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Original Research Article

3, 4-Dihydroxyphenylethanol attenuates cadmium-induced oxidative stress, inflammation and apoptosis in rat heart

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Abstract

Purpose: *To investigate the therapeutic effect of 3, 4-dihydroxyphenylethanol (DOPET) on cadmium (Cd) induced cardiotoxicity in murine model.*
Methods: *Four groups of rats were used in this study (n = 6). The rats were treated with DOPET and Cd for 28 days. Biochemical parameters were determined in plasma and heart tissue homogenates.*
Results: *Cadmium (Cd) significantly increased lipid peroxidation and protein carbonylation. However, DOPET treatment significantly attenuated Cd-induced oxidative stress. Cd intoxication significantly increased cardiac markers {creatine kinase, lactate dehydrogenase (LDH) and cardiac troponin-I} levels in plasma, and reduced the levels of antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase Gpx ,glutathione (GSH) and malndialdehyde (MDA) in heart tissue. These Cd-induced changes in cardiac markers and antioxidants were reversed by DOPET treatment. Cd treatment led to upregulation of protein expressions of pro-inflammatory cytokines (TNF-α and Il-6). However, DOPET supplementation brought about a decrease in the protein expressions of these cytokines. Western blot analysis revealed that Cd induced apoptosis in cardiac tissue, as was evident from alterations in protein expressions of the apoptotic inducers, Bax and cleaved caspase-3, and the anti-apoptotic factor Bcl-2. However, DOPET treatment effectively reversed Cd-induced apoptosis.*
Conclusion: *These results indicate that DOPET exerts cardio-protective effect against Cd-induced toxicity via antioxidant, anti-inflammatory and anti-apoptotic mechanisms.*

Keywords: *Cadmium, Cardiotoxicity, DOPET, Antioxidant, Inflammation, Apoptosis*

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INTRODUCTION

Cadmium (Cd) is a toxic transition heavy metal which acts as a pro-inflammatory agent and a potent carcinogen. As an environmental and occupational toxicant, humans are exposed to Cd in various staple food items like rice, wheat, peanuts, and grains through soil contamination from various chemical industries [1]. Furthermore, tobacco smoke contributes to human exposure to Cd. Cadmium is accumulated in its soluble form, cadmium chloride ($CdCl_2$). It exerts noxious effects in various tissues and organs such as liver, kidney, brain and heart.

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Globally, cardiovascular diseases (CVD) remain the major cause of mortality, despite the advancement in treatment approaches [2]. Environmental changes, high fat diet and lack of exercise remain cardinal factors in the pathogenesis of CVD. Studies have shown that environmental pollution is a key factor in CVD [3]. Increasing evidence have highlighted the relationship between Cd exposure and increased risk of stroke, a CVD [5]. Preclinical studies have shown that Cd insult can lead to cardiotoxicity [5]. Cadmium (Cd) exerts cardiotoxicity at concentrations as low as 0.1 mM [6]. Cadmium-induced cardiotoxicity is mediated through generation of reactive oxygen species (ROS) which in turn decrease the levels of antioxidants [6]. The ROS induce membrane lipid peroxidation, and damage proteins, DNA and other vital biomolecules. Furthermore, Cd-induced ROS increases oxidative stress in the myocardial mitochondria which lead to loss of membrane integrity, activation of caspases and apoptosis of myocytes [7].

The use of plants for the treatment of CVD has continued to attract increasing attention due to their proven efficacy and lack of adverse effects. Polyphenols have shown promising effects in attenuating oxidative stress-mediated cardiac injury. In addition, antioxidants from natural sources have been shown to protect various organs and tissues from Cd-induced oxidative cellular damage [8]. Hydroxytyrosol (3, 4-dihydroxyphenylethanol, DOPET) is a potent polyphenol present in the olive oil [9]. Previous studies have demonstrated the protective effect of DOPET against oxidative stress-mediated cellular death [10]. Moreover, it has been reported that DOPET exerts protective effect on heavy metal-induced neurotoxicity [11]. Earlier reports showed the protective influence of the antioxidant potential of DOPET against Cd-induced oxidative insult [12]. Recently, it was shown that DOPET exerted cardio-protective effect against ischemic/reperfusion and isoproterenol-induced myocardial infarction through its free radical scavenging potential [13]. The current study was undertaken to investigate the protective effect of DOPET against Cd-provoked oxidative cardiotoxicity by assessing various biochemical parameters.

EXPERIMENTAL

Drugs and chemicals

Hydroxytyrosol (DOPET) and Cd were procured from Sigma Chemicals, USA. The other reagents used in the study were analytical grade, and were purchased from Merck, USA.

Animals

Male Sprague Dawley Wistar rats aged 6 weeks and weighing about 180-200 g were utilized for the study. The animals were obtained from Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China. The rats were housed in polypropylene cages under standard laboratory conditions at a temperature of 25 °C and alternating 12 h light/12 h dark cycle. The animals were allowed free access to water and pelleted rat feed. All animal studies were carried out as per the protocol outlined in Care and Use of Laboratory Animals, NIH. The study received approval from the Ethical Committee for Animal Care and Use, Huazhong University of Science and Technology, Wuhan, Hubei, China (Approval no: TK2016321).

Experimental design

Three groups of rats were used (6 rats per group). Group 1 rats were treated with normal saline, and served as control, while group 2 rats received cadmium chloride ($CdCl_2$) at a dose of 5 mg/kg body weight (b.wt.) in normal saline, via the intra-gastric route. Group 3 rats were given DOPET (10 mg/kg b.wt) orally, while in group 4, the rats were pretreated with DOPET (10 mg/kg; b.wt) 1 h before administration of $CdCl_2$ (5 mg/kg b.wt). All treatments were given daily for 28 days.

After the final doses of DOPET and Cd, the rats were subjected to overnight fast. Thereafter, they were anaesthetized using phenobarbital sodium at the dose of 35 mg/kg, i.p. and sacrificed through cervical dislocation. Blood was withdrawn from the jugular vein in heparinized tubes and the plasma was separated for use in the measurement of cardiac marker enzymes. The heart tissue was excised, cleaned from adherent tissues, washed in ice-cold saline and blotted. Then, a 10 % (w/v) tissue homogenate was prepared by homogenizing 100 mg of cardiac tissue in chilled Tris-HCl buffer. The homogenate was used for the assays of various biochemical markers of Cd-induced cardiac damage.

Assay of cardiac markers

Creatinine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) levels were measured in plasma using standard kits from Nanjing Jiancheng Bio-Engineering Institute, Nanjing, China. Furthermore, plasma cardiac troponin (cTn) cTn-I was estimated with ELISA kits (Biocheck ELISA kit, USA) according to manufacturer’s protocol.

Evaluation of lipid peroxidation and protein carbonyl content

The lipid peroxidation (LPO) index, malondialdehyde (MDA) was estimated spectrophotometrically at 532 nm using the method of Ohkawa *et al* [14]. The protein carbonyl content was quantified using the method of Levine *et al* [15].

Assay of antioxidants

The cardiac level of antioxidants CAT, SOD, GPx and GSH were measured using assay kits (Nanjing Jiancheng Bio-Engineering Institute, Nanjing, China) in line with the instructions on the kit manuals.

Determination of plasma pro-inflammatory cytokines

Plasma levels of pro-inflammatory cytokines TNF-α and IL-6 were estimated using ELISA kit in accordance to the instruction provided in the protocol by the manufacturer (Sigma Aldrich, USA).

Western blot analysis of pro-inflammatory cytokines and apoptosis markers

Heart tissue lysate samples were collected using RIPA lysis buffer kit (Applygen Technologies Inc., China). Next, 20 µg of protein was subjected to 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After immunoblotting, the separated proteins were transferred to a nitrocellulose membrane, washed, and incubated with anti-IL-6, anti-TNF-α, anti-Bax, anti-cleaved caspase-3 and anti-Bcl-2 antibodies. Following incubation with primary antibodies, the nitrocellulose membranes were incubated with peroxidase-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz, USA). Visualization of proteins was done using ECL detection kit (Applygen Technologies Inc, China) and the imaging and analysis of the protein bands were done with Gel Doc XR system (Bio-Rad, USA).

Statistical analysis

The data are presented as mean ± standard error of the mean (SEM). One-way analysis of variance, followed Tukey's comparison test using SPSS version 18.0 was performed for comparison amongst the groups. Values of $p < 0.05$ were assumed statistically significant.

RESULTS

Effect of DOPET administration on cardiac markers

The effect of DOPET treatment on levels of cardiac markers in the Cd-toxified rats is shown in Table 1. There were significant elevations in the plasma concentrations of cardiac markers CK-MB, LDH and cardiac troponin I (cTn-I) ($p < 0.05$). Treatment with DOPET effectively reduced the plasma levels of the cardiac markers to normal.

Effect of DOPET on cardiac lipid peroxidation and protein oxidation

As shown in Figure 1 A, the Cd-intoxicated rats showed significantly increased lipid peroxidation as evident from increased MDA content, relative to the control group. However, DOPET administration reversed the Cd-induced increases in MDA to normal levels ($p < 0.05$). The cardiac tissue level of protein carbonyl, an end product of protein oxidation was significantly increased in the Cd-intoxicated group, when compared to the control group. However, DOPET treatment reversed the Cd-induced protein oxidation and restored the cardiac tissue protein carbonyl to normal level (Figure 1 B).

Table 1: Effect of DOPET and Cd on plasma cardiac markers

Group	LDH (IU/L)	CK-MB (IU/L)	cTnI (µg/ml)
Control	145.34 ± 5.43	103.76±4.32	1.23±0.23
Cd	567.98 ± 10.76 ^{a*}	456.32±9.76 ^{a*}	3.43±0.56 ^{a*}
DOPET	148.76±4.65	105.87±4.32	1.25±0.26
DOPET + Cd	176.65±4.67 ^{b*}	112.43± 6.78 ^{b*}	1.41±0.35 ^{b*}

Values are shown as mean ± SEM (n = 6). Analyses were done by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc comparison test. The comparison were made between Cd group and control (a); and between Cd group and DOPET+ Cd group (b); $^*p < 0.05$

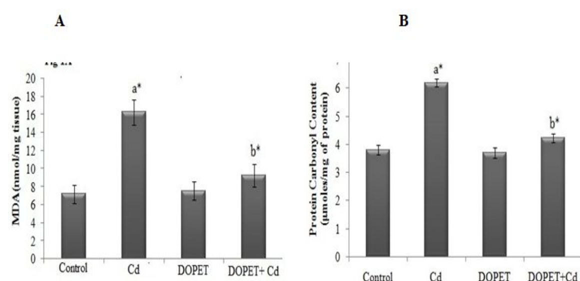

Figure 1: Effect of DOPET on lipid peroxidation and protein carbonyl level in heart tissue. Cd induced oxidative stress and caused marked increase in MDA level and protein carbonyl content in cardiac tissue. DOPET treatment effectively decreased the lipid peroxidation and protein carbonyl levels. A: MDA; B: Protein carbonyl content. Data are shown as mean ± SEM (n = 6). Comparison was made between Cd group and control (a), and between b-DOPET+ Cd group and Cd group (b); *$p < 0.05$

Effect of DOPET on antioxidant status in Cd-induced cardiac oxidative stress

There were significant decreases in cardiac tissue levels of SOD, CAT, GPx and GSH in the Cd-intoxicated rats, when compared to the control group ($p < 0.05$). However, DOPET treatment significantly reversed the Cd-induced decreases in these antioxidants, relative to the Cd group ($p < 0.05$). The results are shown in Table 2.

Effect of DOPET on pro-inflammatory cytokine levels in Cd-induced cardiotoxicity

The plasma levels of pro-inflammatory cytokines (TNF-α and Il-6) were significantly elevated in Cd group, when compared to the control group. The administration of DOPET significantly reduced the Cd-induced increases in pro-inflammatory cytokine levels to normal (Figure 2).

Effect of DOPET on protein expressions of TNF-α and Il-6 in cardiac tissue

The protein expressions of TNF-α and Il-6 were significantly upregulated in Cd-toxified rats, when compared to the control rats (Figure 3 A). Furthermore, the relative protein levels of TNF-α, Il-6 and IL1- β were significantly increased in the Cd group, relative to the control group. However, administration of DOPET significantly decreased the protein expressions of pro-inflammatory cytokines, when compared to the Cd group ($p < 0.05$; Figure 3 B).

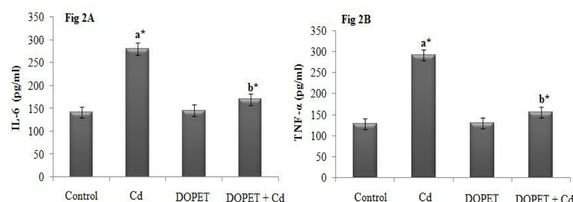

Figure 2: Effect of DOPET on plasma levels of pro-inflammatory cytokines in Cd-induced cardiotoxicity. Cd-treated rats showed elevated levels of IL-6 (A) and TNF-α (B). DOPET-treated rats showed reduced levels of pro-inflammatory cytokines. The values are shown as mean ± SEM (n = 6). Analysis was done by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc comparison test. Comparison was made between Cd group and control (a), and between b-DOPET+ Cd group and Cd group (b); *$p < 0.05$)

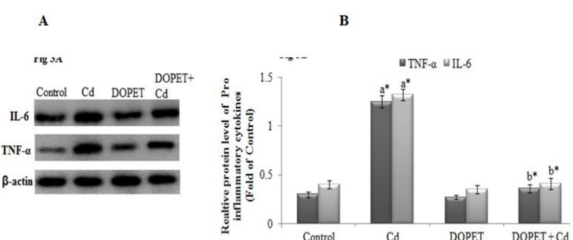

Figure 3: Effect of DOPET on protein expressions of pro-inflammatory cytokines in Cd-induced cardiotoxicity. A: Cd group showing up-regulated expressions of pro-inflammatory cytokines; DOPET treatment effectively down-regulated the protein expressions. B: Significantly reduced relative protein expressions of pro-inflammatory cytokines in DOPET-treated, Cd-exposed rats ($p < 0.05$). Values are expressed as mean ± SEM (n = 6). Analyses were done using one way analysis of variance (ANOVA) followed by Tukey's post-hoc comparison test. Comparison was made between Cd group and control; and between b-DOPET+ Cd group and Cd group; *$p < 0.05$

Table 2: Effect of DOPET and Cd on antioxidant levels in heart homogenate

Group	SOD	CAT	GPx	GSH
Control	10.34±0.87	7.54±0.45	20.54±1.24	14.27±1.26
Cd	5.98±0.56 ^{a*}	3.12±0.23 ^{a*}	12.56±1.12 ^{a*}	7.15±0.82 ^{a*}
DOPET	11.54±0.76	7.28±0.38	19.86±1.54	13.58±1.12
DOPET+ Cd	8.87±0.62 ^{b*}	6.98±0.36 ^{b*}	18.34±1.28 ^{b*}	12.54±1.42 ^{b*}

Values are shown as mean ± SEM (n = 6). Analyses were performed using ANOVA followed by Tukey's post-hoc comparison test. Comparison was made between Cd group and control (a), and between b-DOPET+ Cd group and Cd group; *$p < 0.05$. Units: SOD: U/mg protein; CAT: µmoles/H_2O_2/min/mg protein; GPx: µmoles NADPH oxidized /min/mg protein; GSH: nmol/mg protein

Effect of DOPET on apoptotic protein expressions in cardiac tissue

The protein expressions of the apoptotic markers Bax and cleaved caspase-3 were upregulated, while Bcl-2 was downregulated in Cd-toxified rats, when compared to the control group (Figure 4 A). However, DOPET treatment significantly downregulated the protein expressions of Bax and cleaved caspase-3, and upregulated the protein expression of Bcl-2, when compared to the Cd group (Figure 4 B).

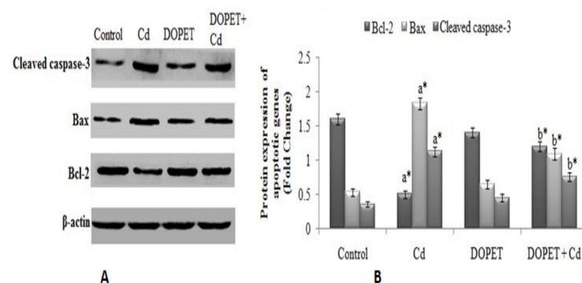

Figure 4: Effect of DOPET on the protein expressions of apoptotic markers in Cd-induced cardiotoxicity. A: Cd treatment led to upregulation of the expressions of Bax and cleaved caspase-3, whilst Bcl-2 protein expression was down-regulated. DOPET treatment effectively reversed the altered protein expressions. B: Relative protein expressions of apoptotic genes in Cd treated rats. The values are shown as mean ± SEM (n = 6). Analyses were done with one way analysis of variance (ANOVA) followed by Tukey's post-hoc comparison procedure. Comparison was made between Cd group and control (a), and between b- DOPET+ Cd group and Cd group; $^*p < 0.05$

DISCUSSION

Oxidative stress, a condition due to excess production of ROS and loss of cellular antioxidant defense mechanism, is a cardinal factor in an array of cardiovascular diseases. The current study investigated cardio-protective effect of 3, 4-dihydroxyphenylethanol (DOPET) against Cd-provoked oxidative cardiotoxicity. Cadmium (Cd) mediates its toxic effects by generation of ROS which bring about alterations in metabolic pathways involved in energy metabolism, protein synthesis and antioxidant network. Furthermore, Cd affects DNA by altering gene control and repair [21]. It triggers peroxidation of lipids present in cell membrane due to excessive ROS release, thereby decreasing the level cellular antioxidants available for neutralization of free radicals [16].

The clinical diagnosis of the pathological symptoms of Cd toxicity relies on measurement of cytoplasmic enzymes released into the blood stream during oxidative membrane damage. Thus, increases in the levels of plasma marker enzymes are indicative of tissue damage and loss of membrane integrity. Creatine kinase (CK-MB) and LDH are the cardiac marker enzymes used to assess the severity of cardiac damage during oxidative free radical attack. During this process, the cardiac membrane integrity is compromised, leading to leakage of enzymes into the blood stream [17].

Troponin is a cardiac protein located in the thin filaments of striated muscles present in heart tissue. It is composed of three subunits namely troponin T (cTn-T), troponin I (cTn-I) and troponin C. Troponin I (cTn-I) is clinically used to assess the degree of myocardial injury [17]. In the present study, Cd-intoxication caused significant elevation in the level of plasma marker enzymes CM-BK, LDH and cTn-I. This is in agreement with previous reports. However, treatment with DOPET mitigated the Cd-mediated increases in levels of plasma cardiac marker enzymes and cardiac troponin. This effect is due to the free radical-quenching and membrane-stabilizing potential of DOPET [13].

Polyunsaturated fatty acids (PUFA) which are present in all tissues and organs are vulnerable to free radical damage due to their high degree of unsaturation. Oxidative deterioration of polyunsaturated fatty acids is a noxious process involved in cell injury during Cd exposure. In the present study, Cd-provoked cardiotoxicity was due to increased generation of free radicals in heart tissue, which was evident from the marked increases in the level of MDA, a reliable marker of lipid peroxidation. Cadmium (Cd)-induced lipid peroxidation has been reported in previous studies. Oxidative damage by ROS results in protein oxidation, the end product of which is protein carbonyl. The protein carbonyl content (PCC) is a reliable marker of the extent of protein oxidation. In the present study, administration of DOPET to Cd-intoxicated rats reduced LPO and protein oxidation, and thus protected proteins and lipids from oxidative attack [18].

The non-enzymatic antioxidant, thiol or sulfhydryl (-SH) group is involved in detoxification of Cd through formation of a complex with Cd (Cd-SH). Reduced glutathione (GSH), a thiol compound forms a complex with Cd through covalent bonding [19]. In the current study, the Cd-intoxicated rats had significantly reduced level of GSH, which might be due to utilization of GSH to quench the free radicals released by Cd-mediated lipid peroxidation, as well as the high affinity binding of Cd to thiol groups. However, treatment with DOPET reversed the Cd-induced

decrease in GSH, most likely due to the free radical scavenging potential of DOPET.

Oxidative stress is a pathological condition in which the physiological antioxidant network losses the ability to remove toxic free radicals form the cells. Catalase and SOD and are the mainstay antioxidant enzymes that protect the cells from oxidative damage. Superoxide dismutase (SOD) is involved in the removal of highly reactive superoxide ion, while catalase mediates the detoxification of toxic H_2O_2. Thus, SOD and catalase guard the cells/organs from free radical attack. In this study, the cardiac levels of SOD, catalase and GPx were decreased following Cd intoxication. However, DOPET treatment effectively increased the antioxidant enzyme levels via its antioxidant activity [20]. The Cd-toxified rats showed elevated levels of TNF-α and IL-6. Cadmium activates monocytes and tissue macrophages to secrete pro-inflammatory cytokines [21]. Treatment with DOPET significantly reduced plasma levels of TNF-α and IL-6, thereby mitigating the Cd-induced inflammation [7,22].

Studies have shown Cd-induced toxicity results in apoptotic cells [23]. The anti-apoptotic protein Bcl-2, is a key modulator that regulates the intrinsic apoptotic pathway by preventing the release of cytochrome-c from the mitochondria following caspase activation [24]. Caspases are a family of proteases involved in the initiation and activation of apoptotic pathway, with caspase-3 as the key mediator. The proteins Bcl-2 and Bax participate in the caspase activation. Caspase-3 participates in the downstream signalling of the mitochondrial pathway, and its activation results in irreversible apoptosis [25].

In the present research, it was found that the pro-apoptotic factors Bax and cleaved caspase-3 were upregulated, whereas BCl-2 was decreased in the immunoblot obtained from the Cd group. However, administration of DOPET attenuated the expressions of these apoptotic proteins. In addition, it was found that treatment with DOPET increased the anti-apoptotic Bcl-2 expression, indicating that amelioration of apoptosis may be a potent therapeutic target in Cd-induced cardiotoxicity.

CONCLUSION

The findings of this study show that DOPET exerts cardio-protective effect against Cd-induced cardiotoxicity by regulation of cardiac marker enzymes, inhibition of lipid peroxidation, and enhancement of antioxidant status, anti-inflammation and anti-apoptotic pathways.

DECLARATIONS

Acknowledgement

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Conflict of interest

No competing interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. YH and HG prepared the protocol and carried out the study. BL prepared the manuscript and ML performed the statistical analysis

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