any commercial company may have a negative impact on the decision to be made during the evaluation process of the study or no moral support.

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