The Effects of Caffeic Acid Phenethyl Ester on DNA-Turnover Rates and Nitric Oxide Level in Doxorubicin-Induced Myocardial Injury¶

DOKSORUBİSİNİN YOL AÇTIĞI MYOKARDİYAL HASARDA KAFEİK ASİT FENETİL ESTERİN DNA-TURN OVER HIZI VE NİTRİK OKSİT SEVİYESİNE ETKİLERİ

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Summary

Purpose: Doxorubicin (DXR), an important chemotherapeutic agent for cancers, has severe cardiotoxic effects. This work was designed to determine whether the doxorubicin-induced cardiotoxity via changes in purine catabolism, nitric oxide (NO) system and collagen formation and is prevented by caffeic acid phenethyl ester (CAPE).

Materials and Methods: Male Sprague-Dawley rats (60 days old) were divided into three groups. One group was untreated and the others were treated with DXR or DXR+CAPE, respectively. DXR was administered by a single i.p. injection (20 mg/kg). CAPE was administered i.p. 10 µmol/kg/day two days before DXR treatment for 12 days. Hydroxyproline (OH-P) formation was determined in myocardium. The changes in purine catabolism and NO system were determined by the activities of xantine oxidase (XO) and adenosine deaminase (ADA) and NO level in the heart tissue, respectively.

Results: DXR treatment without CAPE increased OH-P level significantly in myocardial tissue. The rats treated with CAPE produced significant decrease in OH-P level in comparison with DXR group. The activities of XO and ADA were significantly higher in DXR-treated rats in comparison with control and DXR+CAPE-treated rats. DXR treatment increased tissue NO level in myocardium and CAPE prevented this increase. There was no significant difference in NO levels between control and DXR plus CAPE-treated rats.

Conclusion: The protection of heart tissue by CAPE againts DXR-induced myocardial injury was demonstrated by decreased OH-P level upon CAPE administration to the rats. Increased XO and ADA enzyme activities may indicate high DNA turn over rates in heart tissue due to DXR-toxicity. CAPE may prevent DXR-induced increased DNA turn over rates and preserve myocardium from injury. İncreased NO level, as a free radical, may be regarded as an index of myocardial damage due to DXR, Furthermore, CAPE inhibited excessive NO production and prevented pro-inflammatory effects of NO and so might protect tissue from injury due to high inflammatory reaction as indicated previously in the literature.

Key Words: Doxorubicin, CAPE, Nitric, Oxide, Hidroxyproline

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Özet

Amaç: Kansere karşı etkili bir kemoterapötik ajan olan doksorubisin ciddi kardiyotoksik etkilere sahiptir. Bu çalışma, doksorubisinin indüklediği kardiyotoksisitenin oluşmasında pürin katabolizması, nitrik oksit (NO) sistemi ve kollajen oluşmasında, değişikliklerin olup olmadığı ve kafeik asit fenetil esterin (CAPE) kardiyotoksisiteyi engelleyip engellemediğini saptamak üzere planlandı.

Materyel ve Metod: Erkek Sprague-Dawley sıçanlar (60 günlük) üç gruba ayrıldı. Birinci gruba tedavi verilmedi ve diğerleri sırasıyla doksorubisin ve doksorubisin+CAPE ile tedavi edildiler. Doksorubisin (20 mg/kg) tek doz i.p. olarak uygulandı. CAPE i.p. olarak 10 µmol/kg/gün dozunda doksorubisin tedavisinden iki gün önce başlanarak 12 gün uygulandı. Myokardiyal dokuda hidroksiprolin (OH-P) oluşumu belirlendi. Pürin katabolizmasında ve NO sistemindeki değişiklikler sırasıyla kalp dokusu ksantin oksidaz (XO) ve adenozin deaminaz (ADA) aktiviteleri ile NO seviyesi ölçümleriyle tespit edildi.

Bulgular: CAPE olmadan doksorubisin tedavisi myokardiyal dokuda belirgin OH-P artışına yol açtı. XO ve ADA aktiviteleri doksorubisin uygulanan grupta kontrol ve doksuribisin+CAPE uygulanan gruplara göre anlamlı arttı. Doksorubisin tedavisi myokardiumda doku NO seviyesini arttırdı ve CAPE bu artışı engelledi. Kontrol ve doksorubisin+CAPE uygulanan sıçanlarda NO seviyesi açısından anlamlı bir fark yoktu.

Sonuç: Doksorubisinin indüklediği myokardiyal hasara karşı CAPE'nin kalp dokusunu koruması sıçanlara CAPE uygulanmasıyla azalan OH-P seviyesiyle gösterildi. Doksorubisin toksisitesinden dolayı kalp dokusunda artan XO ve ADA aktiviteleri DNA turn over hızının arttığının belirtisi olabilir. CAPE doksorubisinin indüklediği artmış DNA turn over hızını azaltabilir ve myokardiyal dokuyu hasardan koruyabilir. Bir serbest radikal gibi artan NO seviyesi doksorubisinin yol açtığı myokardiyal hasarın bir göstergesi olarak kabul edilebilir. Ayrıca, CAPE aşırı NO üretimini durdurarak ve NO'nun proenflamatuvar etkilerini engelleyebilir ve bu şekilde literatürde de belirtildiği gibi enflamatuvar reaksiyondan dolayı olan ikincil hasardan dokuyu korumuş olabilir.

Anahtar Kelimeler: Doksorubisin, CAPE, Hidroksiprolin

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Doxorubicin (DXR), a most effective anticancer agent that causes severe cardiotoxicity through reactive oxygen species (ROS) has limitation in clinical usage (1,2). It has been shown that the DXR induced disarrangement of the Z-disc structure, the lack of the thin filaments and the disruption of the cytoskeleton architecture (3). These processes suggest possible involvement of proteins and purine catabolism in DXR cytotoxic activity. Doxorubicin causes lipid peroxidation and damages to adjacent organelles, and DNA (4).

The generation of peroxynitrite is one of the important toxic products during cellular injury induced by oxidative stress (5). It was demonstrated that cardiac NO is increased during the development of doxorubicin-induced cardiomyopathy (6).

Tokudome et al explained that interstitial collagen accumulation of myocardium was high in DXR-treated rats. They also demonstrated that the decrease in interstitial collagen accumulation was beneficial in preventing doxorubicin-induced myocardial damage (7). Hydroxylation of proline is one of the important steps in the collagen formation, and assessment of hydroxyproline level may give valuable information about accumulation of collagen in the extracellular medium of the tissue (8).

Caffeic acid phenethyl ester (CAPE) is an active component of propolis obtained from honeybee hives (9). It has been demonstrated that CAPE displays antioxidant, immunomodulatory and anti-inflammatory activities (10-12). Previous studies have demonstrated that CAPE prevents oxidative injury induced by ischemia-reperfusion injury in kidney, spinal cord and brain (11,13,14).

The aim of this study, thus, was to investigate the *in vivo* effects of CAPE against DXR-induced cardiotoxicity and the changes in purine catabolism, nitric oxide (NO) system and formation of hydroxyproline (OH-P), as an index of collagen synthesis in the tissue.

Materials and Methods

Male Sprague Dawley rats (60 days old) (n=9 per group) were used in the experiments. The animals were housed in quiet rooms with 12:12-h light-dark cycle (7 am to 7 pm) and the experiments were performed in accordance with "Guide for the Care and Use of Laboratory Animals, DHEW Publication No. (NIH) 85–23, 1985" and approved by local ethical committee at Medical School of Inonu University.

Rats were randomly assigned to one of the three groups: untreated control rats; animals treated with single i.p. injection of DXR (20 mg/kg, prepared with saline) (1); animals treated for 12 days with i.p. injections of CAPE (10 μmol/kg/day, water extract) (11) beginning from two days before single i.p. injection of DXR (20 mg/kg). The control and DXR groups' rats were also treated with i.p. saline instead of DXR or CAPE treatment during experimental procedure, respectively.

At the 10^{th} day of DXR-treatment, the animals were killed by decapitation, and then hearts were rapidly excised and stored at–70^{o} C until the study. After weighing the heart, homogenate and supernatant samples were prepared (13), and the following determinations were made on the samples using commercial chemicals supplied by Sigma. The tissue samples were homogenized in four volumes of ice-cold Tris-HCl buffer (50 milimolar, pH 7.4) containing 0.50ml/l Triton X-100 with a homogenisator (IKA Ultra -Turrax T 25 Basic) for 2 minutes at 13000 rpm. The homogenate was then centrifuged at 5000g for 20 minutes to remove debris. The clear upper supernatant was taken and used in the assays. Protein measurements were made at all stages according to the Lowry's method (15). All procedures were performed at +4°C.

Xanthine oxidase (XO, E.C. 1.2.3.2) activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbancy at 293 nm, according to Prajda and Weber's method (16).

Tissue adenosine deaminase (ADA, E.C. 3.5.4.4) activities were estimated by a method, which is based on the direct measurements of the formation of ammonia, produced when ADA acts in excess of adenosine (17).

Nitric oxide (NO) has a half-life of only a few seconds, because it is readily oxidized to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-) which serves as index parameters of NO production. The method for tissue nitrite and nitrate levels was based on the Griess reaction (18).

The heart tissue hydroxyproline (OH-P) levels were determined by the method of Woessner (19) after some pieces of samples were dried, weighed, digested in nitric acid/perchloric acid solution for three hours.

Data were analysed by using a commercially available statistics software package (SPSS® for Windows v. 9.0, Chicago, USA). One-way ANOVA test was performed. Post Hoc multiple comparisons were done with LSD. Results were presented as means ± SEM. *P* values <0.05 were regarded as statistically significant.

Results

The results are summarized in Table 1.

The level of OH-P was higher in DXR-treated rats than that of control (p<0.001). DXR plus CAPE group had almost %63 OH-P level of the DXR group and it was significantly lower than the OH-P level of the DXR group (p<0.001). On the other hand, there was no significant difference between the levels of OH-P between control and DXR plus CAPE groups.

The activity of XO was increased in DXR-treated group in comparison with control group (p<0.001). The CAPE treatment resulted in significant decrease in rats heart tissue in XO activity compared to DXR alone treatment (p<0.001). Doxorubicin group's rats had higher ADA activity in heart tissue than control group's rats (p<0.001). The activity of ADA was decreased in DXR plus CAPE group in comparison with DXR group (p<0.001). There was no significant difference in XO and ADA activities between the control and the DXR plus CAPE groups.

The myocardial NO level was 1.77 times higher in DXR-treated group than in control group (p<0.001). DXR plus CAPE-treated group had significantly lower NO level than DXR-treated group (p<0.001). The NO level was not significantly different in DXR plus CAPE group compared to control group.

Discussion

The importance of oxidative stress inducing genetic toxicity is widely accepted and subsequently has been extensively studied (20). Many studies have been done to examine DXR-induced cardiotoxicity and to prevent its toxicity. It is known that DXR causes high production of oxygen free radicals in myocardium (21). The formation of oxygen free radicals damages cellular structures by lipid peroxidation. This process explains the pathological picture of DXR induced myocardial damage characterized by disruption of heart mitochondrial and sarcoplasmic reticular formation in myocardial compartments (1,21). Our previous study

Table 1. The activities of xanthine oxidase (XO), adenosine deaminase (ADA), and the levels of nitric oxide (NO) and hydroxyproline (OH-P) in control, doxorubicin (DXR) and DXR plus caffeic acid phenethyl ester (CAPE) groups

	OH-P (mg/g dry tissue)	XO (U/g prot)	ADA (U/g prot)	NO (μmol/g wet tissue)
1-Control (n=9)	0.999 ± 0.061#	0.191 ± 0.025#	0.081 ± 0.009#	0.662 ± 0.068#
2- DXR (n=9)	1.529 ± 0.116*	0.555 ± 0.029*	0.138 ± 0.007*	1.172 ± 0.088*
3-DXR+CAPE (n=9)	0.966 ± 0.068#	0.266 ± 0.028#	0.080 ± 0.010#	0.553 ± 0.070#

Mean values ± SEM;
*p<0.001 in comparison with control group;
# p<0.001 in comparison with DXR group.

demonstrated that DXR resulted in lipid peroxidation in plasma and erythrocytes (22). Zhou and Kang demonstrated that metallothionein, an antioxidant, directly interacts with ROS and plays a significant role in protection against oxidative injury by DXR in remote organelles (4). *N*-acetylcysteine which is a sulfydryl containing agent, ameliorates acute high dose DXR-cardiotoxicity (1). Korac and Buzadzic demonstrated that oral supplementation with antioxidants, selenium and vitamins E, C and A, for 15 days prevents toxic effects of DXR in the skin (23). It was recently demonstrated that erdosteine, an antioxidant with its active metabolites, prevented the lipid peroxidation in heart tissue to be exposed to DXR treatment (24).

The effects of DXR on the myocardium are complex. During DXR-induced cardiotoxicity, purines are degraded to hypoxanthine, and xanthine dehyrogenase is converted to XO. Xanthine oxidase catalyses the conversion of hypoxanthine to uric acid with the release of the superoxide radical anions and then to other radicals. Xanthine oxidase consumes molecular oxygen as an electron acceptor. Subsequently, XO is reoxidized under physiological conditions through two, one-electron reductions of molecular oxygen to produce two superoxide anion radicals, which can then form hydrogen peroxide. On the contrary, xanthine dehydrogenase favours to utilize NAD^+ as an electron acceptor to generate NADH through a direct two-electron reduction (25-27). The present study demonstrated that DXR administration alone caused high XO activities. It was parallel to the findings of the literature that DXR induces reactive oxygen species production by increasing XO activities. Furthermore, DXR is a toxic agent to all cellular structures including proteins, DNA. Previous studies have demonstrated that CAPE exhibits antioxidant property. At a concentration of 10 micromolar, it completely blocks production of reactive oxygen species in human neutrophils and the xanthine/XO system (11). Our study demonstrated that CAPE treatment prevented the high XO activity and formation of free radicals, which are toxic to cellular structures. Similar to XO activity, ADA activity also was increased in DXR administered rats. The DXR is a highly toxic to genetic material and these high enzyme activities may point out that there might be genetic damage in myocardial tissue. CAPE treatment prevented the increase in ADA activity of myocardium like XO activity. By this way, CAPE may preserve genetic material from DXR induced destruction.

Doxorubicin toxicity was also due to high NO level. Nitric oxide reacts with superoxide anion to form peroxynitrite, which is toxic to cellular components (5). In the present study, it was demonstrated that CAPE inhibited the high NO production during DXR-administration. In this way, CAPE may exert its anti-inflammatory effect by inhibiting the inducible nitric oxide synthase (9), and antioxidant effect by an additional way, inhibition of endothelial nitric oxide synthase (28).

It was explained that increased interstitial collagen accumulation of myocardium is related with myocardial damage (7). Doxorubicin caused collagen accumulation. Simultaneous administration of temocapril, an angiotensin-converting enzyme inhibitor, with DXR was beneficial in preventing DXR-induced myocardial damage and inhibited interstitial collagen accumulation (7). In our study, we demonstrated the protective effect of CAPE on the increased OH-P formation due to DXR-induced cardiomyopathy.

In conclusion, because DXR is an important chemotherapeutic agent and its cardiotoxicity results in limitation in clinical use, CAPE may be an important candidate to prevent this cardiotoxicity. High DNA turnover rates in myocardium due to DXR-cardiotoxicity may be prevented by CAPE. CAPE also exerts a role in the inhibition of excessive NO production and prevents myocardium from hazardous effects of NO. Furthermore, CAPE may be beneficial to prevent collagen accumulation in myocardium interstitial area and protect myocardium from injuries. However, it should be emphasised whether CAPE helps to preserve the electrical activities of heart and prevent myocardial dysfunction due to DXR-induced cardiotoxicity.

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